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Sphingomonads involved in the biodegradation of xenobiotic polymers

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Sphingomonads involved in the microbial degradation of xenobiotic polymers are introduced. The metabolism of polyethylene glycol was the primary focus of the study. Several others, including polyvinyl alcohol, polyethylene and polyaspartate were also studied. It is suggested that these xenobiotic polymers are metabolized by intracellular enzymes located in the periplasmic space or bound to membranes, indicating that transport of these polymers through outer membranes is requisite for their metabolism. Involvement of specific membrane structures of sphingomonads such as unusual sphingolipids is suggested for membrane transport of xenobiotic compounds, especially hydrophobic materials.

Keywords: sphingomonads; xenobiotic polymers; polyethylene glycol; membrane transport; periplasmic enzymes

Introduction

Interest in environmental issues is still growing and there is increasing demand to develop materials which do not burden the environment significantly. Awareness of the waste problem and its impact on the environment has awakened new interest in the area of degradable polymers. Biodegradation is necessary for water-soluble or water-miscible polymers because they eventually enter streams, which can neither be recycled nor incinerated. Even for water-insoluble polymers, so-called plastics, biodegradability becomes more and more important, because many can not be recycled or incinerated, but remain in the environment.

Studies on biodegradation of synthetic polymers and oligomers have shown close relationships between biodegradability and chemical structure. The synthetic polymers that biodegrade tend to have structures similar to those found in naturally occurring polymers, suggesting that microbial populations produce enzymes that do not discriminate between polymers of similar structure [16]. Examples of synthetic polymers that biodegrade include poly(vinyl alcohol); polyethers, among which polyethylene glycols are manufactured in the largest quantities; poly(lactic acid); aliphatic polyesters; polycaprolactone; and polyamides. Several oligomeric structures which biodegrade are known: oligomeric ethylene, styrene, isoprene, butadiene, acrylonitrile, and acrylate. Hydrolysis and oxidation are the primary processes involved in polymer degradation [19].

Depolymerization processes for polymers can be divided into two groups: endogenous and exogenous. Polymers having hydrolyzable groups on the polymer chains are endogenously hydrolyzed at random, resulting in much faster depolymerization than polymers without hydrolyzable groups. Examples are polyesters, polycaprolactone, polyamides and polylactic acid; they are hydrolyzed by esterases and other hydrolases. These hydrolases are produced extracellularly and solubilize a solid block of synthetic polymers with high molecular weights into low molecular weight compounds, which can be incorporated into cells as carbon and energy sources. Polyethers are depolymerized oxidatively and exogenously by C_2-unit liberation [14]. Poly(vinyl alcohol) is a unique polymer the degradation of which is carried out by primary oxidation and secondary hydrolysis. However, depolymerization is quite different from assimilation of polymers. Assimilation is the incorporation process of low molecular weight products produced by depolymerization into the central metabolic pathways, yielding energy and recycling depolymerized products into cell constituents.

As described above, hydrolytic processes are endogenously catalyzed by hydrolases, which are often found extracellularly, resulting in an easy attack on polymers, irrespective of their molecular size. On the other hand, oxidation processes are often linked with respiratory and energy-producing systems of degradative bacteria. Respiratory systems are generally membrane-bound and consequently most oxidoreductases linked with them are likewise membrane-bound. In these cases, polymers have to make contact with membrane-bound enzymes in the periplasmic spaces of Gram-negative bacteria. The fact that no Gram-positive bacteria have been reported to be involved in this type of degradation is likely due to the differences of cell surface structures between Gram-negative and -positive bacteria as well as their lack of appropriate metabolic enzymes. Many fungi are reported to produce extracellular enzymes (mostly hydrolases) which degrade xenobiotic and biotic polymers, but there are no reports on other eucaryotic cells that are capable of intracellular degradation of polymers.

The fact that sphingomonads are responsible for the metabolism of xenobiotic hydrophobic compounds and some polymers strongly suggests an interrelationship between penetration of the compounds and the specific cell membrane structure. The genus *Sphingomonas* was proposed by Yabuuchi *et al* [45] for Gram-negative yellow-pigmented rods which are motile with single polar flagella,

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and which are nonmotile and nonfermentative. Sphingomonads contain a unique sphingoglycolipid with the long-chain base, dihydrosphingosin, in the outer membranes, instead of the lipopolysaccharides found in most Gram-negative bacteria.

