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original scientific paper

Production of Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by *Comamonas testosteronii A3*

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Summary

The bacterial strain **Comamonas testosteronii** *A3 was investigated for its potential for the production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymers. A screening experiment with accumulation media containing different carbon sources and precursors for 4-hydroxybutyrate production yielded copolymers with mole fractions of 4-hydroxybutyrate above 90%. The medium composition was optimized in shaking-flask experiments for a maximum of polymer production and a maximum of 4-hydroxybutyrate content in the polymer. The kinetics of growth and polyhydroxyalkanoate production were determined in a batch bioprocess. A possible explanation for the production of copolymers with unusually high 4-hydroxybutyrate contents by species from the* **Comamonas** *genus is discussed.*

Keywords: polyhydroxyalkanoate, poly(3-hydroxybutyrate-co-4-hydroxybutyrate), 4-hydroxybutyrate, *Comamonas testosteronii*

Introduction

It has long been known that many bacteria are able to produce polyhydroxyalkanoates (PHAs) as intracellular carbon and energy reserve materials. More recently these materials have attracted industrial interest as biodegradable thermoplastics which, in some fields, might replace conventional petrochemical plastics *(1–4)*. It has been shown that bacteria can incorporate a vast variety of monomers in the polymer, including medium chain length carboxylic acids, halogenated and unsaturated organic acids and phenyl hydroxy acids *(5,6)*.

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3-HB-co-4-HB)] copolymers, which were discovered in 1987 *(7)* are of especially high industrial potential. Even low 4-hydroxybutyrate (4-HB) contents in the polymer lead to a significant drop in crystallinity, making the material less stiff and brittle, and better suited for industrial purposes than a poly(3-hydroxybutyrate) [P(3-HB)] homopolymer. Polymers with high 4-HB contents became very strong thermoplastic elastomers *(8–10)*. In previous papers the production of P(3-HB-co-4-HB) copolymers with high 4-HB concentrations by *Comamonas acidovorans* was reported *(9,11)*. Recently we tested a variety of microorganisms from the rRNA superfamily III for their ability to produce P(3-HB-co-4-HB) in order to find strains for the possible industrial production of these copolymers *(12)*. In this article the production of P(3-HB-co-4-HB) with unusually high 4-HB contents by *Comamonas testosteronii* is reported.

Materials and Methods

Bacterial strain and culture maintenance

Comamonas testosteronii A3, DSM 676 (previously *Pseudomonas testosteronii* A3) was originally isolated by Brilon *et al. (13)* and provided by Prof. H. J. Knackmuss, Stuttgart. For culture maintenance, colonies that had grown on solid media for 3 to 4 days at 30 °C, were kept at 4 °C, and transferred to new plates every 4 to 6 weeks. For long-term maintenance the strain was stored in liquid nitrogen.

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Media and conditions for growth and PHA accumulation

The complex LB medium for growth consisted of 10 g/L of tryptone, 5 g/L of yeast extract and 5 g/L of NaCl. Solid media also contained 1.5% agar-agar. A mineral-salt buffer was used as the basis for the preparation of defined media for growth and PHA-accumulation. It consisted of 4.5 g/L of $Na_2HPO_4 \cdot 2H_2O$, 1.5 g/L of KH_2PO_4, 0.20 g/L of $MgSO_4 \cdot 7H_2O$, 1 mL/L of trace-element solution SL-6, 0.02 g/L of $CaCl_2 \cdot 7H_2O$, and 0.05 g/L of NH_4-Fe(III)-citrate. Each liter of the trace-element solution SL-6 contained 100 mg of $ZnSO_4 \cdot 7H_2O$, 300 mg of H_3BO_3, 200 mg of $CoCl_2 \cdot 6H_2O$, 6 mg of $CuSO_4$, 20 mg of $NiCl_2 \cdot 6H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$, and 25 mg of $MnCl_2 \cdot 2H_2O$. All used chemicals were of analytical grade. All experiments were carried out at 30 °C.

