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Characterization of a Soil Metagenome-Derived Gene Encoding Wax Ester Synthase S

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A soil metagenome contains the genomes of all microbes included in a soil sample, including those that cannot be cultured. In this study, soil metagenome libraries were searched for microbial genes exhibiting lipolytic activity and those involved in potential lipid metabolism that could yield valuable products in microorganisms. One of the subclones derived from the original fosmid clone, pELP120, was selected for further analysis. A subclone spanning a 3.3 kb DNA fragment was found to encode for lipase/esterase and contained an additional partial open reading frame encoding a wax ester synthase (WES) motif. Consequently, both pELP120 and the full length of the gene potentially encoding WES were sequenced. To determine if the *wes* gene encoded a functioning WES protein that produced wax esters, gas chromatography-mass spectroscopy was conducted using ethyl acetate extract from an *Escherichia coli* strain that expressed the *wes* gene and was grown with hexadecanol. The ethyl acetate extract from this *E. coli* strain did indeed produce wax ester compounds of various carbon-chain lengths. DNA sequence analysis of the full-length gene revealed that the gene cluster may be derived from a member of Proteobacteria, whereas the clone does not contain any clear phylogenetic markers. These results suggest that the *wes* gene discovered in this study encodes a functional protein in *E. coli* and produces wax esters through a heterologous expression system.

Keywords: Lipid metabolism, soil metagenome, wax ester synthase, *wes*

Introduction

Wax esters (WEs) are oxoesters of long-chain fatty acids esterified with long-chain fatty alcohols. These neutral lipids are important compounds that are used for various purposes, including cosmetic formulations (moisturizer and cleanser) and removal of foams produced while manufacturing antibiotics such as penicillin. WEs are widespread in nature and found in plants, animals, and microorganisms. They are frequently extracted from honeybees, sperm whales, and the jojoba plant. However, limited supplies of WEs result in high costs and are the major obstacle to their commercial availability [19, 25]. In order to overcome these difficulties, a number of chemical and biological methods have been attempted to produce WEs. Biological WE production is preferred, as it is a safer and simpler method. In comparison, chemical catalysts are not safe to use, can cause equipment corrosion, consume large amounts of energy consumption, and cause the breakdown of esters [14, 25].

WEs accumulate as energy storage products in the proteobacteria *Acinetobacter*, *Pseudomonas*, and *Marinobacter*, and the actinobacteria *Mycobacterium*, *Streptomyces*, and *Rhodococcus* [2, 18, 20, 32, 37]. Industrial mass production of wax in microorganisms and plants is thought to yield significant economic profits. The biosynthesis of WEs has been well characterized in *Acinetobacter baylyi* strain ADP1, where the enzyme wax ester synthase/acyl-coenzyme A

(acyl-CoA)-diacylglycerol acyltransferase (WS/DGAT) is responsible for WE production [24]. WE production via WS/DGAT in microorganisms entails the reduction of acyl-CoA to fatty aldehyde by acyl-CoA reductase, followed by the reduction of fatty aldehyde to fatty alcohol by fatty aldehyde reductase [30]. Finally, an acyl chain from fatty acyl-CoA is transferred to a fatty alcohol, thus forming a wax ester [20, 39]. The WS/DGAT in *A. baylyi* strain ADP1 is a bifunctional enzyme that synthesizes not only WEs but also triacylglycerol [24].

Although various microorganisms are known to express WS/DGAT and produce WEs, we searched soil metagenome libraries for lipid metabolism in microorganisms and selected a clone with the potential for WE production potential. A metagenome includes all the genomes of the microbial community in a certain sample [15]. Since approximately 99% of the microbes in nature cannot be cultured under standard conditions, the metagenomic approach has been developed to tap into the genetic resources of these unculturable microbes [3, 16, 31]. Over the years, there have been several reports about diverse antibiotics and enzymes discovered through the metagenomic approach [4, 8, 9, 12, 21, 27, 28, 31, 36].

