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NOTE

Taxonomic implications of synthesis of poly-β-hydroxybutyrate and other poly-β-hydroxyalkanoates by aerobic pseudomonads

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Whereas poly-β-hydroxybutyrate (PHB) production by *Pseudomonas* species is rare, synthesis of medium-chain-length poly-β-hydroxyalkanoates (mcl-PHAs) other than PHB, has been observed in fluorescent and non-fluorescent species. Contrary to original reports, *Pseudomonas corrugata* and *Pseudomonas ficuserectae* accumulate mcl-PHAs and not PHB. The taxonomic implications of these characteristics are discussed.

Keywords: poly-β-hydroxybutyrate, poly-β-hydroxyalkanoates, *Pseudomonas* taxonomy

Poly-β-hydroxybutyrate (PHB), a polymer discovered by Lemoigne in *Bacillus megaterium* (Lemoigne, 1926), occurs in a variety of bacteria, among them many species of aerobic pseudomonads (Stanier *et al*., 1966). Granules of the polymer accumulate preferably during growth in media of high C/N ratio and can be observed best under phase-contrast optics after staining with Sudan Black.

PHB was detected in aerobic pseudomonads of the genera *Burkholderia* and *Ralstonia* (group II), the family *Comamonadaceae* (group III) and genus *Brevundimonas* (group IV) (Palleroni *et al*., 1973; Palleroni, 1993; Kersters *et al*., 1996). Most strains of *Pseudomonas* and those of group V (*Xanthomonas* and *Stenotrophomonas*) do not synthesize PHB, which confirms the close positions of these organisms in the γ-*Proteobacteria*. Among the exceptions are some weak accumulator strains of *Pseudomonas pseudoalcaligenes* (Stanier *et al*., 1966), strain DSM 50338 of *Pseudomonas viridiflava* (Timm & Steinbüchel, 1990), *Pseudomonas corrugata* (Scarlett *et al*., 1978) and *Pseudomonas ficuserectae* (Goto, 1983).

In the 1960s and 1970s, synthesis of medium chain length poly-β-hydroxyalkanoates (mcl-PHAs) by bacteria was reported, but the evidence presented failed to elicit much interest (Steinbüchel & Valentin, 1995). The situation changed in the following decade and PHAs composed of a large variety of monomeric units were described. At least 92 different monomers are now known to be present in natural PHAs (Steinbüchel & Valentin, 1995; He *et al*., 1998).

As with PHB, accumulation of mcl-PHAs can be elicited in media of high C/N ratio and the polymer also can be clearly seen under phase-contrast after staining with Sudan Black. Granules of PHB and PHA cannot be differentiated by simple observation, and the identification may involve either GC analysis (Timm & Steinbüchel, 1990) or a solubility test [mcl-PHAs are soluble in acetone, whereas PHB is not (Abe *et al*., 1994)].

The first report of mcl-PHA formation by a *Pseudomonas* species, *Pseudomonas oleovorans*, described the production of a polymer containing 3-OH-octanoate by assimilation of medium and long chain length fatty acids (de Smet *et al*., 1983). This capacity is also expressed by growth on alkanes (Lageveen *et al*., 1988), but not on carbohydrates (Timm & Steinbüchel, 1990).

Further studies established that synthesis of PHAs is a common feature of fluorescent pseudomonads (Huisman *et al*., 1989; Timm & Steinbüchel, 1990), which suggested the possibility of using this characteristic for taxonomic purposes (Huisman *et al*., 1989). In contrast to *P. oleovorans* and another fluorescent species, *Pseudomonas resinovorans*, *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* KT2442 and *Pseudomonas* sp. NCIMB 40135 can synthesize PHAs from carbohydrates (Timm & Steinbüchel, 1990; Haywood *et al*., 1990; Ramsay *et al*., 1992).

Abbreviations: mcl, medium chain length; PHA, poly-β-hydroxyalkanoate; PHB, poly-β-hydroxybutyrate.

