3 Polythioesters

Dipl.-Biol. Tina Lütke-Eversloh¹, Prof. Dr. Alexander Steinbüchel²
¹ Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, D-48149 Münster, Germany; Tel.: +49-251-8339854; Fax: +49-251-8338388; E-mail: tina.luetke.eversloh@uni-muenster.de
² Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, D-48149 Münster, Germany; Tel.: +49-251-8339854; Fax: +49-251-8338388; E-mail: steinbu@uni-muenster.de

1 Introduction ...................................... 64
2 Historical Outline ................................. 65
3 Synthetic PTEs .................................... 65
4 Precursor Substrates for the Biosynthesis of PTEs ............. 65
5 Biotechnological Production of PTEs ............................ 66
  5.1 Biosynthesis of Poly(3HB-co-3MP) .......................... 66
  5.2 Biosynthesis of Poly(3HB-co-3MB) .......................... 67
  5.3 Biosynthesis of Homopolymers of 3MP, 3MB, and 3MV ......... 67
  5.4 Biosynthesis of PTEs Consisting of 3MO .................... 67
  5.5 Isolation and Purification .............................. 68
6 Biosynthetic Pathway .................................. 68
  6.1 Metabolic Pathway in R. eutropha .................. 68
  6.2 Metabolic Pathway in Recombinant E. coli .......... 70
  6.3 PTE(CH) Biosynthesis in Recombinant R. eutropha ...... 70
  6.4 Catalytic Mechanism of Polymerization .............. 71
7 Analysis of PTEs .................................... 72
8 Physical Properties of PTEs ............................ 74
1 Introduction

A wide range of bacteria are known to synthesize polyhydroxyalkanoates (PHAs) as intracellular storage compounds. These PHAs are accumulated as insoluble inclusions in the cytoplasm, and the PHA content can contribute up to more than 90% of the cellular dry weight (Anderson and Dawes, 1990). These microbial polyesters reveal interesting thermoplastic and/or elastomeric properties, that are similar to those of synthetic polypropylene which is produced from petrochemicals. Many technical applications have been developed for PHAs, e.g., in industry, agriculture, or medicine, which take into account the clear advantages of the biodegradability of these compounds and their origin from renewable resources (Hocking and Marchessault, 1994). Since the discovery of poly(3-hydroxybutyrate) (poly(3HB), PHB) (Lemoigne, 1926), bacterial polyesters have attracted much interest during recent decades, and the demand for novel biomaterials remains very high (Steinbüchel, 1991).

To date, more than 140 different hydroxyalkanoates (HAs) have been described as PHA constituents; these comprise different carbon chain length, and might also contain various substituents at different positions (Steinbüchel and Valentin, 1995). This large variety of different PHAs is attributed to the
unspecificity of the PHA synthases – the key enzymes which catalyze the biosynthesis of these molecules (Rehm and Steinbüchel, 1999).

The first microbial polymers containing thioester linkages were described only recently, these having been synthesized by a PHA-accumulating bacterium (Lütke-Eversloh et al., 2001a,b). Besides proteins and several complex polysaccharides, these are the only organic biopolymers which contain sulfur. Moreover, they contain sulfur not in the side chains, but in the backbone as part of the covalent linkage of the constituents, thereby establishing a novel class of biopolymers referred to as polythioesters (PTE).

2 Historical Outline

The first studies on synthetic PTEs were reported only 50 years ago (Marvel and Kotch, 1951), representing a relatively young class of polymers which are analogous to polyesters. The chemical synthesis of high-molecular weight PTEs is rather complicated as compared with the preparation of polyesters (see below).

