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ORIGINAL ARTICLE

Transcriptome analysis of *Pseudomonas* sp. from subarctic tundra soil: pathway description and gene discovery for humic acids degradation

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Abstract

Although humic acids (HA) are involved in many biological processes in soils and thus their ecological importance has received much attention, the degradative pathways and corresponding catalytic genes underlying the HA degradation by bacteria remain unclear. To unveil those uncertainties, we analyzed transcriptomes extracted from *Pseudomonas* sp. PAMC 26793 cells time-dependently induced in the presence of HA in a lab flask. Out of 6288 genes, 299 (microarray) and 585 (RNA-seq) were up-regulated by > 2.0-fold in HA-induced cells, compared with controls. A significant portion (9.7% in microarray and 24.1% in RNA-seq) of these genes are predicted to function in the transport and metabolism of small molecule compounds, which could result from microbial HA degradation. To further identify lignin (a surrogate for HA)-degradative genes, 6288 protein sequences were analyzed against carbohydrate-active enzyme database and a self-curated list of putative lignin degradative genes. Out of 19 genes predicted to function in lignin degradation, several genes encoding laccase, dye-decolorizing peroxidase, vanillate *O*-demethylase oxygenase and reductase, and biphenyl 2,3-dioxygenase were up-regulated > 2.0-fold in RNA-seq. This induction was further confirmed by qRT-PCR, validating the likely involvement of these genes in the degradation of HA.

Keywords Biodegradation · Degradation pathway · Humic substances · Low temperature · Soil bacteria

Introduction

Humic substances (HS), primarily humic acids (HA) and fulvic acids (FA), are ubiquitous and extremely important natural organic compounds found throughout the environment, including that of the Arctic and Antarctic. HS are the largest constituent (60–80%) of the total soil organic matter (Stevenson 1994; Gramss et al. 1999; Wang et al. 2016) and are involved in many biological processes in soils such as plant nutrition and degradation and transport of hydrophobic organic chemicals. HS are formed by a condensation reaction (humification) of biomolecules (primarily lignin, its transformation products, and small organic matter) originating from the decay and transformation of plants and other dead organisms (Grinhut et al. 2007). Therefore, HS are considered to be modified lignins and are extremely resistant to biodegradation due to their structural complexity, which is intrinsic in the lignin structure.

Over the past a few decades, the Arctic and Antarctic regions are undergoing rapid warming and the rising temperature has led to various changes in environment and biological responses such as enhanced microbial metabolic activity. Although soil microbes play a critical role in soil nutrient cycling as decomposers, little is known about their responses to the current rapid warming in cold terrestrial ecosystems (Park et al. 2015; Newsham et al. 2016). For example, tundra ecosystems are important for their high accumulation of organic carbon attributed to low microbial degradative activity at low temperatures. Consequently, soil microbes could mineralize more organic carbon, such as HS, and significantly affect the carbon dynamics of the tundra ecosystem (Lee et al. 2013).

Indigenous soil bacteria play a critical role in HS degradation because they are both abundant and diverse, possessing

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numerous versatile catabolic pathways that may be involved in this process (Van Trump et al. 2006; Park et al. 2015). Indeed, many soil bacteria have been isolated under various physiological conditions (Dari et al. 1995; Esham et al. 2000; Park and Kim 2015) and characterized based on their ability to metabolize HA (a principal component of HS). However, despite the fact that HA biodegradation by soil *Streptomyces* strains was reported to be associated with extracellular peroxidase (Dari et al. 1995), the precise mechanisms underlying HA transformation and degradation remain unknown.

Various approaches have been used to explore the metabolic pathways for the degradation of organic compounds at both the biochemical and genetic levels. These approaches include the identification of metabolites accumulated by degradation-defective mutants, characterization of degradative enzymes, bioconversion experiments for metabolic intermediates, and DNA-stable isotope probing (Kim et al. 2002; Kim et al. 2004; Sul et al. 2009). Recently, techniques for global transcriptional profiling, including DNA microarray and RNA sequencing (RNA-seq), have been applied to the detection of catalytic enzyme-coding genes and the identification of pathway-specific genes (Im et al. 2007; Cruz et al. 2015). We predict that the application of these methods to the bacterial biodegradation of HS will allow the identification of novel enzymes, catalyzing various oxidation and cleavage reactions, which are involved in numerous aspects of this degradation process.