In this paper, I will introduce sphingomonads involved in the biodegradation of polymers. As sphingomonads metabolize polymers intracellularly in their periplasmic space, their specific membrane structures are possibly involved in the transport of xenobiotic polymers through bacterial outer membranes.

Microbial degradation of polyethylene glycols

Polyethylene glycols [PEGs, $H(OCH_2\text{-}CH_2)_nOH$)] are important constituents of many industrial products (eg nonionic surfactants) which after use may ultimately be found in wastewater. In principle, the properties of PEG impose large constraints on enzymatic degradation, since it is a large rather hydrophilic molecule (most applications require a large size molecule), the membranes of the organism form a barrier for substrate uptake, and the size of the active site of the enzyme attacking PEG must be rather large. Since PEG when dissolved in water has a random coil formation, the alcohol groups at the termini are randomly distributed in the space taken up by the macromolecule, resulting in difficulty in making contact with the enzyme. From a chemical point of view, aliphatic ether bonds are very stable. Moreover, PEG is a true xenobiotic compound since no close relatives exist in nature [2,14].

Ogata *et al* [29] isolated many Gram-negative PEG-utilizing bacteria, which were divided into several groups according to their ability to utilize different molecular weight ranges of PEGs: PEG 400, 600, 1000, 4000 and 20 000. PEG 400–1000 was degraded by a variety of Gram-negative bacteria, eg *Pseudomonas vesicularis*, *P. solanacearum*, *Alcaligenes xylosoxidans* and *Enterobacter diversus* [41]. PEGs with average molecular weights of more than 4000 were specifically assimilated by two new species of *Sphingomonas*. One was a PEG 4000-utilizer and was identified as *Sphingomonas macrogoltabidus* and another was a PEG 20 000-utilizing *Sphingomonas terrae* (Figure 1) [41]. *S. terrae* was able to assimilate PEG in coculture with associates such as *Rhizobium* sp, *Agrobacterium* sp, and *Methylobacterium* sp. The symbiosis between *S. terrae* and concomitant associates was analyzed: *S. terrae* has all the enzymes necessary for the metabolism of PEG, but its growth is inhibited by glyoxylate at an early stage of cultivation, although glyoxylate is an end product introduced into the central metabolic pathways, as shown in Figure 2 [13]. This could be explained by the fact that one mole of PEG 6000 (average molecular weight of 8000) yields approximately 200 moles of glyoxylate, which accumulates in the medium and can be toxic to cells. Glyoxylate was removed faster by the concomitant associates than by *S. terrae*, resulting in the efficient degradation of PEG.

Interestingly, PEG-phthalate polyester-utilizing consortia were independently obtained by enrichment culture and were composed of a phthalate ester-utilizing *Comamonas acidovorans* and a PEG-utilizing *Sphingomonas* sp [17,18], respectively, as shown in Figure 3. Newly isolated *Sphingomonas* sp K-1 assimilated PEG up to 4000 and *Sphingomonas* sp N-6 assimilated PEG up to 20 000. This is the first report that a pure culture of *Sphingomonas* can assimilate PEG up to 20 000 [18]. These sphingomonads did not produce any extracellular enzymes, but their washed cells degraded PEG, suggesting that PEG is incorporated into cells and degradation occurs in the periplasmic space. The transport properties of the outer-membrane most probably determine the maximum size of the PEGs admitted for metabolism (Figure 4) [17].

On PEG media, the sphingomonads exhibited different cell sizes, cell surface structures, physiological characteristics (susceptibility to lysozyme, Congo Red staining etc) and cell compositions from those grown on nutrient broth (unpublished data). This might strongly suggest the adaptation of the organisms to PEGs with respect not only to enzyme induction, but also to their membrane structures and transport systems. Three kinds of enzymes are involved in aerobic metabolism of PEG: PEG dehydrogenase, PEG-aldehyde dehydrogenase and PEG-carboxylate dehydrogenase (ether-cleaving) [11–15]. All of these enzymes are membrane-bound and can be solubilized by surfactants, suggesting that substrates have to pass the outer membrane to make contact with the enzymes. PEG dehydrogenases were purified from *S. terrae* [11] and *S. macrogoltabidus* [46]. Originally the enzyme was claimed to be a quinoprotein. However, the gene for the enzyme from *S. terrae* was successfully expressed in *E. coli*, (unpublished data) which does not produce PQQ. No PQQ could be detected in the purified preparations and no stimulation of the activity by addition of PQQ was observed. Instead, flavin was extracted from the cloned enzyme and was identified as FAD (unpublished). Interestingly, quinohemoprotein alcohol dehydrogenase isolated from *Comamonas testosteroni* [2,7], *Rhodopseudomonas acidophila* [47] and *Ralstonia eutropha* [48], which are not able to grow on PEGs, was able to oxidize PEGs. Thus, PEG seems to be a non-specific substrate for primary alcohol dehydrogenases. Adventitious oxidation of PEG could take place with any alcohol dehydrogenase having an active site of adequate size for PEG molecules.