The first report on the biosynthesis of PTEs was published in 2001 (Lütke-Eversloh et al., 2001a). This study can be regarded as a milestone, because the ecological advantages of this approach seem obvious in comparison with that for synthetic PTEs. PTEs representing copolymers of 3-mercaptopropionate (3MP) and 3-hydroxybutyrate (3HB) were described in the first reports, though later copolymers of 3-mercaptopbutyrate (3MB) and 3HB were also described (Lütke-Eversloh et al., 2001b). Meanwhile, evidence for the biosynthesis of 3-mercaptovalerate (3MV) -containing copolymers, as well as of homopolymers of 3MP, 3MB, and 3MV, was also obtained (T. Lütke-Eversloh and A. Steinbüchel, unpublished data). On the basis of the recent discovery of biopolymers, the technical applications of biotechnologically produced PTEs can be evaluated, taking into consideration the advantages of their biodegradability and availability from renewable resources.

3 Synthetic PTEs

Synthetic PTEs may be prepared using a variety of different methods. The chemical preparation of PTEs was first published in 1951, and described PTE syntheses from dithiols with adipyl chlorides or terephthalyl chlorides (Marvel and Kotch, 1951). For the condensation of diolefines with dithiocarboxylic acids, mostly biallyls are polymerized with dithio adipic acid. In addition to the free radical polymerization of unsaturated thioesters, dimercaptans can also be condensed with dibasic acid derivatives. These and other, less common methods have been summarized in a review by Sandler (1996). More recently, syntheses of aliphatic PTEs by anionic and cationic ring-opening polymerizations of thiolactones (Sanda et al., 1999; 2000), as well as by ring-opening polycondensation of 2-stanna-1,3-dithiacycloalkanes with dicarboxylic acid chlorides (Kricheldorf et al., 2000; Al-Masri et al., 2001) have been described. As yet, no commercial uses of PTEs have been described, though this may in part be due to the complex preparation methods involved.

4 Precursor Substrates for the Biosynthesis of PTEs

To date, the biosynthesis of copolymers containing 3MP or 3MB besides 3HB has
been reported (Lütke-Eversloh et al., 2001a,b). Current studies indicate also the incorporation of 3MV into polymers synthesized by wild-type bacteria (T. Lütke-Eversloh and A. Steinbüchel, unpublished data), adding a further example of 3-mercaptoalkanoates (3MA) as PTE constituents (Figure 1). With regard to the chemical structure of the precursor substrates, the $\beta$-position of the sulfhydryl group seems to be a prerequisite for incorporation into bacterial polymers.

With the exception of 3-mercaptopropionic acid, 3MAs are commercially not available. The chemical synthesis of 3MAs proceeds via the corresponding acetylmercaptoalkanoic acid, which can be synthesized by the addition of thioacetic acid to a 2-alkenoic acid (Schjänberg, 1941). 3MAs are then obtained by alkaline hydrolysis of the respective acetylmercaptoalkanoic acids, and are subsequently purified by extraction and distillation (Figure 2).

5 Biotechnological Production of PTEs

When Ralstonia eutropha was cultivated in the presence of 3MAs, 3MA-containing copolymers were synthesized. The biosynthesis of 3MP-containing polymers by R. eutropha was also achieved when 3’,3’-thiodipropionic acid (TDP) was fed as the sulfur-containing precursor substrate. R. eutropha is not capable of using either 3MAs or TDP as sole carbon sources for growth, and thus a second utilizable carbon source, such as gluconate or fructose, must be added as cosubstrate. Due to the toxic effects of 3MAs at concentrations greater than 0.2% (v/v) in the culture medium, the addition of 3MAs must be carried out in suitable portions during fed-batch fermentation.

5.1 Biosynthesis of Poly(3HB-co-3MP)

The Gram-negative polyhydroxyalkanoate (PHA)-accumulating bacterium R. eutropha synthesizes 3MP-containing PTEs, when either 3MP or TDP are provided as carbon sources in addition to gluconate (Lütke-Eversloh et al., 2001a). In contrast to 3MP, TDP revealed less growth-inhibiting properties, and was preferred as a substrate for microbial synthesis of PTEs in concentrations of up to 1.5% (w/v) in the culture medium. Several fed-batch fermentations with R. eutropha strain H16 were carried out at the 30 L-scale for the biotechnological production of 3MP-containing polymers (Lütke-Eversloh et al., 2002). First, bacterial growth was achieved in mineral salts medium (MSM) (Schlegel et al., 1961) by feeding with sodium gluconate and ammonium chloride, and revealing cell densities of up to 12 g cellular dry matter per liter. In the second stage of the cultivation, TDP was added for polymer accumulation, whereas
the polymer content contributed up to 30% of the cellular dry matter.