Pseudomonas sp. PAMC 26793 is a bacterium that was isolated from Alaskan tundra soil based on its ability to efficiently degrade HA and deposited to Polar and Alpine Microbial Collection (PAMC) established by Korea Polar Research Institute. Notably, PAMC 26793 shows significant differences in its ability to grow on various monocyclic aromatics, which are putative HA metabolites. Previous studies showed that during growth on HA at 15 °C, PAMC 26793 is able to both depolymerize (degradation) and repolymerize (further humification) HA to higher molecular compounds (Park and Kim 2015). Thus, an analysis of the PAMC 26793 transcriptome would provide some essential information on the genetic basis of the HA-degradative mechanisms utilized by this bacterium. Here, we combine microarray and RNA-seq analysis of PAMC 26793 cells induced by HA to identify putative genes involved in HA degradation and metabolism, potentially uncovering bacterial factors that are critical for microbial HS (HA and FA) degradation process.

Materials and methods

Growth characterization on HA and FA

Pseudomonas sp. PAMC 26793 was inoculated into mineral salts base (MSB) broth containing 5 mmol/L glucose and incubated for 3 days at 15 °C with shaking. After culturing, the remaining glucose in cell culture was removed by washing the cells with glucose-free MSB. The cell suspension was then transferred to 50 mL fresh MSB containing an HS component (1 g/L HA or FA) as the sole carbon source, to an absorbance at 600 nm of 0.1. The natural HA and FA ($NHA_{AK\ 1\text{-}75}$ and $NFA_{AK\ 1\text{-}75}$, respectively) extracted from AK 1-75 soil (Park and Kim 2015) were dissolved in 0.1 N NaOH. During culturing for 23 days at 15 °C, at the indicated time intervals, 100 μL samples of the culture were diluted to 10^{-4} in MSB and 100 μL of this dilution was plated on MSB containing 5 mmol/L glucose. After incubation for 5 days at 15 °C, colony forming units (CFU) per milliliter obtained after growth on glucose was calculated.

Cell culture and RNA isolation

PAMC 26793 cells grown in 500 mL MSB/20 mmol/L glucose at 15 °C for 24 h were harvested, washed with fresh MSB, and resuspended in 500 mL MSB. The cell suspension was split into 50 mL aliquots, and each was induced by glucose (5 mmol/L) or $NHA_{AK\ 1\text{-}75}$ (0.5 g/L) and incubated with shaking at 15 °C for 1–8 days in the dark. The induced cells were pelleted (8000*g*, 30 min, 4 °C) and stored at − 80 °C until required. Total RNA from each sample was extracted using TRIzol reagent (Invitrogen, USA) or the RNeasy Midi Kit (QIAGEN, USA) with on-column DNase treatment, according to the manufacturer's instructions. The resulting pellet was dissolved in RNase-free water, and the concentration and integrity of the RNA were determined by spectrophotometry and electrophoresis, respectively. Total isolated RNA was stored at − 80 °C until use.

Microarray and data analysis

Microarray assays were performed using a custom-designed, Agilent-based platform, with 4 × 44 K probes per slide. A total of 6288 coding sequences from the PAMC 26793 genome (GenBank assembly accession no. GCA_000313235.1) was downloaded from the EzBioCloud database, and 60-mer oligo probes were designed. The target cRNA preparation was performed using an Agilent's Low Input Quick Amp WT Labeling kit, two-color (Agilent Technology, USA), according to the manufacturer's instructions. The amplified and labeled cRNA was purified on an RNeasy mini spin column (QIAGEN) and then fragmented and hybridized onto the assembled 44 K microarray at 65 °C for 17 h.

The hybridized images were analyzed by an Agilent DNA microarray scanner, and data quantification was performed using Agilent Feature Extraction software, version 9.3.2.1. Data normalization and the selection of up- and down-regulated genes were performed using GeneSpringGX 7.3.1 (Agilent Technology). Since a cutoff expression ratio higher

than 2.0 is generally used to determine up- or down-regulated changes (Cruz et al. 2015; Yang et al. 2016), PAMC 26793 genes showing greater than a 2.0-fold change in expression were selected and considered to be differentially expressed.

RNA sequencing and data analysis

RNA-seq and the associated data analysis were conducted by ChunLab (Korea). A Ribo-Zero rRNA removal kit (Epicentre, USA) was used for ribosomal RNA depletion, and the libraries for Illumina sequencing were made with a TruSeqStranded mRNA Library Prep Kit (Illumina, USA). RNA sequencing was then performed on the Illumina HiSeq 2500 platform, using single-end 50 bp sequencing.