Various esterases (E.C. 3.1.1.3) and lipases (E.C. 3.1.1.1) have been used to catalyze the production of novel biosynthetic materials, biodiesel, and important biochemical compounds in biotechnology and industry [12, 21, 22, 23]. One lipase was reported to produce wax by combining highly concentrated fatty alcohols with fatty acids [41]. It is also known that wax can be biosynthesized from the chemical combination of breakdown byproduct fatty acids and various types of fatty alcohols in microbes [24]. In this study, we used clones from previously identified soil metagenomes to identify lipolytic activity [21, 27, 29] and those from newly identified as lipolytic clones from another constructed metagenomics library. One of the clones showed lipase/esterase activities potentially linked with a gene encoding putative acyltransferase. To the best of our knowledge, this study is the first to describe a gene from the soil metagenome encoding the WS/DGAT protein and to determine its wax ester-producing activity in *E. coli*.

Materials and Methods

Bacterial Strains and Culture Conditions

E. coli DH5α and EPI300 strains were routinely cultured at 37°C either on Luria-Bertani (LB) agar, and in LB broth or M9 broth supplemented with the appropriate antibiotics. The *E. coli* strains were supplemented with 25 μg/ml chloramphenicol and 100 μg/ml ampicillin.

Metagenomic Library Used to Select Lipolytic Activity

We previously constructed metagenomic libraries in a fosmid using forest topsoils [21], plant rhizosphere soils [27], rice paddy soil [26], and alluvial soils [40]. A number of lipolytic active clones were selected from those libraries in LB agar medium supplemented with 1% tributyrin, as previously described [29]. Along with previously selected clones, newly selected metagenomic clones based on tributyrin hydrolysis were subjected to subcloned genes encoding esterase or lipase by a shotgun cloning strategy, as previously described [21, 29]. Based on the DNA sequences of the lipolytic subclones, we investigated any genes linked with those encoding esterase/lipase, which will be involved in lipid metabolism. One of the previously identified subclones, pULP120E [21], was shown to carry a partial gene encoding putative acyltransferase activity upstream of a gene encoding hormone-sensitive esterase. Therefore, we further analyzed the gene with its original fosmid clone to characterize the gene function as it related to neutral lipid biosynthesis.

General DNA Manipulation and DNA Sequencing

Plasmid preparation, restriction endonuclease digestion, DNA ligation, transformation of plasmid DNA, agarose gel electrophoresis, and other standard recombinant DNA techniques were carried out as described by Sambrook *et al*. [34]. DNA sequencing and primer synthesis were performed commercially at the DNA sequencing facility of MacroGen (Seoul, Korea). Complete DNA sequencing of the ELP120 clone, the original fosmid clone of pULP120E, was conducted by shotgun sequencing at MacroGen. A shotgun library was constructed by preparing mechanically sheared DNA at sizes of 1.5 to 4 kb. The sequencing gaps of ELP120 were closed by a primer walking to generate full-length DNA sequences of ELP120. DNA sequences were analyzed with the BLAST program provided by the National Center for Biotechnology Information (NCBI). Open reading frames (ORFs) were analyzed with the ORF Finder software at the NCBI. Of the potential ORFs identified by ORF Finder, non-overlapping sequences longer than 50 amino acids were retained. For overlapping ORFs in different reading frames, we selected those with BLAST homologs. The start codons of the ORFs were determined by the presence of conserved Shine-Dalgarno sequences 4 to 10 bp ahead of the ATG or GUG codon for the individual ORF.

Subcloning the *wes* Gene

The initially selected lipolytically active subclone isolated by Hong *et al*. [21] was pULP120E (GenBank Accession No. EF213583; Fig. 1). The original fosmid clone, ELP120, was used to subclone the upstream DNA carrying a putative acyltransferase gene. ELP120 was purified using a Plasmid Large-Construct Kit (Qiagen, Hilden, Germany) and digested with one of the following

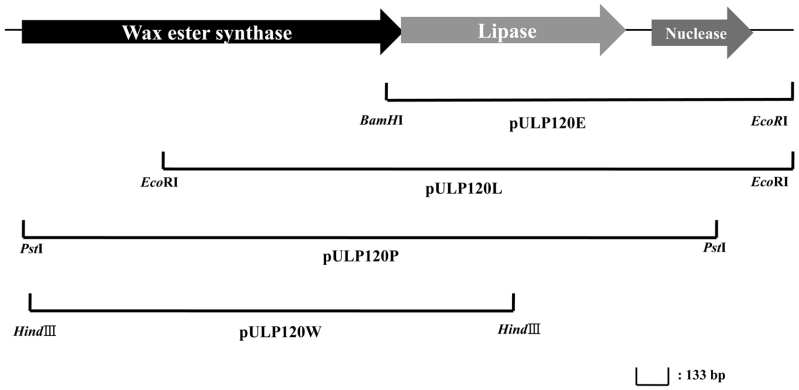

Fig. 1. Gene organization of wax ester synthase, lipase, and nuclease genes in the metagenomic clone pELP120.
Subclones of pELP120, produced by using different enzymes to generate distinct plasmids, are indicated.