Unexpectedly, substrate specificities of PEG dehydrogenases towards PEGs are not in accordance with growth substrate specificities of PEG-utilizing bacteria [15]. Even crude extracts of PEG-utilizing bacteria, which can grow on PEG 400, 1000 and 4000, but not on PEG 6000 and higher, dehydrogenated PEG 6000 or higher. The restrictions for a certain bacterium with respect to the size of the PEG which can be used is not caused by limitations of the active site of the degrading enzymes, but by transport through the outer membrane.

PEG-carboxylate dehydrogenase (ether-cleaving) acted on diglycolic acid and was designated as DGA dehydrogenase. It oxidized the terminal unit of PEG-carboxylic acids (ie the glycolic acid moiety), leading to ether bond splitting and the formation of glyoxylate plus PEG_{n-1}. The enzyme was purified from *S. terrae* [3], but the identity of its cofactor(s), and the substrate oxidation mechanism are not known. Apparently, the presence of a terminal carboxylic acid group is crucial for oxidation to occur at the α-

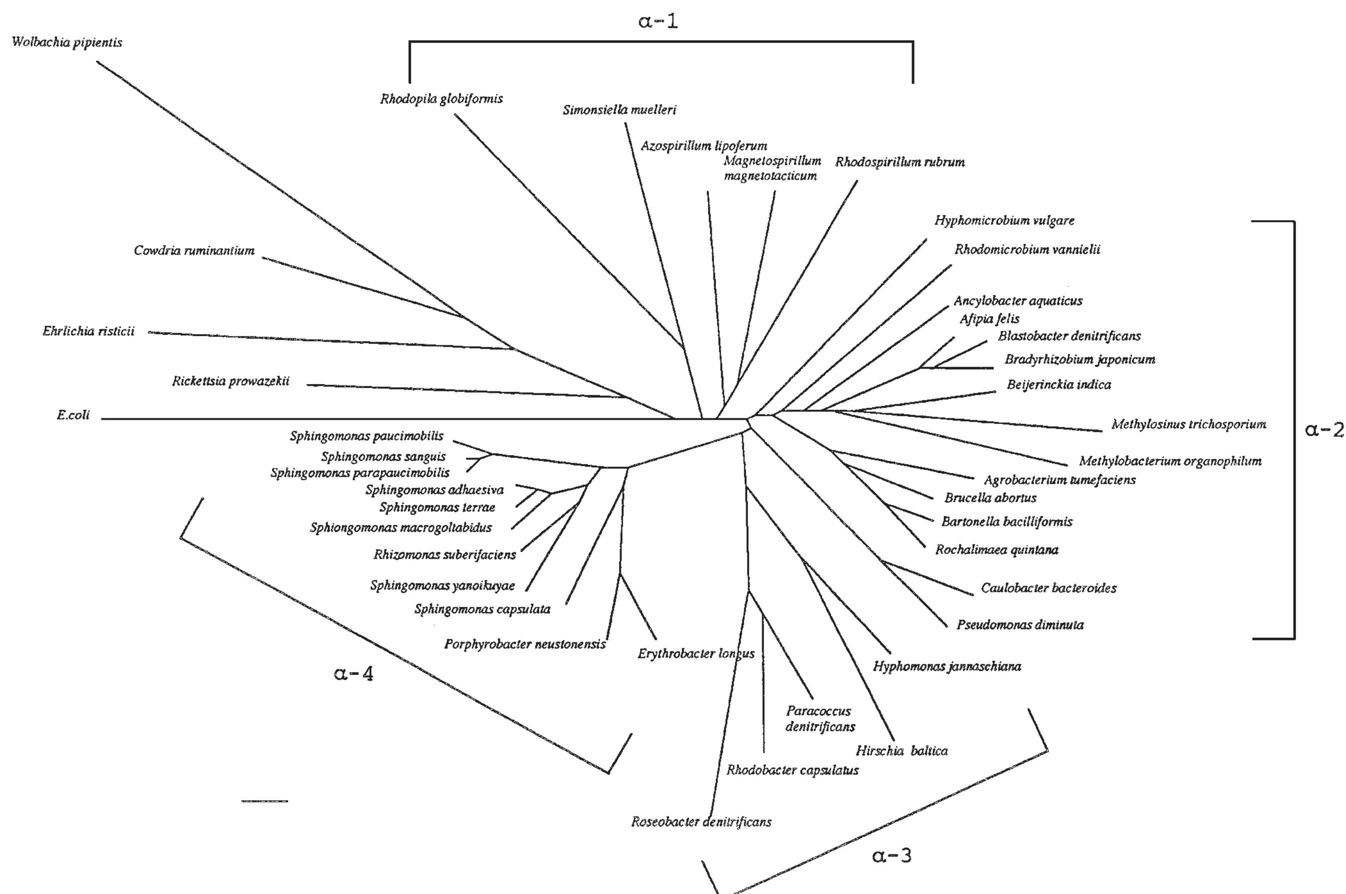

Figure 1 Physlogenetic tree showing the relationships of *Sphingomonas* species and other members of the alpha subclass of the *Proteobacteria* [42]. Bar = 0.01 K_{nuc} unit.

position. However, the presence of an ether bond is not essential since glyoxylic and glycolic acids as well as DGA are also substrates. Therefore, the oxidation mechanism could be similar to that of the peroxisomal, flavoprotein glycolate oxidase (EC 1.1.3.1) or to that of flavoprotein α-hydroxy acid dehydrogenases, proceeding *via* hydride transfer or *via* deprotonation, followed by electron transfer or covalent catalysis taking place in the adduct formed from the (carbanion) substrate molecule and the cofactor. Since no O_2 consumption has been detected in the conversion of PEG-dicarboxylic acid by DGA dehydrogenase *in vitro*, the process must be accompanied by attack of an H_2O molecule so that rearrangement of the oxidized ether bond can take place with formation of a terminal aldehyde group (in glyoxylate) and a terminal alcohol group (in PEG_{n-1}).