The sequence data for the PAMC 26793 reference genome was downloaded from the EzBioCloud database, and quality-filtered reads were aligned to the reference genome sequence using Bowtie2. The relative log expression (RLE) method was then applied to total read count normalization of RNA-seq data (Anders and Huber 2010). The evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database was used to cluster genes into functionally related groups, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze metabolic pathways. All ORFs on the reference genome were translated to amino acids using the CLgenomics program (ChunLab), and these data were searched for lignin degradation enzymes against the carbohydrate-active enzyme (CAZy) database (Cantarel et al. 2009) using carbohydrate-active enzymes annotation tools (CAT; http://mothra.ornl.gov/cgi-bin/cat/cat.cgi) (Park et al. 2010). Visualization of mapping results and differentially expressed gene analysis were performed using the CLRNASeq program (ChunLab).

mRNA quantification by real-time PCR

Total RNA was extracted from PAMC 26793 cells using TRIzol reagent (Invitrogen), and reverse transcription was performed using SuperScript II RTase (Invitrogen), according to the manufacturer's instructions. The cDNA was then amplified using the following primer pairs: (5′ → 3′) D109_01855 (forward, CAGTGCGATTACCGCCTGAT; reverse, TGGAGGCGTGAACGTAGCTT), D109_01860 (forward, ACGTGCGGCGTTTGTACAG; reverse, TGCTCATC GAAATGCAGGAA), D109_16180 (forward, CAACTTCC GGAAACGATTGA; reverse, GGTAAATGTCGGCC ATGAAC), D109_20410 (forward, ACCAGATCAGCTCG CAGTTG; reverse, AGGCACTGGCTGATGATCTC), and 16S rRNA (forward, GGGAACTGCATTCAAAACTG; reverse, TTCACCGCTACACAGGAAAT).

Following reverse transcription, real-time quantitative reverse transcription polymerase chain reaction (qRT-RCR) was performed on a StepOnePlus Real Time PCR System (Applied Biosystems, USA) using the SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions. Thermal cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 and 30 s at optimal Tm (59 °C). The data were analyzed using StepOne software v2.2.2 (Applied Biosystems). The expression levels of each mRNA were normalized to the 16S rRNA gene as an endogenous control and fold inductions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results

Characterization of HS degradation in PAMC 26793

Pseudomonas sp. PAMC 26793 was cultured in MSB containing $NHA_{AK\ 1\text{-}75}$ or $NFA_{AK\ 1\text{-}75}$, and growth on each substrate was measured by calculating CFU values over time (Fig. 1). In both conditions, when incubated at 15 °C, PAMC 26793 viability increased for up to 10-day post-inoculation and then slowly decreased, whereas it did not grow in MSB medium lacking either substrate. As shown in Fig. 1, we observed that PAMC 26793 showed a faster growth rate with higher total CFU values on HA ($83.5 \pm 1.5 \times 10^6$ CFU/mL after 10 days) compared with FA ($44.5 \pm 5.7 \times 10^6$ CFU/mL after 10 days), suggesting that natural HA are more readily utilized by this organism.

Microarray analysis to identify genes differentially expressed in the presence of HA

We then performed a preliminary transcriptional profiling on several samples that were induced by HA for various times. Total RNA was isolated from PAMC 26793 cells induced by $NHA_{AK\ 1\text{-}75}$ for 1–8 days and subjected to microarray analysis using custom-designed arrays. Differential expression

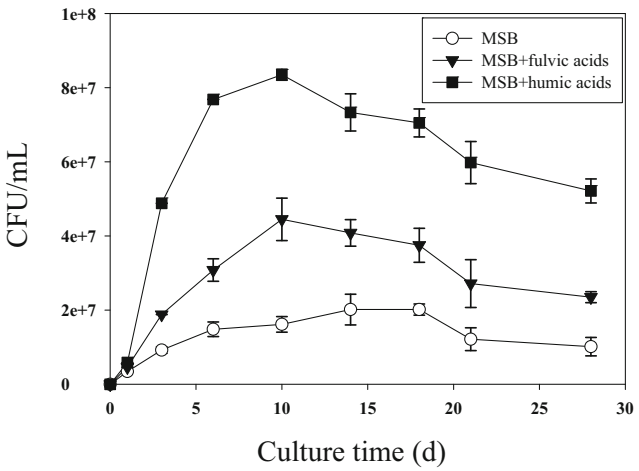

Fig. 1 Viability over time of *Pseudomonas* sp. PAMC 26793 grown on humic acids (HA) and fulvic acids (FA) at 15 °C

