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Microbial degradation of natural and of new synthetic polymers

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1. SUMMARY

In landfills, deposited waste material is usually faced with strictly anoxic conditions. This means that the design of new biodegradable polymers must take into consideration that degradation should be possible especially in the absence of molecular oxygen. Poly-β-hydroxybutyrate is depolymerized by the anaerobic fermenting bacterium *Ilyobacter delafieldii* through an extracellular hydrolase. Monomers are degraded inside the cells through classical β-oxidation. Polyalkanoates containing odd-numbered or branched-chain acid monomers should he degraded in an analogous manner; in most cases the final mineralization of these residues requires special pathways. A comparison of the chemistry of natural polymer biodegradation leads to the conclusion that synthetic biodegradable polymers should be designed in the future to contain linkages which can be cleaved by extracellular hydrolytic enzymes. Recent findings on aerobic and anaerobic bacterial degradation of synthetic polyethers suggest that natural evolution of new depolymerizing enzymes, perhaps from existing hydrolases, could be possible in a reasonable amount of time, provided that the monomers are likely energy sources for a broad variety of microbes.

2. INTRODUCTION

In nature as well as in synthetic chemistry, structural components are made up of polymers that can be rigid or flexible, and it is the aim of the constructor to arrange them in a way that prevents microbial attack for a maximum of time. Living plant cells are surrounded by polysaccharides which give the cell its shape and also protect it from attack from saprophytes or pathogens. The enormous variability of polysaccharide structures and the high specialization of polymer-degrading enzymes mean that polymeric plant tissues, stable for the life span of the plant, can be degraded entirely after plant death.

Biodegradable synthetic polymers are expected to fulfil similar functions: they should be rigid for the period of use, and they should decompose once the material is no longer needed. Biodegradation as opposed to chemical or physical waste treatment has the advantage that such systems usually do not require complicated processing of the material, and that the degradation process itself is autoregulatory and does not need to be controlled in any way. The compost heap is an example of such a microbial waste degradation

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plant that is easy to maintain and produces a useful raw material from wastes through an enormous variety of degradative activities.

Approaches to biodegradation of synthetic organic polymers can include the time-dependent or laboratory-mediated evolution of enzymes or pathways able to deal with novel xenobiotic compounds. An alternative approach is to design and use polymers which are constructed from the very beginning to be susceptible to microbial attack. Paper (and paper products) has long been a biodegradable polymer able to be utilized for many purposes, produced from renewable resources, and able to be degraded by existing microbial activities.

Nearly no natural compound produced by plant or animal cells exists that cannot be degraded through the action of bacteria or fungi. The latter are important in the degradation of ligninaceous or otherwise structured polymeric materials such as tissues, leather, wood, paper etc. [1]. Due to their organization in mycelia, they can transport nutrients over long distances, thus enabling degradative activities in microhabitats of insufficient nutrient supply. It should not be forgotten that, especially in soil, higher animals such as collembolons, ants, termites, woodlice, and snails also contribute to the overall degradation process by grinding or mechanically destroying supramolecular particles.

In contrast to the compost heap, landfills are nearly entirely anoxic habitats in which a complex microbial community degrades the various substrates mainly by fermentation, usually coupled to methane formation as the terminal process. Therefore, the construction of biodegradable synthetics should aim to allow an oxygen-independent degradation which could operate in such a system as well. This concerns not only the depolymerization step itself, but also the terminal mineralization of the monomers formed. The limits and principles of anaerobic degradation activities have recently been summarized elsewhere [2,3] and the reader is referred to the references in these and other review papers.

Here we want to summarize some aspects of our present knowledge on the biodegradation of natural and some synthetic polymers and to identify the limits and challenges of such degradative activities. We present some new data on fermentative degradation of PHB by a strictly anaerobic bacterium, as well as some considerations on polymer biodegradation strategies on the basis of anaerobic polyethylene glycol degradation studied in our laboratory.

3. CHEMISTRY OF MICROBIAL CLEAVAGE REACTIONS

The most important type of enzymatic polymer cleavage reaction is hydrolysis. Especially glycosidic bonds, but ester and peptide linkages as well, are subject to hydrolysis through nucleophilic attack on the carbonyl carbon atom. Polysaccharides, fats, PHB, gelatin, keratin etc., but also the synthetic polymers polylactate or polymalate [4], are all degraded through such reactions. Most bacterial or fungal hydrolases are very specific and react only with very few substrates of very similar chemical structure. Since the outer layers of bacterial and fungal cells are made of polysaccharides as well, non-specific polysaccharide-hydrolysing enzymes would be a hazard for the whole microbial degradative community. Polymer-degrading hydrolases are excreted by the producing cell into the surrounding milieu to allow direct contact with the polymeric substrates. Such hydrolases could basically also attack synthetic polyesters, polyamides, or polyurethanes – provided that the enzymes were less specific or modified, and the substrate sufficiently easily accessible.