Several other aerobic bacteria (mostly *Pseudomonas* spp) are able to metabolize larger PEGs. Several metabolic pathways have been proposed, but detailed studies have mostly been done using sphingomonads [2,14,16].

Biodegradation of other polymers

Biodegradation of xenobiotic polymers by sphingomonads has been studied almost exclusively with PEGs. However, several other examples have recently appeared. In this section, new sphingomonads are introduced and their metabolic enzymes are discussed.

Polyvinyl alcohol (PVA)

PVA is produced in large quantities and widely used in industry. PVA is the only carbon–carbon backbone polymer that is biodegradable and has recently attracted attention as a water-soluble biodegradable polymer. Two distinct oxidative degradation pathways have been demonstrated for the metabolism of PVA, as shown in Figure 5. One of the pathways is a combination of a secondary alcohol oxidase (PVA oxidase) and a β-diketone hydrolase; both enzymes were purified and characterized by Sakai *et al* [24,31,44]. PVA oxidase is an extracellular enzyme and is able to depolymerize PVA together with β-diketone hydrolase. The other pathway in *Pseudomones* sp VM15C, is the combination of a PQQ-dependent PVA dehydrogenase and a hydrolase [32,37]. PVA dehydrogenase is membrane-bound, but it has not yet been purified. Its gene was cloned and expressed in *E. coli* [38]. More recently, Hatanaka *et al* isolated *Pseudomonas* sp 113P3 as a PVA-utilizing bacterium, which required PQQ as an essential growth factor and produced a high level of PVA dehydrogenase [6]. PVA dehydrogenase was purified from cell-free extracts [6]; the enzyme was soluble and periplasmic (personal communications from Dr Tsuji, a coauthor for [6]). As the microbe did not produce any PVA oxidase which contributes to depolymerization of PVA, the original PVA molecules (polymerization degree of 1700 and a corresponding average molecular weight of 76 500) need to enter into the