When considering the “natural” metabolism of *R. eutropha* strain H16, poly(3HB) biosynthesis could not be suppressed under these cultivation conditions, and this in turn led to the synthesis of copolymers which also contained certain amounts of 3HB, poly(3HB-co-3MP) (Figure 3). Details of the metabolic pathway will be discussed in Section 6. Characterization of the polymer samples obtained from different fermentations of *R. eutropha* revealed 3MP contents of up to more than 40 mol% (see Section 8).

5.2 Biosynthesis of Poly(3HB-co-3MB)

In addition to poly(3HB-co-3MP), *R. eutropha* has also been shown capable of synthesizing poly(3HB-co-3MB) as a sulfur analogue to poly(3HB), when 3MB was provided as substrate (Lütke-Eversloh et al., 2001b) (Figure 4).

Interestingly, the 3HB contents of these PTEs were significantly reduced as compared with those of poly(3HB-co-3MP), which contained only 30 mol% 3HB, or less.

5.3 Biosynthesis of Homopolymers of 3MP, 3MB, and 3MV

In contrast to *R. eutropha*, recombinant nonpolymer-accumulating bacteria could provide the opportunity to synthesize homopolymers, if the respective PHA biosynthesis genes were successfully expressed in such organisms. Currently, a recombinant strain of *Escherichia coli* is under investigation as a biotechnological tool for PTE biosynthesis. Initial studies have indicated that different homopolymers may be synthesized using this *E. coli* strain, consisting of either 3MP, 3MB, or 3MV, if the respective mercaptoalkanoic acids were provided as precursor substrates to the fermentation process. The accumulated PTEs were identified microscopically as hydrophobic inclusions within the cytoplasm, revealing PTE contents of up to 35% of the cellular dry matter. The purified PTEs were analyzed using nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and infrared (IR) spectroscopy (T. Lütke-Eversloh and A. Steinbüchel, unpublished data).

5.4 Biosynthesis of PTEs Consisting of 3MO

In the case of 3MP-, 3MB-, and 3MV-containing polymers, these PTEs consisted of 3MAs with carbon chain lengths of between C₃ and C₅; these are designated as PTEs of short chain length (PTE_SCL), as referred to for the analogous nomenclature of PHAs (Rehm and Steinbüchel, 1999). Other PHA-accumulating bacteria, primarily *Pseudomonas* species, synthesize PHAs of medium chain length (PHA_MCL) due to the substrate specificities of PHA_MCL synthases towards carbon chain lengths from C₆ to C₁₂ (Rehm and Steinbüchel, 1999).
Current investigations have revealed that the PHA_MCL synthase from *Pseudomonas mendocina* catalyzed the incorporation of 3-mercaptooctanoate (3MO) into poly(3MO), which was the first example of PTE_MCL biosynthesis. For this, a metabolically engineered PHA-negative mutant PHB/C0 of *R. eutropha*, which harbors plasmid pBBR1::phaC1 expressing the PHA synthase of *P. mendocina* (Hein et al., 2002), was fed with gluconate and 3MO as substrates (U. Remminghorst, T. Lütke-Eversloh and A. Steinbüchel, unpublished data). So far, the biosynthesis of PTE_MCL has not been shown to occur in wild-type bacteria. Due to the unspecificities of PHA_MCL synthases in general, catalysis of the formation of thioester linkages has been suggested, and has indeed been demonstrated in recombinant bacteria. However, the detection methods used may have limited the discovery of PTE_MCL—possibly due to the predominance of the naturally occurring PHAs—in wild-type strains.