Ether linkages are cleaved by aerobic bacteria through monooxygenase reactions which transform the comparably stable ether linkage through hydroxylation to a hemiacetal structure of low stability [5,6]. By similar hydroxylations, alkyl–nitrogen linkages are converted into aldimine hydrates which decompose easily in water, e.g. in aerobic breakdown of nitrilotriacetate [7]. Monooxygenases also oxidize long-chain hydrocarbons to primary or secondary alcohols; the former ones are easily mineralized further. Such enzymes should basically also be able to attack polyethylenes and polystyrenes. However, mono-

oxygenase reactions depend on reduced electron donors as cofactors which have to be regenerated by the cellular metabolism. This means that such enzymes are found (so far as is known) only inside microbial cells and are apparently not excreted into the surrounding medium. Unfortunately, the only report existing on polystyrene degradation does not allow reliable conclusions with respect to the underlying depolymerization reactions [8].

A special case of oxygen-dependent decomposition is lignin degradation by white-rot fungi. For cleavage of the extremely heterogenous assembly of linkages in this natural polymer, the organisms produce extracellularly via hydrogen peroxide a highly reactive low-molecular radical which splits the various C–C or C–O–C linkages in a random and very non-specific manner [9]. It should be emphasized that creation of the radical requires a lot of bioenergetic input by the fungus which is barely covered by the subsequent mineralization of the breakdown products.

An alternative cleavage reaction for ethers and alkyl–nitrogen linkages is observed in nitrate-dependent degradation of polyethylene glycol [10] or nitrilotriacetate [11]: oxygen-independent dehydrogenations and water additions lead to formation of aldehydes through mechanisms that are so far not entirely understood. Strictly anaerobic fermenting and sulfate-reducing bacteria are able to convert the ether linkage in polyethylene glycol through a corrinoid-dependent hydroxyl shift reaction into an unstable hemiacetal linkage which releases acetaldehyde as first identifiable product [12,13]. In a similar manner, triethanolamine, but not triethylamine or nitrilotriacetate, are cleaved and degraded by strictly anaerobic bacteria (unpublished results from our lab).

4. CONDITIONS FOR MICROBIAL DEGRADATION REACTIONS

Most cleavage enzymes mentioned above (monooxygenases, dehydrogenases, corrinoid-dependent enzymes) require co-substrates for reaction and operate therefore exclusively inside microbial cells. They are thus not suitable for degradation of high molecular mass polymers which would need to enter the cytoplasm before depolymerization. Exceptions are pyrroloquinoline quinone-dependent dehydrogenases which can be localized outside the cell in the periplasmic space; however, such enzymes have so far been found to react only with low molecular mass substrates [14]. For degradation of polymeric synthetics, it would therefore be more advisable to employ preferentially hydrolytic enzymes. The extracellular lignin-degrading enzymatic apparatus of the white-rot fungi also appears to be a suitable mechanism, but requires molecular oxygen for activity.

For self-regulation of a biodegradation process, it is important that the microorganisms catalysing it have also some advantage from it. There will be no positive increase of activity with increasing polymer availability if the polymer-degrading (e.g. hydrolase-producing) bacterium cannot make any use of the monomers produced. Many monomers of synthetic polyesters and polyamines (higher dicarboxylic acids of 6 and more carbon atoms, diamines, Ω-aminocarbonic acids, Ω-hydroxocarbonic acids) can be degraded and mineralized by microorganisms, but only by a few specialists. For instance, only recently were bacteria isolated and described which can fermentatively degrade diamines such as putrescine [15] or higher dicarboxylic acids ([16]; Matthies and Schink, in preparation). It cannot be expected that such bacteria, which can hardly grow at the expense of the monomeric substrates, will produce hydrolases for degradation of an artificial polymer that does not exist in nature. Under these circumstances, there will be no positive selection for polymer degradation even if such reactions are basically possible.

For optimization of microbial degradation it has to be postulated finally that hydrolysing enzymes or the microorganisms involved can come into intimate contact with the substrate. This requires that the substrate is sufficiently wettable to allow direct contact in an aqueous phase. Furthermore, the substrate should be structured in such a way as to allow access to single polymer strands for processing. This is a well-known problem in natural cellulose degradation: disperse

areas of cellulose tissue and technically pretreated celluloses are much more rapidly degraded than tightly packed, crystalline substrate domains [17].

