Journal of Biotechnology 270 (2018) 30–38

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Soil metagenome-derived 3-hydroxypalmitic acid methyl ester hydrolases suppress extracellular polysaccharide production in *Ralstonia solanacearum*

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ARTICLE INFO

Keywords:
3-Hydroxy palmitic acid methyl ester hydrolases
Quorum quenching
Ralstonia solanacearum
Soil metagenome

ABSTRACT

Autoinducers are indispensable for bacterial cell–cell communication. However, due to the reliance on culture-based techniques, few autoinducer-hydrolyzing enzymes are known. In this study, we characterized soil metagenome-derived unique enzymes capable of hydrolyzing 3-hydroxypalmitic acid methyl ester (3-OH PAME), an autoinducer of the plant pathogenic bacterium *Ralstonia solanacearum*. Among 146 candidate lipolytic clones from a soil metagenome library, 4 unique enzymes capable of hydrolyzing the autoinducer 3-OH PAME, termed ELP86, ELP96, ELP104, and EstDL33, were selected and characterized. Phylogenetic analysis revealed that metagenomic enzymes were novel esterase/lipase candidates as they clustered as novel subfamilies of family I, V, X, and family XI. The purified enzymes displayed various levels of hydrolytic activities towards 3-OH PAME with optimum activity at 40–50 °C and pH 7–10. Interestingly, ELP104 also displayed N-(3-oxohexanoyl)-L-homoserine lactone hydrolysis activity. Heterologous expression of the gene encoding 3-OH PAME hydrolase in *R. solanacearum* significantly decreased exopolysaccharide production without affecting bacterial growth. mRNA transcription analysis revealed that genes regulated by quorum-sensing, such as *phcA* and *xpsR*, were significantly down-regulated in the stationary growth phase of *R. solanacearum*. Therefore, metagenomic enzymes are capable of quorum-quenching by hydrolyzing the autoinducer 3-OH PAME, which could be used as a biocontrol strategy against bacterial wilt.

1. Introduction

Bacteria are known to produce a variety of lipolytic enzymes such as lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1), which are involved in diverse biological metabolic processes, from routine lipid metabolism (Winkler et al., 1990) to cell-to-cell signaling (Arpigny and Jaeger, 1999; Spiegel et al., 1996). These enzymes have a variety of biotechnological potentials (Jaeger and Eggert, 2002), making them suitable candidates for use in pharmaceutical, food, and detergent industries (Bornscheuer, 2002; Hasan et al., 2006). Esterases preferentially hydrolyze water-soluble esters containing short-chain carboxylic acids, whereas lipases prefer long-chain acylglycerides. Most lipolytic enzymes carry a conserved Gly-Xaa-Ser-Xaa-Gly motif as a characteristic α/β hydrolase (Nardini and Dijkstra, 1999). These enzymes have been classified into sixteen families based on conserved sequence motifs and biological properties (Arpigny and Jaeger, 1999; Lenfant et al., 2013).

Most bacterial lipolytic enzymes discovered so far have been traced back to culturable microbes. Considering that more than 99% of microorganisms are not culturable, one can imagine the biotechnological potential and utility of the yet-unexplored microbial diversity (Amann et al., 1995; Hugenholtz and Pace, 1996). Functional bioprospecting from metagenomes has already been proven to be an efficient and promising technique to explore novel microbial potential (Handelsman et al., 1998; Lee and Lee, 2013; Elend et al., 2006; Lee et al., 2010; Lim et al., 2005; Tao et al., 2012; Uchiyama and Miyazaki, 2009; Yu et al., 2011). Recently, there has been an increasing trend to search for novel and unique lipolytic enzymes, which usually involves screening for *p*-

Abbreviations: 3-OH PAME, 3-hydroxypalmitic acid methyl ester; pNP, p-nitrophenyl acyl ester; AHL, N-acyl homoserine lactones; EPS, extracellular polysaccharide; LB, Luria-Bertani; CPG, casamino acid-peptone-glucose; TTC, triphenyltetrazolium chloride; GC, gas chromatography; DCM, dichloromethane; NCBI, National Center for Biotechnology Information; RT-PCR, reverse transcription PCR; OHHL, N-(3-oxohexanoyl)-L-homoserine lactone; BSA, bovine serum albumin