analysis of the PAMC 26793 genes represented on the oligonucleotide array revealed that upon exposure to HA, 299 (4.8%), 23 (0.4%), 92 (1.5%), and 30 (0.5%) genes of the total 6288 genes increased by at least 2.0-fold (gene expression ratio ≥ 2.0) at 1, 2, 4, and 8 days, respectively, compared with control cells induced by glucose. Conversely, 675 (10.9%), 369 (5.9%), 594 (9.6%), and 181 (2.9%) genes were down-regulated by at least 2.0-fold (expression ratio ≤ 0.5) at these same time points (Supplementary Table S1). These results suggest that genes involved in the degradation of high-molecular weight, complex HA maybe significantly induced by PAMC 26793 cells, and 1-day exposure to HA is sufficient to produce to the highest number of changes in the gene expression profile. Among the 299 genes up-regulated in cells induced by HA for 1 day, 39% (117 out of 299) could be assigned to orthologous groups using the eggNOG database. These genes include inorganic ion transport and metabolism (eggNOG category P, 15 genes), amino acid transport and metabolism (E, 14 genes), transcription (K, 13 genes), and cell wall/membrane/envelope biogenesis (M, 11 genes). However, a large portion (61%) of these 299 up-regulated genes are of unknown function and, as such, are hypothetical proteins not assigned in the eggNOG database.

RNA-sequencing analysis to identify genes differentially expressed in the presence of HA

Our microarray analyses of the PAMC 26793 transcriptome suggested that HA induce expression of a number of genes involved in general housekeeping functions. To confirm the results of this analysis and further investigate the specific processes and functions influenced by HA, RNA-seq analysis was performed using the mRNA extracted from PAMC 26793 cells induced with HA for 1 day. Differential gene expression analysis revealed that 585 (9.3%) and 445 (7.1%) genes of the total 6288 genes on PAMC 26793 genome were significantly up-and down-regulated, respectively, after 1-day induction by HA as compared to control cells, using a twofold cutoff (DESeq2 P value ≤ 0.05).

We then performed a functional classification of the global transcriptome using eggNOG and found that HA induce considerable changes (up-regulation) in the expression of genes involved in the transport and metabolism of amino acids (eggNOG category E, 72 genes), inorganic ions (P, 34 genes), and carbohydrates (G, 35 genes), as well as transcription (K, 66 genes) (Fig. 2, Supplementary Table S2). Critically, in both our microarray and RNA-seq global transcriptomic analyses, we observed an up-regulation of genes involved in the transport and metabolism of small molecule compounds in HA-induced cells. Because HA are composed of heterogeneous biopolymers, we expect that these genes are involved in the uptake and further metabolism of HA-derived compounds. However, in both of our analyses, a large portion (34.9% of the total 585 up-regulated genes in RNA-seq analysis) of genes affected by HA were annotated as general function (R), unknown function (S), or function not assigned (NA).

Targeted transcriptome analysis for HA degradation

To detect the specific genes involved in lignin and/or HA degradation, all 6288 predicted protein sequences from the PAMC 26793 genome were analyzed against the CAZy database. Based on this analysis, a total of 836 proteins were assigned to five main enzyme classes: glycoside hydrolases (GH, 38.0%), glycosyltransferases (GT, 37.0%), carbohydrate esterases (CE, 12.2%), non-catalytic carbohydrate-binding modules (CBM, 8.4%), and auxiliary activities (AA, 3.2%). Within the AA class, which includes the eight families of lignin degradation enzymes, a catalase-peroxidase (D109_01850) and two laccase (D109_05705 and D109_24245) genes were detected in the PAMC 26793 genome. However, their expression changes in HA-induced cells were not significant (ratio of 0.8–1.0 in RNA-seq analysis) compared with those in glucose-induced cells, indicating that none are likely to be involved in HA degradation. Additionally, these protein sequences were subjected to KEGG annotation to search for lignin degradation pathways and enzymes; however, this was unsuccessful, possibly because the genome sequence is poorly mapped to the KEGG pathways.

As an alternative to the CAZy database, we made a curated list of the bacterial and fungal genes that are known to take part in the different metabolic pathways for lignin and lignin-derived compound degradation (Bugg et al. 2011a, b). Using this gene collection as a reference, 19 PAMC 26793 genes were selected as likely to be involved in the degradation of lignin and lignin-derived compounds, and the changes in expression of these genes in our transcriptomic profile are shown in Table 1. It is noteworthy that of the 11 up-regulated genes, deferrochelatase/peroxidase (D109_11515), multi-copper polyphenol oxidoreductase laccase (D109_16180), and dye-decolorizing peroxidase (DyP, D109_27265) were significantly expressed in HA-induced cells compared with glucose-induced cells. Bacterial laccases and laccase-like multi-copper oxidases have been discovered that can oxidize β-*O*-4 linkages in phenolic compounds (e.g., lignin and humic compounds), resulting in the production of small molecular weight lignin-derived compounds (Freedman and Zak 2014). More recently, bacterial DyPs were characterized to initiate the lignin depolymerizationvia C_α–C_β bond cleavage in β-aryl ether structures within lignin polymer, producing vanillin as a main intermediate (Ahmad et al. 2011; Lin et al. 2016). Two genes catalyzing a central metabolic process during lignin degradation were also significantly expressed in HA-induced cells: vanillate *O*-demethylase oxygenase and reductase (D109_01855 and D109_01860), which converts vanillic acid