For PHA-accumulation experiments in rolling-shaker cultures, 300-mL shaking flasks containing 70 mL of complex LB medium were inoculated with colonies from solid media. The cultures were incubated for 24 hours under reciprocal shaking at 130 rpm. After this growth phase, the biomass was harvested by a 20-min centrifugation at 4 °C and $7000 \times g$. The pellets were resuspended in the mineral-salt buffer and transferred to test tubes containing different accumulation media (mineral-salt buffer plus 10 g/L of carbon source and 3 g/L of precursor for P(4-HB) production), yielding a final volume of 10 mL. A 36-hour incubation phase on a rolling shaker was followed by the harvesting of the cells and determination of biomass and PHA.

For PHA-accumulation experiments in shaking flasks, 1000-mL shaking flasks containing 200 mL of complex LB medium were inoculated from overnight liquid cultures and incubated for 18 hours, followed by harvesting and resuspending as described before. The cells were then transferred to 300-mL shaking-flasks with 70 mL of mineral-salt buffer containing 0 to 27 g/L of sodium acetate and 0 to 9 g/L of 1,4-butanediol as precursor for P(4-HB) production. Samples were taken during both the growth phase and the accumulation phase.

Batch production system

The batch production was carried out in a 16-L laboratory bioreactor L1523 (Bioengineering AG, Wald, Switzerland) with 10 L of culture volume. 500 mL of overnight shaking-flask cultures served as inoculum. The temperature was kept at 30 °C and the pH was automatically maintained at 7.0 by addition of sterile aqueous solutions (100 g/L) of NaOH or H_3PO_4. The dissolved oxygen concentration was monitored with a sterilizable oxygen electrode (type IL530, Ingold, Frankfurt/Main, Germany) and kept above 50% of the air saturation value by manually controlling the air flow rate or impeller speed. Mineral-salt buffer containing 13.6 g/L of sodium acetate as carbon source and 2 g/L of $(NH_4)_2SO_4$ as nitrogen source was used as the growth medium.

Determination of cell dry mass (CDW)

5 mL portions of the liquid culture were centrifuged for 15 min in a Hereaus Megafuge 1.0 R centrifuge at $3240 \times g$ and 4 °C, followed by a first washing of the pellet with 2 mL of 0.9% NaCl solution and a second washing with 2 mL of deionized H_2O. The pellet was freeze-dried for 12 h and weighed on an analytical balance.

PHA assay

Following Braunegg's method *(14)* modified by Zeiser *(15)*, PHAs were determined by gas-chromatography. Freeze dried biomass samples were subjected to methanolysis with acidified methanol [$\varphi(H_2SO_4)$=5%] which also contained 1 g/L of hexanoic acid as internal standard. A Hewlett Packard (HP) 5890 Series II gas chromatograph with a 5-m HP1 pre-column, a 25-m HP Ultra 2 capillary column (ID 0.32 mm, film thickness 0.52 mm), and equipped with an auto sampler, was used. The helium flow-rate through the columns was 1 mL/min at 130 °C, and the flame ionization detector was kept at 250 °C and supplied with air, hydrogen, and nitrogen as make-up gas. The injection split ratio was 1:10, and 1-µL injections were made at 250 °C. The oven temperature was initially set to 90 °C, cooled down to 60 °C at the rate of –15 °C/min, heated to 100 °C at the rate of 4 °C/min and finally heated to 290 °C with a rate of 30 °C/min. Biopol™ P(3-HB) samples mixed with sodium 4-hydroxybutyrate were used as PHA standards.

Substrate analysis

The ammonium concentration was determined with an ion sensitive electrode model 95–12, Orion Research Inc., Boston, MA. 1,4-butanediol and acetate were determined by gas-chromatography as tetrahydrofurane and ethylacetate, respectively. 1 mL of ethanol and 1 mL of concentrated H_2SO_4 were added to 1 mL of frozen culture supernatant and heated to 105 °C for 3.5 hours in PTFE-sealed glass vials. After cooling to room temperature, 2 mL portions of methylene chloride were added and the vials were vigorously shaken for 2 minutes. After a short centrifugation step the organic phase was transferred into GC vials. The oven temperature was initially set to 50 °C, cooled down to 40 °C at the rate of -2 °C/min, heated to 150 °C at the rate of 10 °C/min and finally heated to 290 °C at the rate of 30 °C/min. All other GC parameters were the same as for PHA analysis.

