Microbial Degradation of Aliphatic Polyesters

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Summary: Polyester-degrading ability of actinomycetes obtained from culture collections was investigated by the formation of clear zones on polyester-emulsified agar plates. Using 41 genera (43 strains) of actinomycetes with phylogenetic affiliations based on 16S rRNA sequences, poly(L-lactide) (PLA)-degraders were found to be limited to members of family *Pseudonocardiaceae* and related genera. On the other hand, poly(β-hydroxybutyrate) (PHB)-, polycaprolactone (PCL)-, and poly(butylene succinate) (PBS)-degraders were widely distributed in many families.

Keywords: actinomycetes, biodegradation, lipase, polyesters, *Pseudonocardiaceae*

Introduction

Serious problems regarding the treatment of plastic wastes have stimulated the development of material that can be decomposed after disposal to the environment by the activity of microorganisms to carbon oxide and water. The development of biodegradable plastics is considered to be one of the most desirable product innovations in order to resolve the problems on plastic waste.

We have reported that a fungus of *Penicillium* sp. strain 14-3, isolated from soil, almost completely degraded a kind of aliphatic polyester, poly(ethylene adipate) (PEA)[1] and its purified PEA-degrading enzyme was similar to lipase.[2] In 1977, we discovered that lipase and esterase from various microorganisms were able to degrade aliphatic polyesters such as PEA, PCL, etc.[3] According to this finding, aliphatic polyesters now are generally known to be susceptible to biological attack.

We also noted that the melting point (Tm) of plastics had a great effect on enzyme degradation, e.g., enzymatic degradabilities for the same series of polyesters decreased with increasing Tm. In general, the Tm of the polymer is determined by the values of ΔS (the change of entropy in melting) and ΔH (the change of enthalpy in melting). The high Tm of aromatic polyesters is caused by small ΔS value with increase in the rigidity of the polymer molecule based on an aromatic ring. Enzymatic degradation of copolyester (CPE) composed of aliphatic and aromatic polyester, by *Rhizopus delemar* lipase decreased with increasing rigidity (increase in aromatic polyester content) of the CPE molecule.[4] On the other hand, the high Tm of nylon (aliphatic polyamide) is caused by the large ΔH value based on the hydrogen bonds

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among polymer chains. Enzymatic degradation of copolyamide-ester (CPAE), composed of aliphatic polyester and aliphatic polyamide, by *Rhizopus delemar* lipase decreased with an increase in the aliphatic polyamide content of the CPAE molecule.[5] Hence it was found that rigidity of CPE molecular chains, which is related to the ΔS value, and the ΔH value in the CPAE had a great effect, in addition to the chemical structure, on enzymatic degradation. The above described CPE and CPAE are now commercially available as biodegradable plastics under the names of Ecostar (Eastman Chemical Co., USA), Ecoflex (BASF Co., Germany) and BAK (Bayer Co., Germany), respectively. Furthermore, using various polymers such as PCL,[6,7] poly(β-hydroxybutyrate) (PHB),[8,9] poly(β-propiolactone),[10] poly(butylene succinate) (PBS),[11,12] poly(ethylene succinate),[13] polylactide (PLA),[14-16] poly(*p*-dioxanone),[17] polycarbonate,[18] etc, we made clear about their microbial degradation mechanisms.

Various kinds of biodegradable plastics are presently manufactured. Among the biodegradable plastics, aliphatic polyesters, such as PHB, PCL, PBS and PLA, have been widely explored. PHB is a natural aliphatic polyester that is produced by a wide variety of bacteria as an intracellular storage of carbon and energy. On the other hand, PCL and PBS are synthetic aliphatic polyesters that are currently available. PLA is polymerized from lactic acid, which can be prepared effectively by fermentation with renewable resources such as corn and tapioca starches.