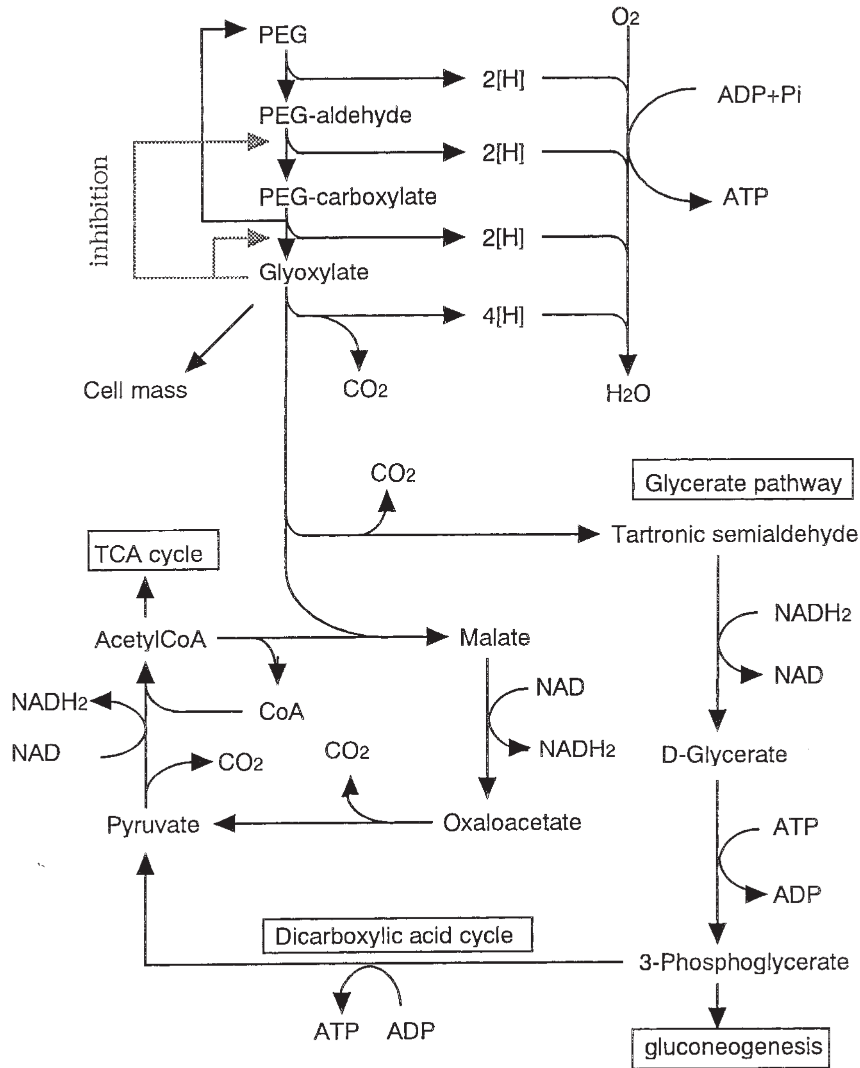

Figure 2 Metabolism of PEG by aerobic bacteria.

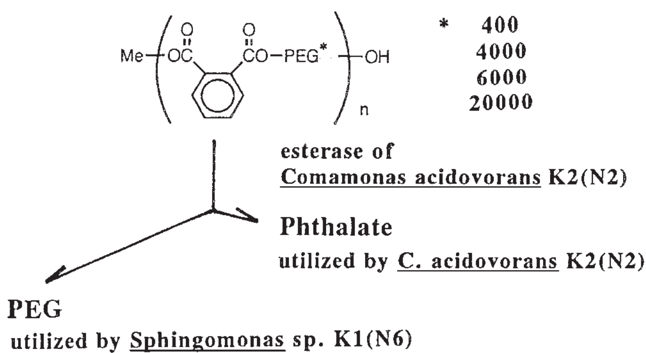

Figure 3 Symbiotic degradation of PEG 20 000-phthalate polyester by phthalate ester- and PEG 20 000-utilizing bacteria.

periplasmic space to make contact with the enzyme. PVA having ethylene blocks introduced in their structures showed much higher V_{max}/K_m values than those of PVA without ethylene blocks, indicating that the hydrophobicity of PVA, caused by an acetyl group and also an ethylene block in the polymer structure, increases the affinity of PVA-DH [5]. The bacterium was reidentified by 16SrRNA homology, suggesting a genus-level match with *Sphingomonas chlorophenolica* (unpublished data). It seems likely that PVA dehydrogenase of a sphingomonad showed higher affinity towards hydrophobic PVA derivatives than PVA itself. The increased hydrophobicity of PVA might increase penetration into the periplasmic space through the outer membrane of a sphingomonad. The enzyme oxidized two hydroxyl groups simultaneously, which was different from the sequential oxidation of two hydroxyl groups previously reported.