Results and Discussion

P(3-HB-co-4-HB) production in rolling culture

Results from rolling-culture experiments with different accumulation media showed that practically no PHA was produced, when glucose or butyrate were used as the sole carbon source during the accumulation phase (Fig. 1).

Using glucose or butyrate together with precursors for 4-HB production gave the same results as using only the precursors alone. In all these cases the mole fraction of 4-HB in the resulting polymers ranged from 86 to 94%. This indicates that only the precursor was used for PHA accumulation.

When sodium acetate was used as the sole carbon source during accumulation, a P(3-HB) homopolyester was produced. Mixtures of sodium acetate with the precursors yielded P(3-HB-co-4-HB) copolymers with 4-HB

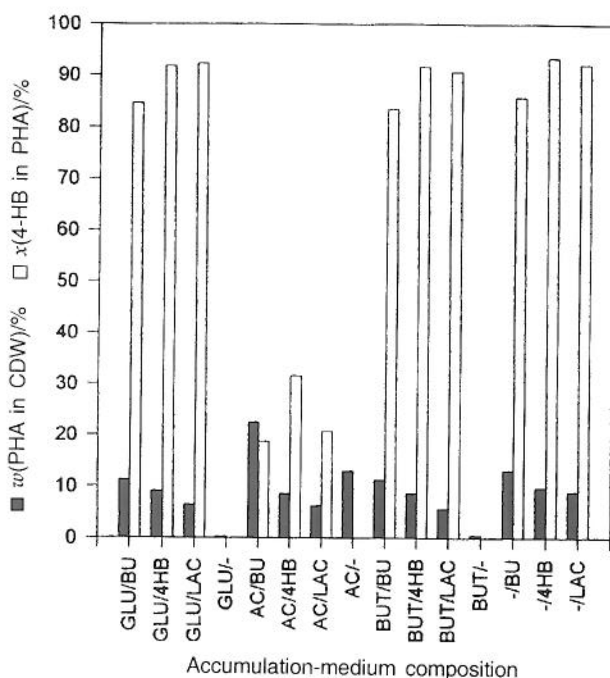

Fig. 1. Production of P(3-HB-co-4-HB) by *Comamonas testosteronii* A3 after 36 hours in nitrogen-free accumulation media in rolling culture. Glucose GLU, sodium acetate AC, sodium butyrate BUT, all 10 g/L; 1,4-butanediol BU, sodium 4-hydroxybutyrate 4-HB, 4-butyrolactone LAC, all 3 g/L.

mole fractions of 19 to 32%. Apparently acetate and precursors were converted simultaneously to 3-HB and 4-HB units respectively.

Under similar accumulation conditions such high contents of 4-HB have so far only been reported for *Comamonas acidovorans (9,12)*. The copolymer compositions differ considerably from those observed with *Alcaligenes eutrophus*, producing P(3-HB-co-4-HB) copolymers with 4-HB contents of less than 40%, even when precursors were used as the sole carbon source during the accumulation phase *(5)*. A pathway for 4-hydroxybutyrate utilization and (P3-HB-co-4-HB) production for *Alcaligenes eutrophus* has been proposed *(16)*, in which 4-hydroxybutyrate is partly converted to 4-hydroxybutyryl-CoA and inserted then into the polymer. The other part is oxidized to succinate, and further converted to acetyl-CoA, which can then be used for the formation of 3-hydroxybutyryl-CoA. Thus low 4-HB contents in the polymer are the result of a high conversion of 4-hydroxybutyrate to 3-hydroxybutyrate via acetyl-CoA. In the case of *Comamonas testosteronii* the remarkably high 4-HB contents (90%) in the polymer suggest that almost no degradation of 4-hydroxybutyrate is taking place.

P(3-HB-co-4-HB) production in shaking flasks

In order to optimize the PHA production for a biomass containing a maximum of polymer with mole fraction of 4-HB of at least 20%, shaking-flask experiments were carried out. Because in rolling-shaker experiments the highest polymer content had been found in a medium containing acetate and 1,4-butanediol, different concentrations of these substances were used in the optimization experiments. As shown in Figs. 2 and 3 the optimum medium composition for a maximum of polymer was at acetate concentrations of 3 g/L, whereas the medium composition for a maximum of 4-HB in the polymer contained no acetate at all. The best overall result was obtained with a medium containing 3 g/L of sodium acetate and 7 g/L of 1,4-butanediol, yielding a biomass with 12% (mass fraction) of copolymer containing 35% (mole fraction) of 4-HB.