5. ENZYMES INVOLVED IN FERMENTATIVE PHB DEGRADATION

Degradation of PHB has so far been studied only with aerobic bacteria, e.g. *Pseudomonas lemoignei* and *Alcaligenes* spp. The immediate products of polymer hydrolysis are monomeric 3-hydroxybutyrate, or mixtures of monomer, dimer, and trimer [18–21]. In addition, enzymes capable of hydrolysing dimers and trimers have also been found [22,23].

The recently isolated *Ilyobacter delafieldii* can grow fermentatively with PHB, producing acetate, butyrate and hydrogen [24]. An extracellular PHB hydrolase was produced only after growth with PHB; no activity was detected in cultures grown with 3-hydroxybutyrate, crotonate, pyruvate, or lactate. The PHB depolymerase activity was largely associated with the PHB granules supplied as growth substrate, and only 16% was detected free in the culture supernatant. In this respect, the enzyme behaves similar to that of *Pseudomonas lemoignei* [25]. Monomeric 3-hydroxybutyrate was detectable as an end-product of depolymerase activity. However, it is still open whether only monomers are released or whether dimers and trimers appear as primary but intermediate depolymerization products.

After activation of 3-hydroxybutyrate by CoA transfer from acetyl-CoA or butyryl-CoA, the resultant 3-hydroxybutyryl-CoA was oxidized to acetoacetyl-CoA and cleaved to acetyl-CoA; ATP was formed via phosphotransacetylase and acetate kinase reactions. The reducing equivalents primarily transferred to NAD could be released as free hydrogen, or used in reductive butyrate formation via crotonyl-CoA. In mixed cultures with hydrogen-utilizing sulfate reducers, the ratio of acetate over butyrate formation increased significantly as compared to the pure culture. Bacteria similar to *I. delafieldii* could, therefore, well be the primary fermenting actors in complete methanogenic PHB degradation reported recently [26]; we have observed methanogenic PHB degradation in several enrichment cultures, especially in those obtained from sewage sludge (Janssen, unpublished).

It appears that anaerobic PHB degradation follows basically the same strategy as aerobic degradation does, and it is obvious that PHB degradation by the isolated fermenting bacteria could be an efficient means of dissolving PHB in anoxic environments such as sanitary landfills.

6. PATHWAYS OF ANAEROBIC DEGRADATION OF ODD-NUMBERED OR BRANCHED-CHAIN FATTY ACIDS

PHAs containing odd-numbered or branched-chain fatty acids have been synthesized biochemically, mainly by feeding such fatty acid residues to PHB-polymerizing bacteria, e.g. under conditions of nitrogen limitation [27]. Hydrolytic cleavage of these polymers will lead to the corresponding 3-hydroxy fatty acids which, after subsequent β-oxidation, will give rise to the formation of odd-numbered and branched-chain fatty acids, although, to our knowledge, studies on this matter are lacking so far. Odd-numbered fatty acids form propionate as terminal product which is oxidized in nearly all cases, including syntrophic methanogenic associations [28,29], through the methylmalonyl-CoA pathway.

Further degradation of the branched fatty acid residues produced should basically proceed through known pathways as well. We have studied the anaerobic degradation of branched-chain fatty acids by fermenting and by sulfate-reducing bacteria. The pathway of neovalerate degradation is a usual β-oxidation which produces propionate from the last acid residue [30]. Anaerobic isovalerate degradation is much more complicated and was studied with a fermenting bacterium. Degradation involves activation to the CoA-derivative, dehydrogenation and carboxylation to 3-hydroxy-3-methylglutaryl-CoA which yields acetyl-CoA and acetoacetate [31]. These bacteria get very little energy from the overall process, grow very slowly (doubling time about 1 week under

optimal conditions) and depend on cooperation with hydrogen-oxidizing methanogenic bacteria. Isobutyrate is degraded anaerobically through two different pathways: whereas sulfate-dependent oxidation follows the pathway basically known from aerobic degradation [32], methanogenic degradation involves isomerization of isobutyrate to butyrate, and subsequent syntrophic oxidation to two acetate residues [32]. The isomerization of isobutyrate to butyrate has been studied recently in our lab with a pure culture of a glutarate-fermenting bacterium. The reaction acts on the CoA-derivatives and requires coenzyme B_{12} as cofactor [33]. Thus, anaerobic degradation pathways are known for all three major types of branched-chain fatty acids, but these degradation processes are slow compared to degradation of straight-chain fatty acids.