Table 1. PHAs synthesized by *Pseudomonas corrugata* and *Pseudomonas ficuserectae* at the expense of two substrates

Abbreviations: CDW, cell dry weight (g l^{-1}) determined by the absorbance at 450 nm (Witholt, 1972); % PHA, percentage weight per cell dry weight (Lageveen *et al*., 1988); C6, 3-OH-hexanoate; C8, 3-OH-octanoate; C10, 3-OH-decanoate; ND, not detectable.

Organism	Substrate	CDW	% PHA	C6	C8	C10
P. corrugata	Octanoate	0·9	61	15	83	2
	Glucose	0·5	14	4	29	67
P. ficuserectae	Octanoate	0·17	3	25	61	14
	Glucose	0·31	0·4	ND	14	86

Whereas the formation of endocellular PHB granules by *P. pseudoalcaligenes* has been confirmed by identification of the monomer 3-OH-butyrate (Timm & Steinbüchel, 1990), such confirmation is still lacking for the reserve material of *P. corrugata* and *P. ficuserectae*. Consequently, the reported capacity for PHB biosynthesis by strains of the two species was re-examined in this study.

P. pseudoalcaligenes strain LMG 1225^T (included as control), *P. corrugata* strain LMG 2172^T and *P. ficuserectae* strain LMG 5694^T were grown in shake flasks in nitrogen-limited minimal medium 0·1N E2 (Huisman *et al*., 1989). For *P. corrugata* and *P. ficuserectae*, the medium was supplemented with either 14 mM octanoate or 0·8 % glucose (final concentrations). *P. pseudoalcaligenes* was grown on 14 mM octanoate. The cells of *P. corrugata* and *P. pseudoalcaligenes* were harvested after 52 h incubation at 28 °C on a rotary shaker; those of *P. ficuserectae* were harvested after 92 h under the same conditions. Cell preparations were stained with Sudan Black (Schaad, 1988) and observed under phase-contrast microscopy for the detection of polymer granules.

The cellular PHA content and composition were determined using a GC 8000 (Fisons) equipped with a 25 m CP-Sil5CB capillary column (Chromopack) as described elsewhere (Lageveen *et al*., 1988). PHA analyses were performed in triplicate, and mean values for *P. corrugata* and *P. ficuserectae* are given in Table 1.

The results in Table 1 clearly show that *P. corrugata* and *P. ficuserectae*, contrary to the original descriptions, do not synthesize PHB, since the polymers give C6, C8 and C10 monomer units on hydrolysis. 3-OH-butyrate monomer units may also be incorporated in small amounts into the PHA polymers, but our analytical results and the presence of Sudan Black-stained inclusions leave no doubt about the nature of the bulk composition of the granules.

In our experiments, *P. pseudoalcaligenes* produced PHB and only traces of 3-OH-octanoate and some 3-OH-decanoate could be detected. According to previous observations, 3-OH-decanoate is not a constituent of the PHA polymer in *Pseudomonas*, but rather part of the rest of the biomass, most likely part of the rhamnolipids (Durner, 1998). The total amount of mcl-PHA found in *P. pseudoalcaligenes* strain LMG 1225^T was less than 0·05 % of its dry weight.

The majority of *Pseudomonas* species that have been examined are devoid of the capability of PHB synthesis. However, fluorescent species have been found to accumulate mcl-PHAs when the strains are grown in the presence of C4 to C18 fatty acids (de Smet *et al*., 1983). The ability to synthesize mcl-PHAs is also found in non-fluorescent *Pseudomonas* species (He *et al*., 1998). In *P. stutzeri*, the polymer may amount to 52 % of the dry weight. After growth in minimal medium with soybean oil, the PHA contains 63 % of a novel monomer (3,6-epoxy-7-nonene-1,9-dioic acid) and minor proportions of C8 and C10 monomers.

Our findings indicate that, from a taxonomic point of view, the inability to accumulate PHB by the majority of *Pseudomonas* species preserves its taxonomic value as a good negative characteristic for use in the differentiation of aerobic pseudomonads at the genus level. When granules of reserve material are observed, it would be very convenient to supplement the observation with the identification of the constituent monomer units after growth under controlled conditions at the expense of various carbon sources.

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