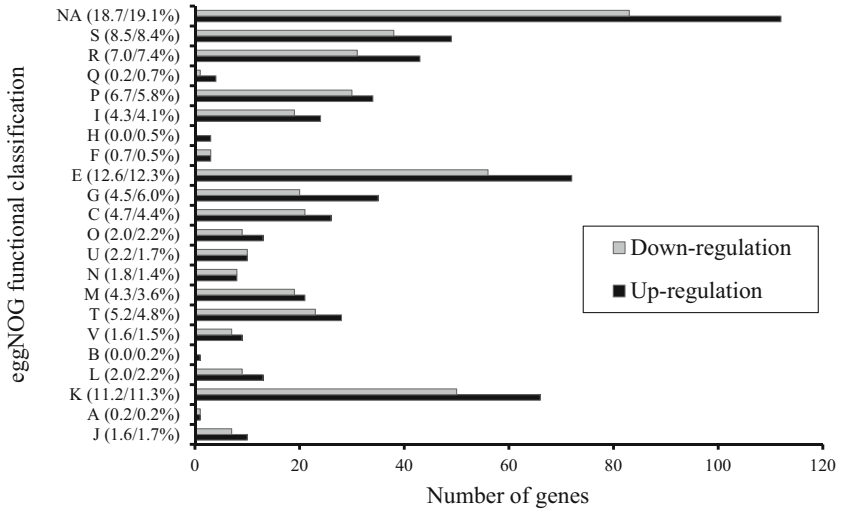

Fig. 2 eggNOG functional classification of genes differentially expressed in HA-induced PAMC 26793 cells. Numbers in parentheses indicate percentage of genes in each category out of the 445 down-regulated genes and the 585 up-regulated genes, which were defined by RNA-seq analysis using a twofold cutoff (DESeq2 P value ≤ 0.05). Categories from eggNOG functional annotation: *J*, translation, ribosomal structure, and biogenesis; *A*, RNA processing and modification; *K*, transcription; *L*, replication, recombination, and repair; *B*, chromatin structure and dynamics; *V*, defense mechanisms; *T*, signal transduction; *M*, cell wall/membrane/envelope biogenesis; *N*, cell motility; *U*, intracellular trafficking, secretion, and vesicular transport; *O*, posttranslational modification, protein turnover, chaperones; *C*, energy production and conversion; *G*, carbohydrate transport and metabolism; *E*, amino acid transport and metabolism; *F*, nucleotide transport and metabolism; *H*, coenzyme transport and metabolism; *I*, lipid transport and metabolism; *P*, inorganic ion transport and metabolism; *Q*, secondary metabolites biosynthesis, transport, and catabolism; *R*, general function prediction only; *S*, function unknown; *NA*, not annotated

to protocatechuic acid. It has been reported that the lignin catabolic pathways in several bacteria lead to the production of vanillin or its oxidation product vanillic acid, which is converted via demethylation to protocatechuic acid. Protocatechuic acid can be degraded by many soil bacteria via oxidative *ortho*- and/or *meta*-ring cleavage using catechol

Table 1 Fold changes in expression, as measured by RNA-seq, for genes predicted to be involved in the degradation of lignin and lignin-derived compounds in *Pseudomonas* sp. PAMC 26793cells induced in the presence of HA (1 day) compared with those grown in glucose