Matsumura *et al* isolated a PVA-utilizing *Alcaligenes faecalis* that did not produce PVA oxidase, but produced PVA dehydrogenase in the culture filtrates and membrane fraction [21]. The membrane-bound enzyme was purified and found to be PQQ-dependent. Surprisingly, without PQQ, the apoprotein catalyzed an aldolase-type cleavage to produce the methyl ketone and an aldehyde [22]. There seem to be several types of PVA-oxidizing enzymes that might be induced by the PVA used for cultivation.

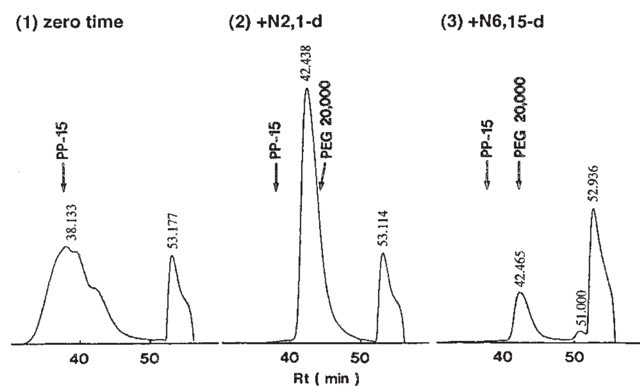

Figure 4 Degradation of the polymer by washed cells [17]. The incubation mixture (100 ml) contained PEG-20 000-phthalate polyester, 1.0% and potassium phosphate buffer (pH 7.0), 0.05 M. The incubation mixture was incubated with washed cells of *Comamonas acidovorans* N2 grown on dibutyl phthalate. The incubation mixture was then centrifuged to remove cells. The supernatant was resuspended with washed cells of *Sphingomonas* sp N6 grown on PEG 20 000. The supernatant was analyzed by GPC. (1) Zero time; (2) 1-day incubation with washed cells of *C. acidovorans* N2; (3) 14-day incubation of the supernatant of (2) with washed cells of *Sphingomonas* sp N6. Abbreviations: PP-15, polyester; Rt, retention time.

Polyethylene (PE)

PE is a plastic that has generally been regarded as biologically and chemically inert. The polymer can be readily recycled if uncontaminated and on incineration has a calorie value similar to fuel oil. However, some PEs used for packaging and agricultural applications can cause a litter problem. Scott concluded in 1975 that an attack on PE by microorganisms is a secondary process [34]. The first process for degradation of PE is oxidation, which reduces the molecular weight of the molecule to the degree required for biodegradation to occur. Based on this theory, he developed the Scott–Gelead System (SGS, prooxidants to PE) to enhance the oxidation of PE molecules [35]. Albertsson *et al* [1] indicated that biodegradation of PE is affected by preliminary irradiation with a UV source, the presence of photodegradative enhancers and antioxidants. More recently Wasserbauer *et al* [36,43] found that PE which has been extracted to remove antioxidants is oxidized rapidly in the presence of *Pseudomonas putida* with the formation of CO_2. This process is inhibited completely by an antioxidant. Potts and his collaborators found that linear paraffin molecules below *ca* 500 molecular weight were utilized by several microorganisms [30]. The biodegradable range of PE molecular weights is still not elucidated.

Capsules of PE films enhanced by SGS are being used successfully for the controlled release of fertilizers in order to reduce pollution by fertilizers in ground waters and associated eutrification of rivers and lakes. To confirm the biodegradability of photofragmented PE capsules and to determine the molecular size limits for PE biodegradation, PE-utilizing microorganisms were isolated from soil samples [20]. PE film including 3% SGS was exposed outdoors for several years, where the primary molecular weight (MW) of 35 500 was degraded to less than 10 000 in 6 months. Photofragmented PE film was broken into pieces, washed to remove fertilizers and ground into approximately 80-μm particles. These particles were used as a sole carbon and energy source for the soil microorganisms. Commercially available PE wax (MW of 1290) was also used as a sole carbon and energy source. Several consortia, which were able to grow on photofragmented PE as well as on PE wax, were obtained, MW below 2000 being the optimal biodegradation limit [20]. Each consortium was composed