restriction enzymes: EcoRI, PstI, and HindIII. The digested DNA fragments of ELP120 were ligated into linearized pUC119 with the same restriction enzyme to generate the secondary shotgun library. Subclones with tributyrin hydrolysis were selected on LB supplemented with ampicillin and tributyrin. The subclones were selected and named after the initial fosmid clones as follows: pULP120L, pULP120P, and pULP120W (Fig. 1). The DNA sequences of all three subclones were determined.

GC-MS Analysis of WEs

E. coli DH5α cells carrying pULP120W or pUC119 were cultured in 100 ml of M9 liquid medium (pH 7.0) containing 0.4% of glucose, 0.01% of thiamine HCl, ampicillin, and 0.3% hexadecanol, at 37°C for 48 h with vigorous agitation, as described previously [25]. *E. coli* cells grown in M9 broth reached the cell density of approximately 3×10^8 cells/ml. The cells were centrifuged and precipitated, and the pelleted cells were sonicated with 10 sec intervals for 2 min twice on ice. The sonicated cell lysates were extracted twice with the same volume of ethyl acetate, and the extracts were dried off by the vacuum evaporator and subsequently solubilized in 500 µl of chloroform, and 2 µl of the solution was used for gas chromatography (GC) analysis. GC analysis was performed preliminarily on an Agilent Technologies 7890A GC system. GC electron impact MS analyses were performed with an Shimadzu QP5050A GC system. GC conditions consisted of an DB-5 column (30 m × 0.32 mm (inner diameter) × 1.5 µm, coated with 5% phenylmethyl silicone), with the injector temperature set to 310°C. The oven was set to a temperature gradient of 40°C/min from 40°C to 200°C, followed by slowing of the gradient from 200°C to 310°C at 3°C/min using helium as a carrier gas. The electron energy of the MS conditions was 70 eV, and the source temperature was set to 170°C. Mass spectra were scanned in a range of *m/z* 40 to 600 at 1 sec intervals. Wax esters were identified on the basis of comparison of their relative retention time and mass spectra with those of standard wax esters. Palmityl palmitate (Sigma-Aldrich Co.) solubilized in the same solvent was used as a standard control for wax ester. For the negative control, *E. coli* DH5α carrying the pUC119 vector was used as described above.

Nucleotide Sequence Accession Number

The nucleotide sequence of the DNA insert of ELP120 was deposited in the GenBank database under the accession number KR057702.

Results and Discussion

Selection of Lipolytic Active Clones from Metagenomics Library

Metagenomic libraries constructed from forest topsoils, plant rhizosphere soils, rice paddy soils, and alluvial soils were previously screened to select lipolytic active clones based on tributyrin hydrolysis. Of the 326,200 metagenomic clones subjected to screening, 31,000 were from forest soil, 107,000 were from rice paddy soil, 142,900 were from plant rhizosphere soil, and 45,300 were from Nakdong River alluvial soils. A total of 155 clones with tributyrin hydrolysis were selected for further subcloning analysis. The frequency of lipolytic activity was one clone positive for tributyrin hydrolysis per 2,104 library clones. Secondary shotgun libraries were generated in pUC119 from the individual lipolytic clones to select the lipolytic subclones with the smallest DNA insert. The DNA sequences of genes encoding lipolytic activity were examined to search for other functionally linked lipid metabolism genes. The subclone pULP120L, which contained the previously characterized pULP120E [21], carried a gene encoding a putative acyltransferase motif and the original fosmid clone pULP120 and was, therefore, subjected to further analysis to investigate if the upstream gene of the