Gene ID	Putative gene product and function (Swiss-Prot accession no., identity)	Ratio[a]
D109_00320	Glutathione *S*-transferase, cleavage of β-aryl ether (P30347, 36%)	1.1
D109_01690	Dye-decolorizing peroxidase (Q47KB1, 36%)	1.0
D109_01850	Catalase-peroxidase (Q4ZNN4, 89%)	0.8
D109_01855	Vanillate *O*-demethylase oxygenase, demethylation of vanillate to protocatechuate (O05616, 78%)	*2.6*
D109_01860	Vanillate *O*-demethylase reductase, demethylation of vanillate to protocatechuate (O54037, 78%)	*2.8*
D109_05705	Laccase, ligninolytic phenoloxidase (Q02497, 27%)	1.0
D109_07990	Anthranilate 1,2-dioxygenase large subunit (O85673, 32%)	1.1
D109_11515	Probable deferrochelatase/peroxidase (P76536, 30%)	*3.6*
D109_16180	Multi-copper polyphenol oxidoreductase laccase (P33663, 71%)	*2.3*
D109_20400	Anthranilate 1,2-dioxygenase electron transfer component (O85675, 62%)	1.4
D109_20405	Biphenyl 2,3-dioxygenase subunit beta (Q52439, 28%)	1.5
D109_20410	Biphenyl 2,3-dioxygenase subunit alpha (Q52438, 27%)	*2.0*
D109_20435	Catechol 1,2-dioxygenase (P07773, 55%)	0.3
D109_22815	Biphenyl-2,3-diol 1,2-dioxygenase (P47231, 25%)	1.0
D109_23100	Homoprotocatechuate 2,3-dioxygenase (Q05353, 62%)	1.7
D109_24245	Laccase, ligninolyticphenoloxidase (Q70KY3, 30%)	0.8
D109_25335	Protocatechuate 3,4-dioxygenase alpha chain (P20371, 45%)	0.6
D109_25340	Protocatechuate 3,4-dioxygenase beta chain (P15110, 62%)	1.0
D109_27265	Dye-decolorizing peroxidase, Tat-dependently exported enzyme (Q47KB1, 30%)	*2.2*

[a] Ratio of RLE values (humic acid-induced/glucose-induced PAMC 26793 cells); changes > 2.0-fold are highlighted in italics

dioxygenase enzymes (Fig. 3). Additionally, one protein (D109_20410) that catalyzes aromatic ring dioxygenation of biphenyl was highly expressed in HA-induced cells. It has been reported that many soil bacteria possessing initial aromatic dioxygenases, such as biphenyl 2,3-dioxygenase, have natural abilities to degrade lignin and its by-products (Bugg et al. 2011b).

Validation of transcriptome analysis by qRT-PCR

To confirm the changes in expression levels of those genes identified using RNA-seq analysis, qRT-RCR was performed with primers specific for the four genes showing significant up-regulation in the presence of HA and predicted to be involved in lignin/lignin-derived compound degradation (polyphenol laccase, vanillate *O*-demethylase oxygenase and reductase, and biphenyl 2,3-dioxygenase). The 16S rRNA gene was used as an endogenous control for gene expression. As shown in Fig. 4, mRNA expression levels of these genes in HA-induced cells were significant (expression fold change > 1.7) compared with those in glucose-induced cells, validating that they are induced in the presence of HA and could be involved in HA degradation.

Discussion

We investigated the global changes in transcription that occur in *Pseudomonas* sp. PAMC 26793 cells when grown with HA as the sole carbon source. To accomplish this goal, we used microarray for its preliminary transcriptional profiling benefits and RNA-seq for its superiority detecting differentially expressed genes. We found a high correlation between gene expression profiles derived from these analyses and, in both cases, observed an up-regulation of PAMC 26793 genes predicted to be involved in the transport and metabolism of small molecular weight compounds in HA-induced cells compared with control cells induced by glucose. Although many of these genes' functions have yet to be identified, they are presumed to be involved in the degradation of HA-derived small molecular weight compounds with one or two aromatic rings that are produced during the HA metabolism. Thus, we predict that some of these genes will be useful for HA-degradative pathway prediction.

Lignin is a complex aromatic heteropolymer formed by radical polymerization of guaiacyl, syringyl, and *p*-hydroxyphenyl units that are linked by β-aryl ether, biphenyl, and heterocyclic linkages (Pollegioni et al. 2015). Due to its structural similarity, lignin has been used as a model