Along with the development of biodegradable plastics, it is also important to gain much knowledge on microorganisms capable of degrading these polymers. We reported our study on the distribution of microorganisms able to degrade four representative aliphatic polyesters, e.g. PHB, PCL, PBS, and PLA. We used the plate count and clear zone methods to evaluate the distribution of polyester-degrading microorganisms in different soil environments and found the degrading microbes decreased in the order of PHB=PCL>PBS>PLA.[11,14,19,20] However, little is known about what family level or the group of microorganisms are responsible for the degradation of polyesters.[21]

A previous study among 25 strains of *Amycolatopsis* showed that they were able to form clear zone on more than one kind of emulsified-polyester plates, e.g. PCL, PHB, PBS and PLA. In addition to polyester-degrading activity, silk fibroin-degrading ability was also observed in these actinomycetes.[15] A similarity in chemical structures of a PLA monomer that is the L-lactic acid unit and L-alanine unit of silk fibroin were noted in our reports.[15,16,22,23]

To obtain further information on the microbial degradation of these commercially available degradable materials, here we attempt to investigate the polyester-degrading activity among

actinomycetes obtained from culture collections with regard to their phylogenetic affiliation. The study was also extended to examine the silk fibroin-degrading activity of these actinomycetes.

Experimental Part

Materials

PLA, LACTY 1012 (number-average molecular weight, $M_n = 3.4 \times 10^5$) was obtained from Shimadzu Co. Ltd. Other biodegradable polyesters were obtained as follows: PHB, ($M_n = 1.4 \times 10^5$) from Mitsubishi Gas Chemicals; PCL, TONE P-767 ($M_n = 6.7 \times 10^4$) from Union Carbide Corporation, and PBS, BIONOLLE 1020 ($M_n = 3.5 \times 10^4$) from Showa High Polymer Co. Silk fibroin powder was obtained from Tosco Co. and washed with hot water (50°C) for 8 h under vigorous stirring to remove the water soluble components (repeated four times). Amino acid analysis showed that the silk powder was composed of glycine, 44.6 (mol-%); alanine, 29.5; and serine 11.7.

Microorganisms

All actinomycetes used were obtained from two culture collections, Japan Collection of Microorganisms (JCM) and Institute for Fermentation, Osaka (IFO). For the study of the phylogenetic positions of the actinomycetes, the published 16S rRNA gene sequences of the tested actinomycete genera in the DDBJ/EMBL/Genebank DNA sequence data libraries were used to construct a phylogenetic tree with the GENETYX 11.0 (Software Development Co., Japan) and CLUSTAL programs. The nucleotide sequence accession numbers used are shown in Figure 2.

Culture Media

Basal medium contained (per liter) 100 mg yeast extract, 200 mg $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot H_2O$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg $Na_2WO_4 \cdot 2H_2O$, 0.5 mg $MnSO_4$, 1600 mg K_2HPO_4, 200 mg KH_2PO_4 and 1000 mg $(NH_4)_2 SO_4$ (pH 7.1). Polyester-emulsified culture plates were prepared by dissolving 1 g polymer pellets in 40 ml chloroform. Using an ultrasonic disruptor (Tomy UD-201, Tomy Seiko Co. Ltd.), the solution was emulsified into 1000 ml basal medium with 100 mg of surface active agent [Plysurf A210G Daiichi Kogyo Seiyaku, $RO(CH_2CH_2O)_nP(=O)(OH)OR'$; R, alkyl or alkylallyl group ; R',-H or $(CH_2CH_2O)_n$ R ; hydrophile-lipophile balance, 9.6]. After sonication, the emulsion was

evaporated in vacuum at 40°C to remove the chloroform. Preparation of silk fibroin culture plates was carried out by dispersing the powder directly into basal medium with no addition of surfactant. Agar plates were prepared by the addition of 2% (wt./vol.) agar and pouring into petri dishes after autoclaving.

Degradation of Emulsified-polyester and Silk Fibroin on Agar Plates

The actinomycetes were streaked-out onto polyester-emulsified agar and silk fibroin agar plates. Degradability was determined by the clear zone formation around the colony on the opaque plates after 30 days of cultivation time at the optimum temperature of their growth.