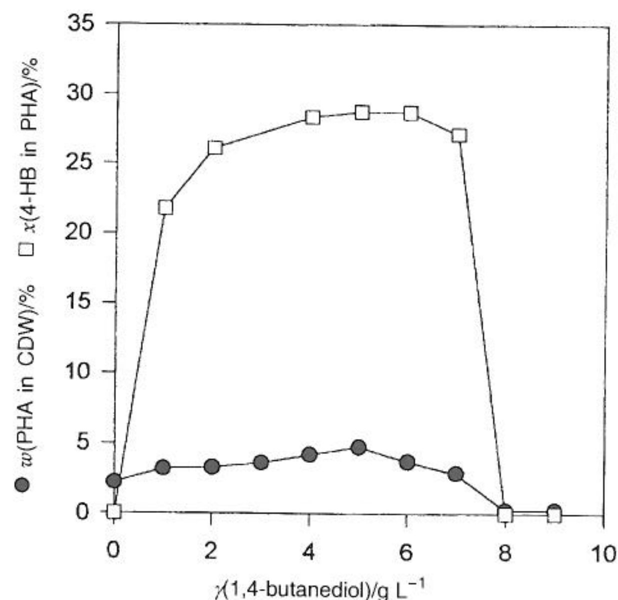

Fig. 2. Production of P(3-HB-co-4-HB) in shaking flasks by *Comamonas testosteronii* A3 after 24 hours in nitrogen-free accumulation media containing 9 g/L of sodium acetate and 0 to 9 g/L of 1,4-butanediol as carbon sources.

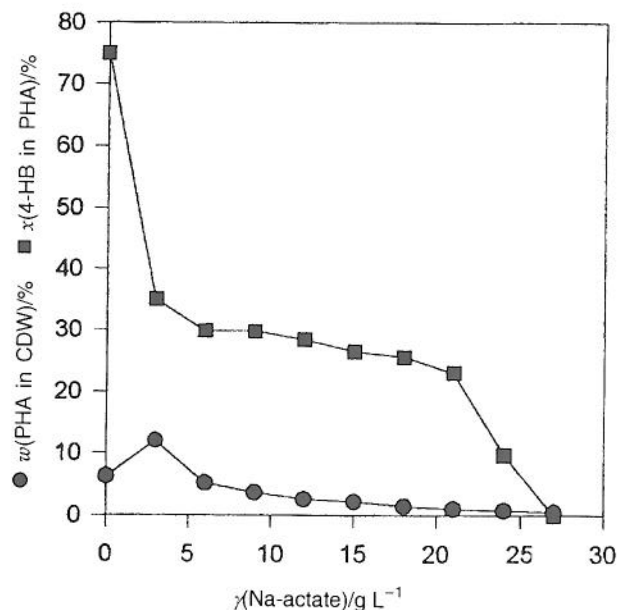

Fig. 3. Production of P(3-HB-co-4-HB) in shaking flasks by *Comamonas testosteronii* A3 after 24 hours in nitrogen-free accumulation media containing 0 to 30 g/L of sodium acetate and 7 g/L of 1,4-butanediol as carbon sources.