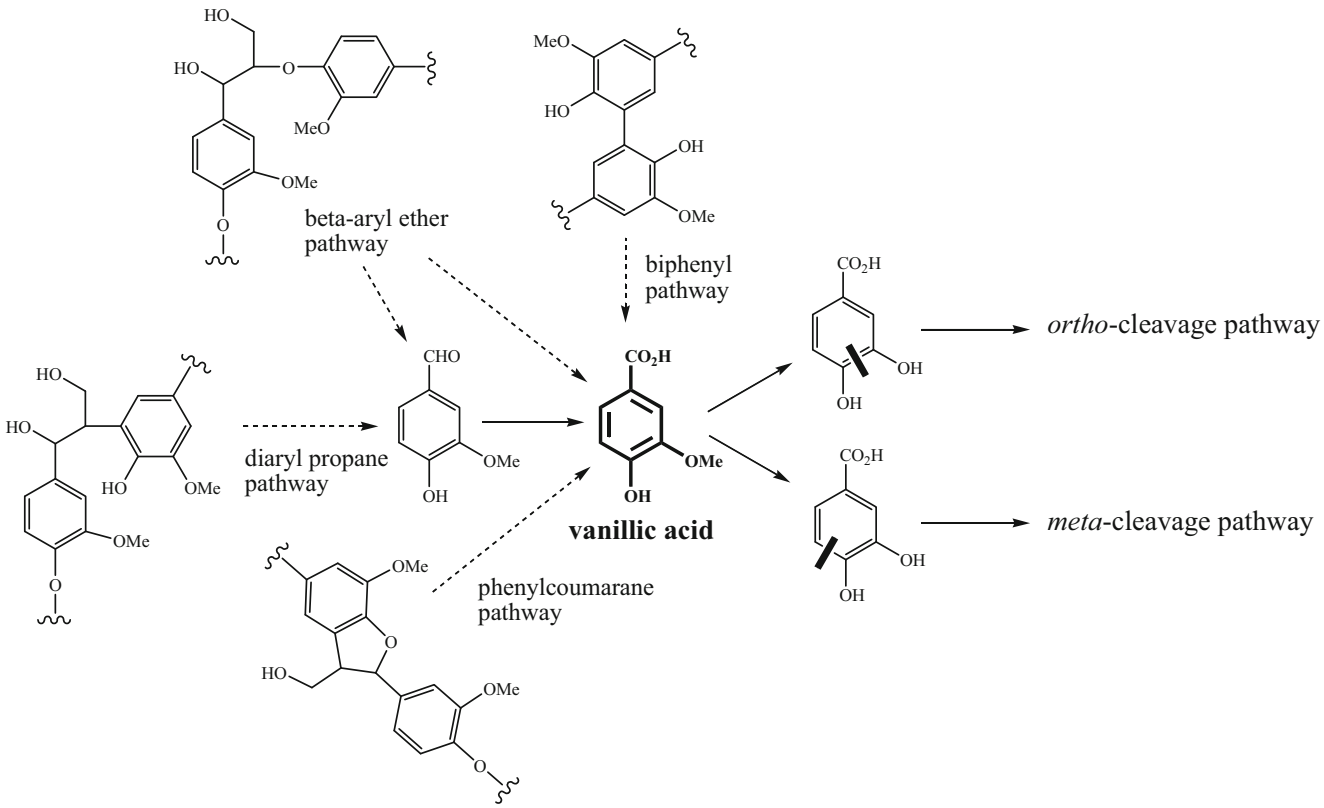

Fig. 3 Catabolic pathways for the degradation of lignin-derived small molecular weight compounds. A central intermediate, vanillic acid, is demethylated to protocatechuic acid, which is further degraded through the *ortho*- and/or *meta*-cleavage pathway. Modified from Bugg et al. (2011a) and Masai et al. (2007)

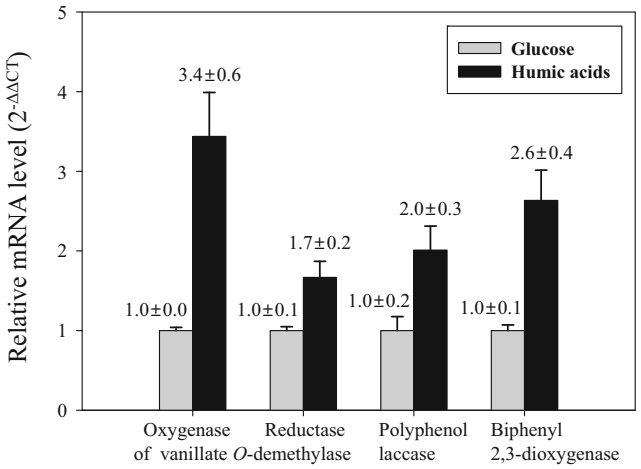

Fig. 4 Real-time quantitative reverse transcription (qRT)-PCR analysis of target gene expression in PAMC 26793 cells induced by HA for 1 day. Relative gene expression in humic acid-induced cells (black bar) is compared with that of cells induced in glucose (gray bar). Normalization using expression of the 16S rRNA gene was performed using the $2^{-\Delta\Delta Ct}$ method

compound for HA in experimental labs for the elucidation of HA degradation pathways. Previous studies using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and Fourier transform ion cyclotron resonance (FTICR) mass spectrometry have suggested that the ligninolytic system of white-rot fungi can lead to HA degradation (Grinhut et al. 2011). Generally, white-rot fungi that secrete extracellular peroxidase and laccase enzymes are known to attack and breakdown lignin, whereas bacteria play a critical role in the mineralization of lignin-derived small molecule compounds. Although the enzymes and genes for bacterial lignin degradation have not been studied as extensively as those of white-rot fungi, there are several reports on the bacterial degradation pathways for the lignin and lignin-derived compounds, such as β-aryl ether, vanillic acid, ferulic acid, biphenyl, diaryl-propane, benzoic acid, and protocatechuic acid (Fig. 3). During lignin degradation and modification, the cleavage of various linkages, including aromatic rings, as well as oxidations and demethylations, is known to be catalyzed by bacterial enzymes, which have been identified from soil pseudomonads and actinomycetes (Masai et al. 2007; Bugg et al. 2011a).