5.5 Isolation and Purification

The PTEs synthesized by *R. eutropha* were identified as water-insoluble granules in the cytoplasm. These granules are most likely coated with a lipid monolayer and different proteins, as in the case of PHA granules (Lundgren et al., 1964; Steinbüchel et al., 1995). The PTEs were isolated using two different methods.

For the isolation of polymers from *R. eutropha*, the cells were lyophilized and subsequently extracted in a soxhlet apparatus using cellulose thimbles in refluxing chloroform. In order to obtain the polymer from the viscous chloroform solution, precipitation with either cold ethanol or methanol was carried out; this resulted in the formation of visible polymer fibers which were separated from the solvent mixture. In order to obtain highly purified PTEs, the precipitation procedure must be repeated several times. The yield of PTE isolation, as well as putative contaminations with compounds such as fatty acids, can be controlled by monitoring the process using gas chromatography (see Section 7).

Due to the poor chloroform-solubility of the homopolythioesters from recombinant *E. coli*, these PTEs were isolated enzymatically. First, the cells were disrupted with sodium dodecylsulfate (SDS) and lysozyme. After treatment with DNaseI, the proteins were digested with proteinase K, and the PTEs obtained were washed several times with water. Finally, the PTEs were extracted with acetone/diethyl ether (2:1, v/v) in order to remove fatty acids (T. Lütke-Eversloh and A. Steinbüchel, unpublished data).

6 Biosynthetic Pathway

Since bacterial PTEs are not synthesized *de novo* from structurally unrelated carbon sources such as fructose, the presence of suitable precursor substrates is necessary. The microbial synthesis of PTEs occurs via the same well-known metabolic pathway by which PHAs are synthesized. Today, many genera of bacteria are known to accumulate PHAs, but *R. eutropha* is presumably the most famous representative, in light of the many academic studies and industrial applications that have been reported (Anderson and Dawes, 1990; Hocking and Marchessault, 1994).

6.1 Metabolic Pathway in *R. eutropha*

*R. eutropha* synthesizes PTEs when 3MAs are provided as precursor substrates. Follow-
ing uptake into the cells, an activation to the corresponding 3MA-coenzyme A thioester is thought to occur, this being most likely catalyzed by an unspecific thiokinase, though a CoA transferase might also fulfill this reaction (Figure 5).

Very few data are available regarding the metabolic fate of 3MAs, but the conversion of 3MP to 3MP-CoA has been demonstrated, for example, in rat heart mitochondria, where it is catalyzed by a medium-chain acyl-CoA synthetase (Cuebas et al., 1985). Strong inhibitory effects of 3MP-CoA on the enzymes of the fatty acid β-oxidation pathway have also been shown (Sabbagh et al., 1985), and this may explain the reduced growth capability of <i>R. eutropha</i> in the presence of 3MAs at concentrations >0.2% (v/v) in the culture broth.

<i>R. eutropha</i> is also able to synthesize 3MP-containing polymers when TDP is provided as a sulfur-containing substrate. The reaction is thought to proceed via the hydrolytic cleavage of TDP into 3MP and 3-hydroxypropionate (3HP), although this putative sulfide hydrolase has not yet been identified. Whilst regarding the metabolic pathway responsible for PTE synthesis in <i>R. eutropha</i>, the PHA biosynthesis system is also used for polymer production, and this can be seen as analogous to that for PHA. However, the most important peculiarity is the catalysis of a completely different linkage type: sulfhydryl groups are covalently bound to car-
bonyl groups, resulting in the formation of thioester linkages.

When using the wild-type strain H16 of *R. eutropha* for biotechnological production, the synthesis of homopolythioesters would appear impossible, and only copolymers of different compositions can be synthesized (see also Table 1). The presence of acetyl-CoA as a central metabolic intermediate also promotes 3HB-CoA biosynthesis, and hence 3HB was found to occur in all polymer samples isolated from *R. eutropha* H16.