Results and Discussion

The phylogenetic positions of the tested actinomycete genera with polyester-degrading ability were investigated using the clear zone method as previously described by Nishida and Tokiwa.[19] Figure 1 shows an example of clear zone formation on an agar plate emulsified with PLA after incubation at 30 °C for 2 weeks.

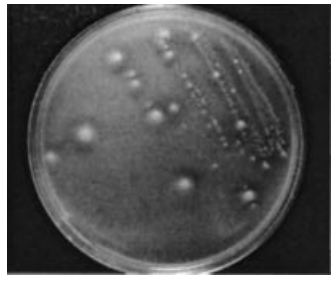

Fig. 1. Colonies of *Saccharothrix waywayandensis* JCM 9114 and clear zones formed on an agar plate with emulsified PLA.

The rationale for selecting the actinomycetes of this study was that (i) All of the strains used to study the relationship between phylogenetic positions and their polyester-degrading abilities are type strains, (ii) They have already been genetically classified and (iii) They are available from the culture collections. Since there were about 600 species in the genus of *Streptomyces*, three of the *Streptomyces* type strains were used to construct the phylogenetic tree. The phylogenetic positions of the tested genera based on 16S rRNA gene sequence data and their polyester-degrading abilities are shown in Figure 2. Interestingly, PLA-degraders were found to be limited to the family *Pseudonocardiaceae* and related genera. *Amycolatopsis*, *Saccharothrix,*

Lentzea, *Kibdelosporangium* and *Streptoalloteichus* listed in this family were found to have PLA-degrading abilities.

Several studies on microbial degradation of polyester have previously been studied by isolating the strains from the natural environment, and then analyzed their phylogenetic affiliations.[10,17,21,24] Suyama et al. studied polyester-degrading bacteria, i.e., PHB-, PCL- and PBS-degrading bacteria, using 16S rRNA gene sequence data and the polyester-degrading abilities of thirty-nine bacteria of classes *Firmicutes* and *Proteobacteria* isolated from soil samples. They reported the characteristic patterns of polyester substrate specificity of those strains but no PLA-degrading bacteria isolates were found.[21]

Out of the 41 genera tested in this study, only 5 genera showed clear zones around their colonies on PLA-emulsified plates. This approach revealed a restricted number of PLA-degraders among actinomycetes. The result in this study demonstrated that there is a relationship between the phylogenetic positions and PLA-degrading ability among the tested actinomycetes. On the other hand, PHB-, PCL-, and PBS-degraders were widely distributed in various families. From our result, it is considered that actinomycetes might play an important role in microbial degradation of polyesters. Additional strains will be investigated in order to substantiate our results, especially among a large number of *Streptomyces*.

Natural aliphatic polyesters are distributed widely in the environments, i.e. PHB (storage substance) in bacteria and cutin (the structural polyester of the plant cuticle) in plants. Since these polymers occur in nature, no doubts that enzymes degrading them are ubiquitous in living organisms. Nishida and Tokiwa found that some phytopathogenic fungi degraded a synthetic polyester PCL and proposed that their cutinase act on PCL as an analogue of cutin.[25] Further, Murphy et. al. confirmed that PCL-depolymerase of a fungal pathogen *Fusarium* is in fact the cutinase.[26] PBS is a kind of polyester in which component monomers are bonded via ester linkages. We found that lipase was able to degrade PBS.[27]

L-Alanine is a major amino acid constituent of silk fibroin within amino acid sequence: (Gly-Ala)$_2$-Gly-Ser-Gly-(Ala)$_2$-Gly-[Ser-Gly-(Ala-Gly)$_n$]$_8$-Try, where n is usually 2.[28] An isolated strain of *Amycolatopsis* used in silk degradation was confirmed able to form clear zones on PLA plate.[22] Surprisingly, many of the *Amycolatopsis* strains used in PLA degradation also formed clear zones on the silk plates.[15] Recently, we confirmed that the purified PLA-degrading enzyme produced by *Amycolatopsis* strain HT-41 has ability to degrade both PLA and silk fibroin powder.[23] Most strains forming clear zones on PLA-emulsified agar plates also formed clear zones on silk fibroin agar plates (Figure 2). It can be anticipated that the strains may regard the repeated L-lactic acid unit of PLA as L-alanine unit of silk fibroin.