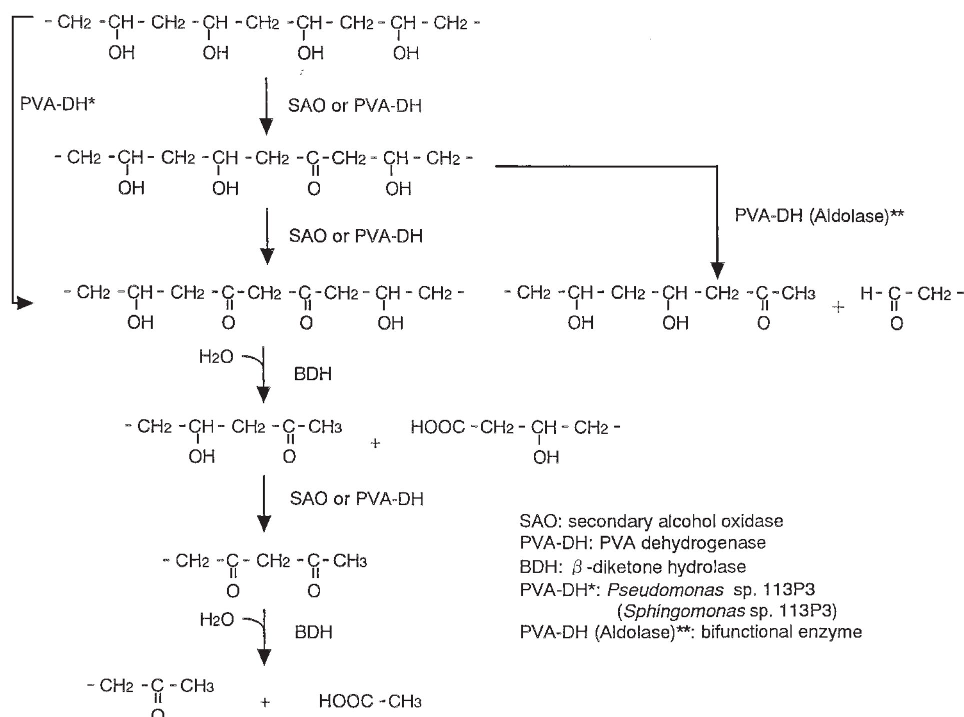

Figure 5 Proposed metabolic pathways for PVA.

of 3–4 major strains that independently assimilated PE waxes. These bacteria were purified and identified, among which was a sphingomonad, *S. macrogoltabidus* FKSH-1, which was deposited as a new species (FERM P-16820) to Patent Microorganism Depository, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan. The sphingomonad was issued Japanese Patent number 98-205923. The metabolic pathways have not yet been elucidated. However, referring to the metabolic pathways for paraffin degradation (Figure 6), we suggest that the pathways for PE wax degradation are similar to those for paraffins. After primary oxidation, paraffins are metabolized through β-oxidation; the enzymes are found in membrane fractions and the substrates need to be incorporated into cells. As suggested for polyethers and PVA, PE wax seems to be transported into the periplasmic space through bacterial outer membranes.

Polyaspartate

This polymer is a chemically synthesized polyamide and is expected to have use in detergents as a chelating agent. A polyaspartate (MW less than 5000)-utilizing sphingomonad was recently isolated by K Tabata *et al*, The Institute of Physical and Chemical Research, Japan, and was deposited as a new species to the Japan Collection of Microorganisms as JCM 10459; its 16SrRNA sequence was registered in Gene Bank with registration No. AB 022601. The hydrolase acting on the polymer is tightly bound to the cells and has not been successfully solubilized (personal communications from Dr K Tabata). Unlike most hydrolases acting on macromolecules, this hydrolase might be working in cells just like dehydrogenases involved in the metabolism of PEG and PVA.

Outlook

Many sphingomonads have been isolated which can degrade polymer. Research on the relationship between degradation mechanisms and membrane structure of sphingomonads has just begun and awaits further characterization. However, from results obtained so far, we can speculate that unique membrane structures derived from unusual complex lipids, sphingolipids, are possibly relevant to the metabolism of polymers.