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https://doi.org/10.1016/j.jbiotec.2018.01.023
Received 18 October 2017; Received in revised form 30 January 2018; Accepted 31 January 2018
Available online 03 February 2018
0168-1656/

nitrophenyl acyl ester (*p*NP)-based hydrolytic activity. Some lipolytic enzymes from soil metagenomes are already reported to have bifunctional catalytic activities (Heath et al., 2009; Rashamuse et al., 2009; Rhee et al., 2005; Tao et al., 2012). Considering the bioprospecting potential of metagenome analysis, one can screen and select for novel lipolytic enzymes of particular interest.

Quorum-sensing is the cell density-dependent cell-to-cell communication process that regulates specific bacterial cell functions (Fuqua et al., 1994) such as virulence (Burr et al., 2006), biofilm formation (Hammer and Bassler, 2003), swarming motility (Givskov et al., 1998), and antibiotic production (El-Sayed et al., 2001). Cell-to-cell communication depends on diffusible and recognizable specific signal molecules such as N-acyl homoserine lactones (AHL) and other autoinducers (Miller and Bassler, 2001). However, a few bacterial species use 3-hydroxypalmitic acid methyl ester (3-OH PAME) as an autoinducer for quorum sensing (Waters and Bassler, 2005). For example, 3-OH PAME, also called a quormone, is the major cell-to-cell signaling molecule in *Ralstonia solanacearum*, which causes a lethal bacterial wilt in solanaceous plants and many other plant families (Hayward, 1991). Recently, a novel quorum sensing molecule (R)-methyl 3-hydroxymyristate [(R)-3-OH MAME] that regulates the production of virulence factors and secondary metabolites was reported from strains of *R. solanacearum* that lack 3-OH PAME-mediated signaling (Kai et al., 2015). The virulence mechanisms of *R. solanacearum* are still not completely understood; however, extracellular polysaccharide (EPS) production has been known to be the major virulence determinant in *R. solanacearum* (Flavier et al., 1997; Genin and Boucher, 2004; Hayward, 1991). 3-OH PAME is an essential factor in cell density-dependent expression of *phcA*-regulated genes, including *xpsR* and other genes associated with EPS production. PhcA is a master transcriptional regulator which positively and negatively regulates a variety of genes involved in pathogenicity, including the *xpsR* gene which further regulates EPS production in *R. solanacearum* (Brumbley et al., 1993; Clough et al., 1997a,b; Flavier et al., 1997; Schell, 1996). Interestingly, lipolytic enzymes from culture-based sources, capable of hydrolyzing 3-OH PAME from *R. solanacearum,* suppressed EPS production in *R. solanacearum* (Shinohara et al., 2007). In addition, quorum-sensing in most proteobacteria relies on AHL (Case et al., 2008), which is quenched by a variety of enzymes such as lactonase (Dong et al., 2000), acylase/amidohydrolase (Park et al., 2005), and oxidoreductase (Uroz et al., 2005). Considering the fact that 2–10% of culturable bacteria in soil showed AHL hydrolysis activity (Park et al., 2005), the potential of the largely diverse unculturable bacteria has remained unknown (Riaz et al., 2008).

Functional metagenomics has been reported as a promising strategy for discovering novel quorum-quenching enzymes such as lactonases (Bijtenhoorn et al., 2011a). However, metagenomics-based screening often faces the problem of failed or weaker expression of foreign proteins in *Escherichia coli*. To overcome this problem, an intracellular screening method has been developed using a *luxI-luxR* system with the *gfp* gene as a reporter. This method has led to the isolation of novel quorum-sensing related enzymes (Williamson et al., 2005). In this study, we report four novel candidate metagenomic esterases, capable of hydrolyzing 3-OH PAME and AHLs, from an industrial waste-contaminated soil metagenome.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions, and DNA manipulation