Batch P(3-HB-co-4-HB) production

In order to investigate the kinetics of growth and P(3-HB-co-4-HB) production by *Comamonas testosteronii* a two-step batch process was carried out. Results are shown in Figs. 4 to 6. From the results presented in Fig. 4 the specific growth rate μ_{γ_r} was calculated, which was 0.15 h^{-1} for the first 17 hours and then gradually increased to 0.35 h^{-1} until at 27 hours the nitrogen source was depleted and the sodium acetate concentration was lowered down to 1.5 g/L, as can be seen in Fig. 5. During the same period the specific acetate consumption rate (calculated as sodium acetate) $\sigma_{Na\text{-}acetate}$ increased from initially 0.70 h^{-1} to 1.80 h^{-1}, and the specific $(NH_4)_2SO_4$ consumption rate $\sigma_{(NH_4)_2SO_4}$ increased from 0.13 h^{-1} to 0.30 h^{-1}. The acceleration of growth towards the end of the growth phase may be due to the relief of inhibitory effects of higher substrate concentrations. The initial inhibition of growth is also reflected by the fact that a considerable amount of P(3-HB) homopolymer (w=5% of the biomass) was produced throughout the first 16 hours of growth phase (Fig. 6). When the growth rate increased the mass fraction of the polymer dropped to 2% of the biomass. During shaking-flask experiments with complex medium the polymer content of the exponentially growing biomass had always stayed below 1%. The overall yield coefficients for the growth phase were $Y_{res.\ biomass\ /(NH_4)_2SO_4} = 1.05$ g/g and $Y_{total\ biomass\ /Na\text{-}acetate} = 0.20$ g/g.

At the time the $(NH_4)_2SO_4$ concentration reached zero, the accumulation phase was started by adding sterile solutions of sodium acetate and 1,4-butanediol to final concentrations of 4.6 and 9.0 g/L, respectively.

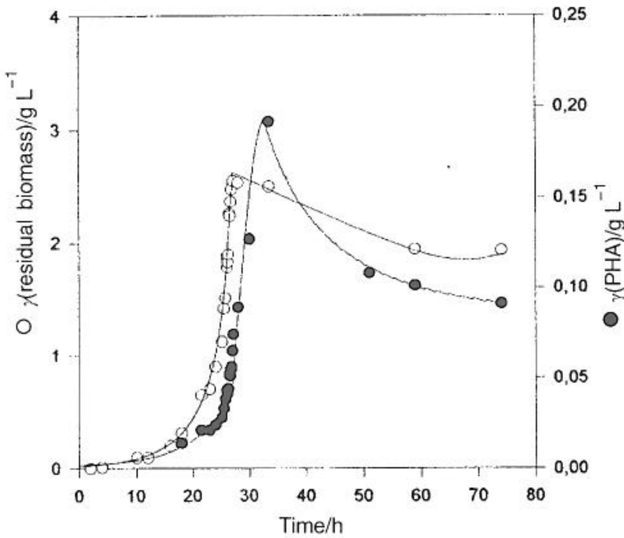

Fig. 4. Residual biomass concentration and PHA concentration during the batch production with *Comamonas testosteronii* A3

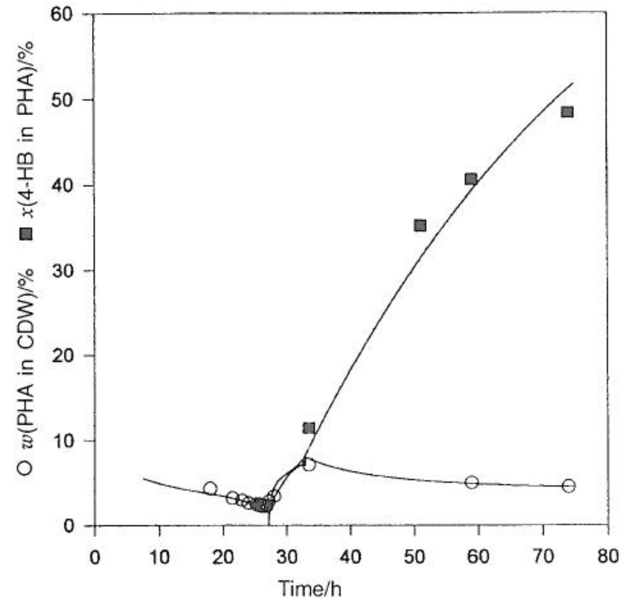

Fig. 6. Polymer content of the biomass and polymer composition during the batch production of PHA with *Comamonas testosteronii* A3