As a hypothesis, it may be regarded that each chemically synthesized polymer is an analogue of a natural substrate. The research on biodegradable plastic is continuing with the aim of achieving harmony between human activities and the natural environment.

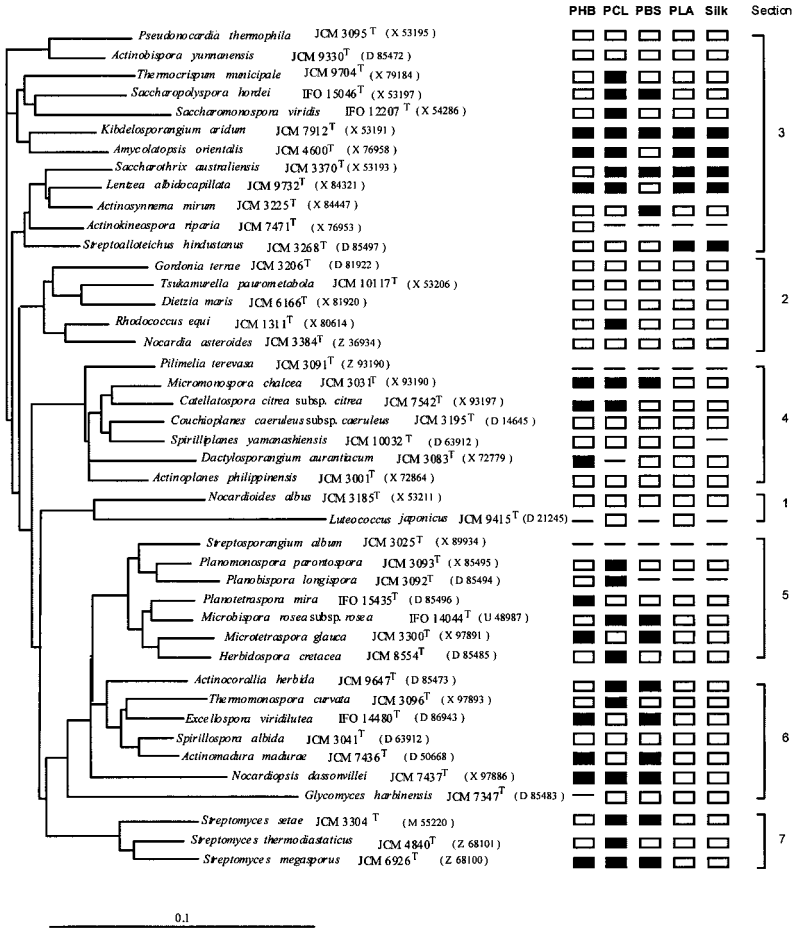

Fig. 2. Phylogenetic positions of actinomycetes and their polyesters, and silk fibroin degradabilities. The phylogenetic tree was constructed based on pairing of 16S rRNA sequences. The nucleotide sequence accession numbers are in the parentheses. The scale bar corresponds to a 10% difference in nucleotide sequence. T=type strain. Symbols: ■ degradation; □ no degradation and – no growth on the tested plates. Section 1: *Micrococcus, Microbacterium* and related genera. Section 2: *Mycobacterium, Nocardia* and related genera. Section 3: Family *Pseudonocardiaceae* and related genera. Section 4: Family *Micromonosporaceae.* Section 5: Family *Thermomonosporaceae.* Section 6: Family *Streptosporangiaceae.* Section 7: Family *Streptomycetaceae.*

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