### 6.2 Metabolic Pathway in Recombinant *E. coli*

In order to biosynthesize PTE homopolymers, a metabolically engineered *E. coli* was used which harbored a plasmid expressing a butyrate kinase, a phosphotransbutyrylase, and a PHA synthase (Liu and Steinbüchel, 2000). The putative metabolic pathway for PTE biosynthesis in this *E. coli* provided a completely different route, because the precursor substrates were activated via phosphorylation and subsequent conversion to the corresponding 3MA-CoA thioester (Figure 6). Interestingly, these enzymes revealed relatively high unspecificities, since as well as different HAs such as 3HB, 4HB, or 4HV (Liu and Steinbüchel, 2000), several 3MAs were converted by these “estranged” enzymes. So far, poly(3MP), poly(3MB), and poly(3MV) have been produced using this alternative PTE biosynthesis system (T. Lütke-Eversloh and A. Steinbüchel, unpublished data).

### 6.3 PTE<sub>MCL</sub> Biosynthesis in Recombinant *R. eutropha*

The PHA-negative mutant PHB<sup>-4</sup> of *R. eutropha* strain H16 is incapable of synthesizing either PHAs or PTEs due to the defective PHA<sub>MCL</sub> synthase. Transformation of this strain with a plasmid expressing a functional PHA synthase compensates for this mutation, and the ability for polymer synthesis can be re-conferred. Whilst complementation of strain PHB<sup>-4</sup> with a PHA<sub>MCL</sub> synthase resulted in a phenotype similar to the wild-type strain H16, complementation with a PHA<sub>MCL</sub> synthase would result in a separate polymer biosynthesis pathway, which is independent of the 3HB-CoA syn-

### Table 1: Characterization of PTE samples obtained from different fermentations of *R. eutropha*

<table>
<thead>
<tr>
<th>Polymer sample</th>
<th>S-content [wt%]</th>
<th>$M_w$</th>
<th>$M_w/M_n$</th>
<th>$T_c$ [°C]</th>
<th>$T_m$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(3HB-co-4.6 mol% 3MP)</td>
<td>2.20</td>
<td>633,000</td>
<td>2.7</td>
<td>52.2</td>
<td>164.2</td>
</tr>
<tr>
<td>Poly(3HB-co-8.7 mol% 3MP)</td>
<td>4.17</td>
<td>749,000</td>
<td>3.6</td>
<td>52.3</td>
<td>162.9</td>
</tr>
<tr>
<td>Poly(3HB-co-15.8 mol% 3MP)</td>
<td>5.84</td>
<td>470,000</td>
<td>3.5</td>
<td>56.9</td>
<td>163.2</td>
</tr>
<tr>
<td>Poly(3HB-co-34.9 mol% 3MP)</td>
<td>12.88</td>
<td>1,120,000</td>
<td>1.1</td>
<td>50.3</td>
<td>168.6</td>
</tr>
<tr>
<td>Poly(3HB-co-33.2 mol% 3MB)</td>
<td>11.65</td>
<td>790,000</td>
<td>2.1</td>
<td>53.3</td>
<td>168.6</td>
</tr>
<tr>
<td>Poly(3HB-co-61.5 mol% 3MB)</td>
<td>20.52</td>
<td>563,000</td>
<td>2.0</td>
<td>nd</td>
<td>225.0</td>
</tr>
</tbody>
</table>

Poly(3HB-co-3MP) and poly(3HB-co-3MB) were synthesized by *R. eutropha* (Lütke-Eversloh et al., 2001a,b). The elemental sulfur (S-) content was analyzed by the method of Grote and Kerkeler (DIN 51768), and the derived molar fraction of 3MP and 3MB, respectively, were calculated. The weight average molecular mass ($M_w$) and polydispersity ($M_w/M_n$) of the polymers were estimated by gel-permeation chromatography relative to polystyrene standards. The crystallization temperature ($T_c$) and melting temperature ($T_m$) of the polymer samples were determined by differential scanning calorimetry (DSC) (Lütke-Eversloh et al., 2002).