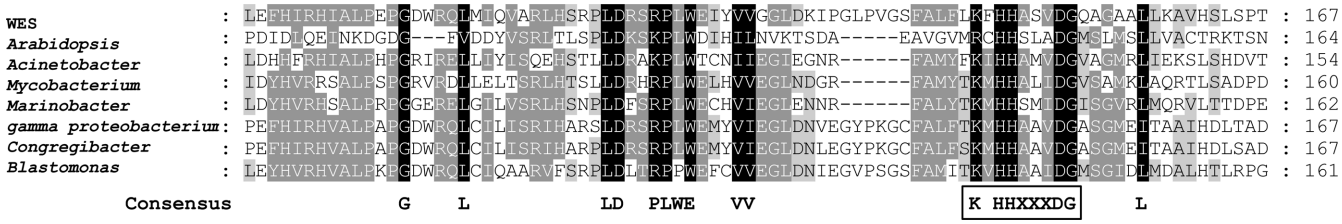

Fig. 2. Multiple sequence alignment analysis of WES from metagenome with WES proteins from bacteria and *Arabidopsis thaliana*. Identical and similar amino acids are marked black and gray, respectively. Only the region corresponding to the active site (87–167 aa) is shown here. The boxed region corresponds to the putative conserved acyltransferase catalytic domain, HHXXXDG [24]. Protein sequences are as follows: *A. thaliana* (NP_200150, AT5G53380); *Acinetobacter* sp. (AF529086); *Mycobacterium tuberculosis* (CCP46561); *Marinobacter hydrocarbonoclasticus* ATCC 49840 (ABO21021); *Gammaproteobacterium* NOR5-3 (WP_009025331); *Congregibacter litoralis* (WP_023659904); *Blastomonas* sp. (WP_023838323).

identified lipase gene encoded acyltransferase activity for WE synthesis.

Characterization of the Metagenomics Clone pULP120

Since pULP120 carried *wes* dissimilar to the known *wes* of cultured bacteria, the DNA sequence of the full-length insert of pULP120 was determined and analyzed to locate any linked genes for lipid metabolism or the origin of this metagenomic fosmid clone. The overall G+C content of pULP120 was 68.46%. A G+C content plot of pULP120 showed the uniform distribution of GC over the insert DNA in a range of 60–80% (data not shown), suggesting that the insert DNA might have originated from one bacterial organism. We defined the potential ORFs and their organization using a BLAST search and ORF Finder (Fig. S1). The DNA is 32,762 bp in length, and 33 ORFs were identified within it. Close relatives of the pULP120 genes and the identities of deduced amino acid sequences are summarized in Table S1. There were no clear phylogenetic marker genes in the insert DNA; therefore, the pULP120 organism of origin was not apparent. However, most of the ORFs were highly similar to Proteobacteria genes, indicative of potential origin from a member of Proteobacteria. Several genes relevant to lipid metabolism were identified, although they were not physically linked with *wes*. Since the *wes* gene was the first ORF in the insert DNA, *wes* expression in the original pELP120 may be due to the transcription read-through by the fosmid-originated promoter in the original pELP120.

Sequence Analysis of the Gene with Lipolytic Active Clones

The purified plasmid of the fosmid clone pULP120 was digested with EcoRI, and the resulting digested DNA fragments were ligated into pUC119 to generate pULP120E (Fig. 1) [21]. DNA sequencing of the insert revealed that it comprises a lipase, a nuclease, and partial wax ester synthase (WES) homologs (Fig. 1). To obtain the full ORF of *wes*, pULP120 was subsequently digested with PstI, and the resulting insert DNA was ligated to the PstI site of the pUC119 vector, resulting in pULP120P (Fig. 1). The HindIII-digested DNA containing the ORF region of *wes* was subcloned to pUC119 (pULP120W), and the insert DNA with the ORF of the lipase was ligated to pUC119 (pULP120L) (Fig. 1). The *wes* gene was predicted to encode a protein consisting of 552 amino acids with a molecular mass of 64.8 kDa. Homologous genes of *wes* were found by a BLAST search via NCBI. A group of conserved hypothetical proteins exhibited the highest homology to the WES protein identified from the soil metagenome. The most homologous protein originated from *Gammaproteobacterium* NOR5-3 and shared 43% identity with the WES discovered (Fig. 2). The identities between the WES and proteins from other organisms are as follows: *Blastomonas* sp. CACIA14H2 (42%), *Congregibacter litoralis* (41%), *Actinetobacter* ADP1 WSD (31%), *Mycobacterium tuberculosis* TGS2 (31%), *Marinobacter hydrocarbonoclasticus* (30%), and *Arabidopsis thaliana* At5g53380 (22%) (Fig. 2).