It is noteworthy that the metabolic enzymes found in sphingomonads involved in the degradation of xenobiotic polymers are not extracellular as is often found with hydrolases, but are intracellularly located in the periplasm as soluble/membrane-bound enzymes. Even for polyaspartate, the enzyme, possibly a peptidase, is tightly bound to the cells. PEG is freely soluble in water as a hydrophilic polymer, but at the same time it is hydrophobic, since it is soluble in alcohol or acetone. The enzymes are localized in the membrane fractions from sphingomonads; the incorporation of PEGs (up to MW 20 000) into cells is strongly suggested by data described above. A PVA-degrading sphingomonad had no extracellular PVA oxidase, but had a periplasmic dehydrogenase, suggesting the incorporation of PVA molecules into the periplasm of cells. The bacterium showed a higher affinity for more hydrophobic PVA derivatives than for PVA itself. PVA is a hydrophilic polymer, but it should include a very small number of acetyl groups derived from the starting material, polyvinylacetate. PVA might have an affinity for bacterial cells through acetyl groups or ethylene segments and might be incorporated in the periplasmic space through hydrophobic segments. PE wax is hydrophobic as a kind of paraffin. It is quite natural that PE wax is incorporated into cells through hydrophobic

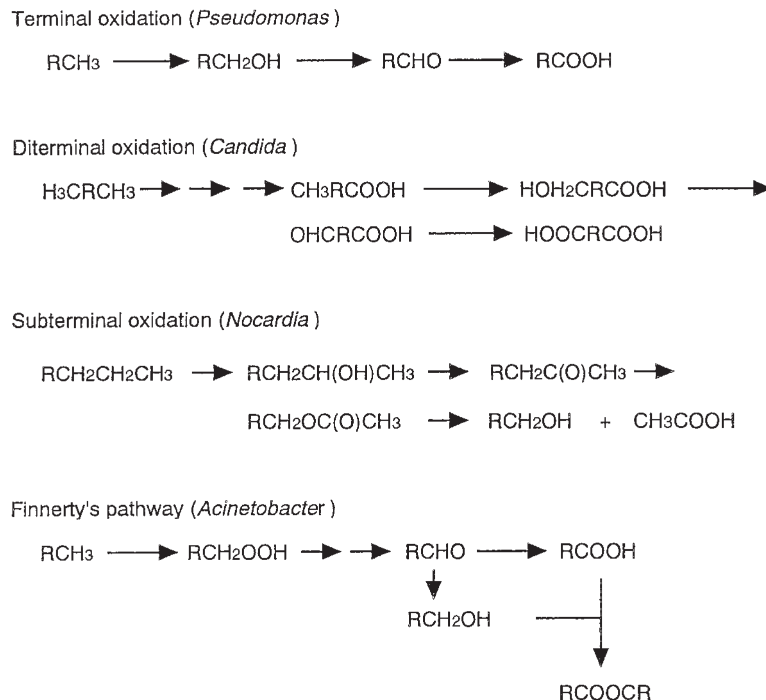

Figure 6 Oxidation pathways for *n*-alkane by microorganisms.

pathways, because oxidized PE wax is possibly metabolized through β-oxidation systems.

Besides the biodegradation of synthetic polymers, many sphingomonads have been isolated as degraders of such hydrophobic materials as polychlorophenol [27,28], polycyclic aromatic hydrocarbons [9,39], γ-hexachlorocyclohexane [25], polychlorobiphenyl [10,40], 2,4-dichlorophenoxyacetic acid [8], dibenzo-*p*-dioxin and dibenzofuran [4,23] and diphenyl ether [33]. These hydrophobic compounds are metabolized in the cells. Not only the enzymes, but also specific membrane structures of these species must be related to their feasibility to metabolize hydrophobic xenobiotics.

As it seems unlikely that xenobiotic macromolecules are incorporated through cytoplasmic membranes, bacteria must have receptors on their cell surfaces for these compounds to regulate degradative genes. Demonstration of the existence of receptors as well as the transport systems of the outer membranes awaits future investigation.

Pseudomonads are considered to be responsible for the microbial degradation of xenobiotics [26]. The degradative enzymes/genes of pseudomonads have been well characterized. Some species of *Pseudomonas* like *P. paucimobilis* and *P. parapaucimobilis* were newly classified as *S. paucimobilis* and *S. parapaucimobilis*. Sphingomonads and pseudomonads are closely located in phylogenetic trees. Sphingomonads as well as pseudomonads must play important roles in the microbial degradation of man-made xenobiotics liberated in the environment. It is another focus as to whether some degradative genes can be transferred among genera and are newly generated by mutation.

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