Escherichia coli strains DH5α, HB101, EPI300, BL21(DE3), and JB525 (MT102 carrying pJBA132; Andersen et al., 2001) were grown at 37 °C in Luria-Bertani (LB) broth or on LB agar containing the appropriate antibiotics. *E. coli* JB525 was kindly provided by Jo Handelsman, University of Wisconsin, Madison, USA. *R. solanacearum* strain GMI1000 was used in these experiments and was routinely cultured in casamino acid-peptone-glucose (CPG) medium (Schaad et al., 2001) or CPG broth supplemented with 0.005% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) (Kelman, 1954) and 1.5% agar. The following concentrations of antibiotics were used for *E. coli* strains: ampicillin, 100 μg/mL; chloramphenicol, 50 μg/mL; kanamycin, 50 μg/mL; tetracycline, 10 μg/mL. pCC1FOS (Epicentre, Madison, USA) and pUC119 were used to construct metagenomic libraries and to subclone the genes encoding for lipolytic and 3-OH PAME hydrolysis activity, respectively. A broad-host-range vector pRK415 (Keen et al., 1988) carrying metagenomic 3-OH PAME hydrolase genes was used to transform *R. solanacearum* GMI1000. Most recombinant DNA manipulation techniques were carried out using previously described standard methods (Sambrook et al., 1989).

2.2. Metagenomic library construction and screening for lipolytic metagenomic clones

Soil samples were collected from the Gam-geon stream (Sasang-Gu, Busan, Republic of Korea) (coordinates; 35.148476, 128.976000) (Khan et al., 2016), which receives combined sewage effluent from various industries, as this area has been highly urbanized by a number of industries since 1968. The Gam-geon stream eventually meets the Nakdong River, which in turn converges to the East Sea, a marginal sea of the Pacific Ocean, and forms a unique ecosystem. Metagenomic DNA was isolated and purified as previously described (Zhou et al., 1996).

The metagenomic libraries were constructed and stored following the protocol described previously (Tao et al., 2011) and metagenomic libraries previously constructed from various soils were also used in this study (Kim et al., 2016a). Lipolytic metagenomic clones were selected on LB agar medium supplemented with 1% tributyrin as previously described (Lee et al., 2004). To emulsify tributyrin in the medium, LB agar medium was sonicated and poured into petri dishes. The metagenomic clone pools were serially diluted with 0.5X diluent (0.5 g NaCl, 0.15 g KH_2PO_4, 0.3 g Na_2HPO_4, 0.1 g $MgSO_4$, and 0.05 g gelatin per liter) and spread onto the medium followed by 4 days of incubation at 37 °C. The metagenomic clones showing a clear zone around a single colony, indicating tributyrin hydrolysis, were selected. Pure cultures of lipolytic clones were processed for fosmid isolation followed by *Bam*HI restriction digestion, and the unique clones were finally selected based on restriction profiles.

2.3. Subcloning of lipolytic clones with 3-OH PAME hydrolytic activity

To identify the genes involved in tributyrin hydrolysis from lipolytic clones, secondary libraries were constructed in pUC119 as previously described (Lee et al., 2004) and unique lipolytic clones were selected. The selected subclones were further tested for 3-OH PAME hydrolysis using gas chromatography (GC). Metagenomic lipolytic subclones were initially grown in LB broth medium for 1 day at 37 °C in a shaking incubator followed by an additional incubation for 1 day at 4 °C in static conditions. Culture supernatants were collected by centrifugation at 13,200 rpm for 10 min and were filtered through a membrane filter with 0.25 μm pores (Corning SFCA type). Filtered supernatants (300 μL) were mixed with the reaction mixture (500 μL of 1.5 mM 3-OH PAME solution in DMSO, 10 mM sodium phosphate buffer, pH 7.0) and incubated for 12 h at 37 °C. To extract the remaining 3-OH PAME, 800 μL dichloromethane (DCM) was added to the reaction mixture and this step was repeated twice to maximize 3-OH PAME extraction. The extracted 3-OH PAME solution in DCM was used for GC analysis to estimate the remaining 3-OH PAME. GC analysis was performed on an Agilent 7890A GC system equipped with a 30 cm long, 0.32 mm diameter, (5%-phenyl)-methylpoly-siloxane coated column (Agilent, USA). The hydrogen carrier flow rate was 3 mL/min, the injector temperature was 300 °C, and the column temperature was adjusted to 150 °C for 5 min followed by an increase of 5 °C/min to 280 °C (Flavier et al., 1997; Shinohara et al., 2007).