7. CONCLUSIONS

It appears from the above considerations that synthetic polymers designed to be biodegradable should be composed from units which are linked by bonds that can be cleaved outside the bacterial cell by hydrolytic enzymes. Such enzymes must be excreted into the medium to be effective in depolymerization. Moreover, the chemical structure of the polymer should be similar to that of natural polymers to enhance the chance of evolution of a polymer-degrading enzyme. The resulting oligo- and monomers should be an easy-to-use growth substrate for a broad variety of bacteria to ensure that production of suitable hydrolytic enzymes is induced in as many different types of bacteria as possible, and to ensure a positive regulation of enzyme production in a natural or semi-natural habitat. Only under such conditions will a positive selection for production of unspecific hydrolytic enzymes be possible. One could speculate that production of such enzymes could be promoted in the laboratory by mutative treatment of a broad variety of bacteria under substrate limitation, e.g. in a chemostat with a synthetic polymer as sole substrate.

The chances for evolution of such new degradative capacities seem to be surprisingly high. We reported above on degradation of the polyether polyethylene glycol (PEG) by several different metabolic types of aerobic and anaerobic bacteria, through at least two entirely different metabolic pathways. Since PEG has been present in the environment in significant amounts for about 40 years only, both degradative pathways must have evolved in a comparably short time. For the anaerobic, corrinoid-dependent pathway, it can be speculated that it was derived from a preexisting diol dehydratase enzyme. However, the polymer-degrading enzyme is definitively different from the typical diol dehydratase, and we could not produce polymer-degrading mutants from ethylene glycol-utilizing wild-type cells by treatment with usual mutagens. Thus, evolution of the PEG-degrading enzyme requires more than one point mutation to evolve from diol dehydratase. Moreover, a polymer uptake system must have evolved in the same time to allow degradation by the intracellular ether-cleaving enzyme, since PEG cannot diffuse through a phospholipid membrane at sufficient rates. To make the total degradation efficient, both systems must have evolved together. The occurrence of this capacity in a broad variety of anaerobic bacteria indicates that this combination may even have evolved more than once in this comparably short time of exposure to PEG.

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REFERENCES

[1] Schmidt, O. and Kerner-Gang, W. (1986) Natural materials. In: Biotechnology (H.-J. Rehm, G. Reed, Eds.), Vol. 8, pp. 557–582. Verlag Chemie, Weinheim.

[2] Zehnder, A.J.B. and Stumm, W. (1988) Geochemistry and biogeochemistry of anaerobic habitats. In: Biology of Anaerobic Microorganisms (A.J.B. Zehnder, Ed.), pp. 1–38. John Wiley and Sons, New York, NY.