Despite the low similarities between WES from the metagenome and those from other organisms, these WES proteins were indicated to have a conserved catalytic domain, HHXXXDG (143–151 aa), which is known for acyl-transferase activities (Fig. 2). The highly conserved HHXXXDG motif may be the catalytic site responsible for ester bond formation (Fig. 2) [24]. It was proposed that the strictly conserved histidine residue (His^{144}) may be the catalytic site for nucleophilic attack on the thioester bond of the fatty acyl-CoA molecule [24].

Wax Ester Synthesis by the Metagenome-Derived Enzyme

Using GC analysis, WE biosynthesis was investigated in *E. coli* cells carrying pUC119 or pULP120W (Fig. 3). Palmityl palmitate was used as a WE control (Fig. 3). In the

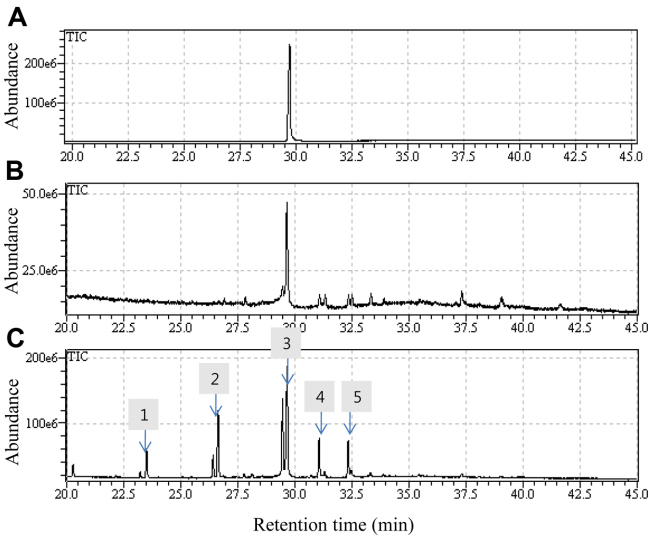

Fig. 3. Gas chromatography analysis of bacterial cell extract of *E. coli* carrying pULP120W or pUC119.
(**A**) A positive control, palmityl palmitate, (**B**) bacterial cell extract of *E. coli* carrying pUC119, and (**C**) bacterial cell extract of *E. coli* carrying the metagenome-derived *wes* gene. The numbers 1 to 5 are selected peaks analyzed by mass spectroscopy and indicated in Fig. 4.