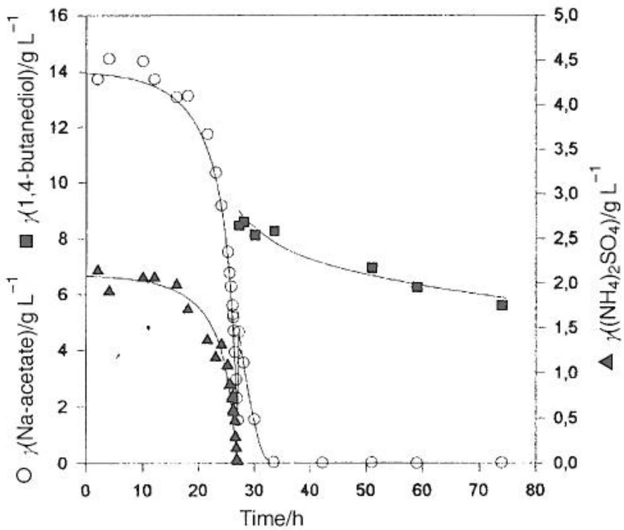

Fig. 5. Concentration of substrates during the batch production of PHA with *Comamonas testosteronii* A3

Within 5 hours the sodium acetate was completely consumed and the polymer content of the biomass increased to 7% (mass fraction) with 12% (mole fraction) of 4-HB in the polymer. The specific PHA production rate π_{PHA} was 0.014 h^{-1}. Once the acetate concentration reached zero, the previously accumulated P(3-HB) was degraded by the cells with a specific consumption rate $\sigma_{P(3\text{-}HB)}$ of 0.005 h^{-1}. During the same time P(4-HB) was inserted in the remaining polymer at a specific production rate $\sigma_{P(4\text{-}HB)}$ of 0.001 h^{-1}. After 47 hours of accumulation time a polymer with a mole fraction of 48% 4-HB was obtained which represented 5% of the dry biomass.

The results indicate that once acetate becomes limiting, the previously stored P(3-HB) serves as the main carbon and energy source, although high concentrations of 1,4-butanediol are available. As already observed during the rolling culture experiments, the diol is apparently not degraded to acetyl-CoA. The inability to meta-

bolize 4-hydroxybutyric acid (and 4-butyrolactone) as carbon and energy source seems to be the reason for the production of copolyesters with high 4-HB contents in *Comamonas testosteronii*.

The yields of P(3-HB) from sodium acetate and P(4-HB) from 1,4-butanediol were, however, extremely poor ($Y_{P(3-HB)/Na-acetate}$ =0.022 g/g, $Y_{P(4-HB)/1,4-butanediol}$ =0.015 g/g), but as a HPLC system for the detection of possible by products was not available, the reason for this could not be further investigated.

List of symbols

1. γ ; total biomass concentration
2. $\gamma_r = \gamma - \gamma_{PHA}$; residual biomass concentration
3. $\mu_{\gamma_r} = \frac{d\gamma_r}{dt} \cdot \frac{1}{\gamma_r}$; specific growth rate
4. $\sigma_{substrate} = \frac{d\gamma_{substrate}}{dt} \cdot \frac{1}{\gamma_r}$; specific substrate consumption rate
5. $\pi_{product} = \frac{d\gamma_{product}}{dt} \cdot \frac{1}{\gamma_r}$: specific product production rate
6. $Y_{product/substrate} = \frac{m(\text{product})}{m(\text{substrate}_{consumed})}$; yield coefficient

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Proizvodnja poly(3-hidroksibutirat-co-4-hidroksibutirata) s pomoću *Comamonas testosteronii A3*

Sažetak

Istražena je sposobnost bakterijskog soja ***Comamonas testosteronii*** *A3 da proizvede kopolimere poli(3--hidroksibutirat-co-4-hidroksibutirata). Pokusi su pokazali da se nakupljanjem tvari u podlozi, koje su sadržavale različite izvore ugljika i prekurzore za proizvodnju 4-hidroksibutirata, proizvode kopolimeri s množinskim udjelom 4-hidroksibutirata iznad 90%. U pokusima na tresilici utvrđen je optimalan sastav podloge za proizvodnju maksimalne količine polimera s najvećim udjelom 4-hidroksibutirata.*

Kinetika rasta i proizvodnja polihidroksialkanoata utvrđena je pri diskontinuiranom vrenju. Raspravljano je o mogućem mehanizmu proizvodnje kopolimera s neuobičajeno velikim udjelom 4-hidroksibutirata s pomoću sojeva iz roda ***Comamonas****.*