[3] Schink, B. (1988) Principles and limits of anaerobic degradation: environmental and technological aspects. In: Biology of Anaerobic Microorganisms (A.J.B. Zehnder, Ed.), pp. 771–846. John Wiley & Sons, New York, NY.
[4] Hutchinson, F.G. and Furr, B.J.A. (1987) Biodegradable carriers for the sustained release of polypeptides. Trends Biotechnol. 5, 102–106.
[5] Bernhardt, F.-H., Staudinger, H. and Ullrich, V. (1970) Eigenschaften einer *p*-Anisat-O-Demethylase im zellfreien Extrakt von *Pseudomonas* species. Hoppe-Seyler's Z. Physiol. Chem. 351, 467–478.
[6] Stirling, D.I. and Dalton, H. (1980) Oxidation of dimethyl ether, methylformate and bromomethane by *Methylococcus capsulatus* (Bath). J. Gen. Microbiol. 116, 277–283.
[7] Cripps, R.E. and Noble, A.S. (1973) The metabolism of nitrilotriacetate by a pseudomonad. Biochem. Z. 136, 1059–1068.
[8] Tsuchii, A., Suzuki, T. and Takahara, Y. (1977) Microbial degradation of styrene oligomers. Agric. Biol. Chem. 41, 2417–2421.
[9] Kirk, T.K. and Farrell, R.L. (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–505.
[10] Kawai, F. (1987) The biochemistry of degradation of polyethers. CRC Crit. Rev. Biotechnol. 6, 273–307.
[11] Egli, Th., Bally, M. and Uetz, T.H. (1990) Microbial degradation of chelating agents used in detergents with special reference to nitrilotriacetic acid (NTA). Biodegradation 1, 121–132.
[12] Schramm, E. and Schink, B. (1991) Ether-cleaving enzyme and diol dehydratase involved in anaerobic polyethylene glycol degradation by an *Acetobacterium* sp. Biodegradation 2, 71–79.
[13] Frings, J., Schramm, E. and Schink, B. (1992) Enzymes involved in anaerobic polyethylene glycol degradation by *Pelobacter venetianus* and *Bacteroides* strain PG1. Appl. Environ. Microbiol. 58, 2164–2167.
[14] Duine, J.A., Frank Jzn, J. and Jongejan, J.A. (1986) PQQ and quinoprotein enzymes in microbial oxidations. FEMS Microbiol. Rev. 32, 165–178.
[15] Matthies, C., Mayer, F. and Schink, B. (1989) Fermentative degradation of putrescine by new strictly anaerobic bacteria. Arch. Microbiol. 151, 498–505.
[16] Matthies, C. and Schink, B. (1992) Fermentative degradation of glutarate via decarboxylation by newly isolated anaerobic bacteria. Arch. Microbiol. 157, 290–296.
[17] Colberg, P. (1988) Anaerobic microbial degradation of cellulose, lignin, oligolignols, and monoaromatic lignin derivatives. In: Biology of Anaerobic Microorganisms (A.J.B. Zehnder, Ed.), pp. 333–372. John Wiley & Sons, New York, NY.
[18] Chowdhury, A.A. (1963) Poly-β-hydroxybuttersäure abbauende Bakterien und Exoenzym. Arch. Mikrobiol. 47, 167–200.
[19] Delafield, F.P., Doudoroff, M., Palleroni, N.J., Lusty, C.J. and Contopoulos, R. (1965) Decomposition of poly-β-hydroxybutyrate by pseudomonads. J. Bacteriol. 90, 1455–1466.
[20] Merrick, J.M., Delafield, F.P. and Doudoroff, M. (1962) Hydrolysis of poly-β-hydroxybutyric acid in bacteria. Fed. Proc. 21, 228.
[21] Tanio, T., Fukui, T., Saito, T., Tomita, K., Kaiho, T. and Masamu, N.E. (1982) An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Eur. J. Biochem. 124, 71–77.
[22] Delafield, F.P., Cooksey, K.E. and Doudoroff, M. (1965) β-Hydroxybutyric acid dehydrogenase and dimer hydrolase of *Pseudomonas lemoignei*. J. Biol. Chem. 240, 4023–4028.
[23] Shirakura, Y., Fukui, T., Tanio, T., Nakayama, K., Matsuno, R. and Tomita, K. (1983) An extracellular D(−)-3-hydroxybutyrate oligomer hydrolase from *Alcaligenes faecalis*. Biochim. Biophys. Acta 748, 331–339.
[24] Janssen, P.H. and Harfoot, C.G. (1990) *Ilyobacter delafieldii* sp. nov., a metabolically restricted anaerobic bacterium fermenting PHB. Arch. Microbiol. 154, 253–259.
[25] Lusty, C.J. and Doudoroff, M. (1966) Poly-β-hydroxybutyrate depolymerase of *Pseudomonas lemoignei*. Proc. Natl. Acad. Sci. USA 56, 960–965.
[26] Budwill, K., Fedorak, P.M. and Page, W.J. (1992) Methanogenic degradation of poly(3-hydroxyalkanoates). Appl. Environ. Microbiol. 58, 1398–1401.
[27] Fritzsche, K., Lenz, R.W. and Fuller, R.C. (1990) Bacterial polyesters containing branched poly(β-hydroxyalkanoate) units. Int. J. Biol. Macromol. 12, 92–101.
[28] Koch, M.E., Dolfing, J., Wuhrmann, K. and Zehnder, A.J.B. (1983) Pathways of propionate degradation by enriched methanogenic cocultures. Appl. Environ. Microbiol. 45, 1411–1414.
[29] Schink, B. (1985) Mechanisms and kinetics of succinate and propionate degradation in anoxic freshwater sediments and sewage sludge. J. Gen. Microbiol. 131, 79–87.
[30] Stieb, M. and Schink, B. (1985) Anaerobic degradation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Arch. Microbiol. 140, 387–390.
[31] Stieb, M. and Schink, B. (1986) Anaerobic degradation of isovalerate by a defined methanogenic coculture. Arch. Microbiol. 144, 291–295.
[32] Stieb, M. and Schink, B. (1989) Degradation of isobutyrate by methanogenic enrichment cultures and by pure cultures of a *Desulfococcus multivorans* strain. Arch. Microbiol. 151, 126–132.
[33] Matthies, C. and Schink, B. (1992) Reciprocal isomerization of butyrate and isobutyrate by the strictly anaerobic bacterium strain WoG13 and methanogenic isobutyrate degradation by a defined triculture. Appl. Environ. Microbiol. 58, 1435–1439.