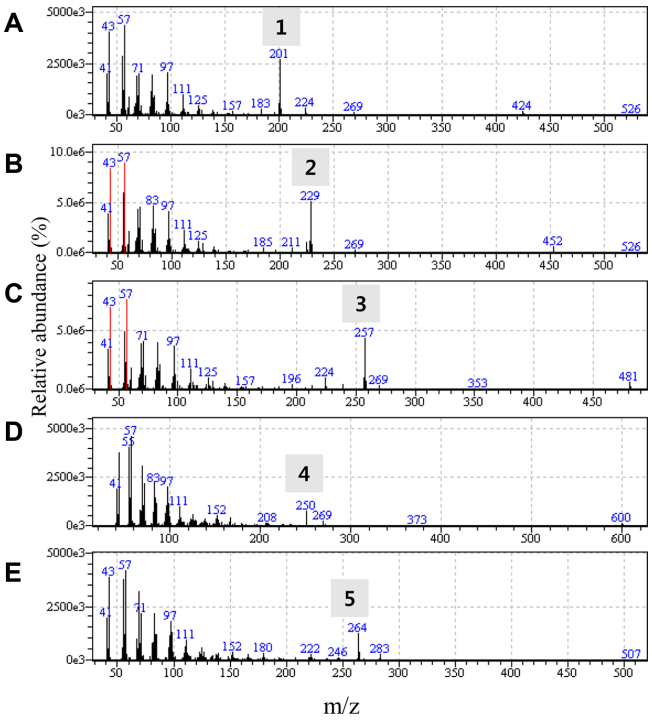

Fig. 4. Wax ester identified by GC-MS.
The numbers 1 to 5 are selected peaks analyzed by MS. (**A**–**E**) Mass spectra of wax esters exclusively from *E. coli* carrying the *wes* gene. 1, Palmityl laurate ($C_{28}H_{56}O_2$, molecular weight (MW) 424.74); 2, palmityl myristate ($C_{30}H_{60}O_2$, MW 452.8); 3, palmityl palmitate ($C_{32}H_{84}O_2$, MW 480.85); 4, palmityl *cis*-10-heptadecenoate ($C_{33}H_{33}O_2$, MW 461.61); and 5, palmityl oleate ($C_{34}H_{55}O_2$, MW 506.89).

cells with WES, unique peaks were observed in retention time between 23 and 33 min compared with the cells with only pUC119 (Fig. 3). The retention time of this peak (30 min) was the same as that of palmityl palmitate (Fig. 3). Further GC-MS analysis and comparison with mass spectra of standard compounds in the NIST08 Mass Spectral Library (NIST, MD, USA) helped identify the peaks as those for wax esters with different carbon-chain lengths, such as palmityl laurate (C_{28}), palmityl myristate (C_{30}), palmityl palmitate (C_{32}), palmityl *cis*-10-heptadecenoate (C_{33}), and palmityl oleate (C_{34}) (Fig. 4). The retention time was correlated with the carbon-chain length of WEs (Fig. 4). This indicated that *E. coli* cells with WES produce WEs of various lengths. Although we tried to overexpress and purify a His-tag fused WES protein using *E. coli* BL21 (DE3) carrying pET30b(+) with *wes*, the overexpressed protein was somehow truncated at its C-terminal to generate two different proteins similar in molecular weight. Since the truncated protein still retained the WES activity weakly, we could not determine the enzymatic activity with WES protein itself (data not shown).

Microorganisms have been reported to synthesize triacylglycerols (TAGs) [42], polyhydroxyalkanoates [38], and WEs [43]. Biosynthesis of WEs has been reported mostly in *Acinetobacter* species, which store WEs weighing up to about 14% of the cellular dry matter [24]. Some species, such as the genera *Moraxella* [6], *Micrococcus* [33], and *Alcanivorax* [5], have been also known for WE biosynthesis. Many of these strains were isolated from assorted petroleum-polluted soils and water habitats, indicating that hydrocarbons may act as a carbon source for storage-lipid biosynthesis in these bacteria [1, 5, 24]. The final step in WE and TAG biosyntheses in bacteria is catalyzed by a WS/DGAT enzyme, which exhibits both wax ester synthase (WS) and diacylglycerol (DAG):acyl-coenzyme A (CoA) acyltransferase (DGAT) activities [24]. The WS/DGAT enzymes in prokaryotes are different from the acyltransferases involved in the formation of TAGs and WEs in eukaryotes [7, 10, 35]. WS/DGAT was first identified in *Acinetobacter* ADP1 [24]. The functional relevance of WS/DGAT-related proteins in lipid accumulation was elucidated in mycobacteria, as lipid accumulation in *M. tuberculosis* could be an essential factor in regulating pathogenesis [13]. In *Marinobacter* species, isoprenoid WEs

have been identified as a means of energy storage [11, 20]. Here, we report a metagenome-derived WS/DGAT homolog with low similarity to known enzymes and a function in WE biosynthesis in *E. coli*.

WE synthesis in microorganisms or plants has enormous potential for the supply of neutral lipids [17, 44]. The overproduction of WE could be also beneficial to the WE-producing organism itself by providing defense against various biotic or abiotic stresses [11]. Our screening of 326,200 members of metagenome libraries yielded 155 lipolytic active clones. Although we identified a clone with WE activity, there might be other industrially relevant clones for lipid metabolism in the lipolytically active clones selected from the soil metagenomes.

WEs have multiple commercial uses, but their commercial production is restricted by limited sources and high costs. Our study provides the possibility that the WEs could be generated for commercial purposes using a novel metagenome-derived *wes* gene and a microbial expression system as an alternative means of production.

Acknowledgments

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