Considering the basic knowledge of microbial lignin degradation described above, we further analyzed the results of our transcriptome analysis using both the CAZy database and a self-customized curated list of genes predicted to be involved in the degradation of lignin and lignin-derived compounds. Our data revealed that genes encoding polyphenol laccase, vanillate *O*-demethylase oxygenase and reductase, and biphenyl 2,3-dioxygenase were up-regulated by more than 2.0-fold in HA-induced cells compared with glucose-induced cells. This induction was validated by qRT-PCR for all four genes (inductions > 1.7-fold). Notably, we also observed that PAMC 26793 showed significant growth on vanillic acid and weak growth on lignin (data not shown).

Additionally, it is worth noting that some bacteria are known to use dye-decolorizing peroxidase (DyP)-based enzymatic system for lignin depolymerization. It is presumed that bacterial lignin depolymerization by DyP is the initial step in lignin degradation, resulting in the formation of various biaryl (e.g., β-aryl ether) and monoaryl aromatic compounds (e.g., vanillic acid). A variety of peripheral pathways are subsequently induced to funnel the various lignin-derived aromatic compounds into a few key intermediates (e.g., protocatechuic acid), which are ring cleaved to central metabolites (e.g., acetyl-CoA and succinyl-CoA) (Ahmad et al. 2011; Lin et al. 2016). In our experiment, a DyP-type peroxidase gene (D109_27265) was 2.2-fold up-regulated in the HA-induced cells. Notably, this DyP contains a highly conserved GXXDG motif and some residues typically conserved among DyP-type peroxidases and is supposed to be secreted via Tat secretion machinery. Although its induction was not confirmed by qRT-PCR, DyP in PAMC 26793 is sufficiently believed to be involved in the initial lignin depolymerization. In another similar induction experiment with *Pseudomonas putida* A514, a plant-associated lignin-utilizing strain, DyP (PputA514_2985) was significantly up-regulated by a protein expression level of $\log_2 E = 6.8$ when grown in the presence of lignin, compared with $\log_2 E = 0$ in glucose-grown cells (control). Expression levels of vanillate *O*-demethylase oxygenase subunit (PputA514_0644) and reductase subunit (PputA514_0645) in A514 cells were $\log_2 E = 12.2$ and 11.6, respectively (Lin et al. 2016). The A514 induction patterns are in a good accordance with those from the PAMC 26793 HA-induced cells: vanillate *O*-demethylase oxygenase and reductase were induced by 2.6- and 2.8-fold, respectively.

Based on the results of these transcriptomic analyses and the general concept of lignin degradation pathways, we predict that PMAC 26793 can degrade HA via catabolic processes similar to the lignin degradation pathways utilized by *Rhodococcusjostii* RHA1 (Ahmad et al. 2011), *Sphingobium* sp. SYK-6 (Bugg et al. 2011b), or *P. putida* A514 (Lin et al. 2016). As in the three bacteria above, extracellular polyphenol laccase and/or DyP secreted from PAMC 26793 could detect any preferred structure containing vanillic acid in HA and breakdown HA by oxidative cleavage, releasing vanillic acid as a main product (a direct attack mechanism). Vanillic acid, which is known as an intermediate resulting from the enzymatic cleavage of the major linkages present in lignin (Sainsbury et al. 2013), is subsequently converted to protocatechuic acid by vanillate *O*-demethylase (oxygenase and reductase) which would be further degraded via the *ortho*- and/or *meta*-cleavage pathway. Alternatively, various small aromatic and aliphatic compounds, which are linked to the HA moiety, could be detached by laccase or DyP and degraded through oxidation reactions by

various dioxygenases, including biphenyl 2,3-dioxygenase (an indirect attack mechanism).

Microorganisms play a critical role in the formation, transformation, degradation, and mineralization of HS. Based on the results of many biochemical and physiological studies, aromatic macromolecules were hypothesized to be degraded by white-rot fungi through a nonspecific, non-stereoselective, and highly oxidative process. Grinhut et al. (2011) confirmed the hypothesis by performing chemical and physical analyses of HA degradation products derived from two white-rot fungi, *Trametes* sp. and *Phanerochaete* sp. However, most of the available studies are focused on observations at the cellular level rather than at the molecular level. To obtain a more comprehensive understanding of this process at the genomic level, in this study, a global transcriptome analysis was used to study the HA degradation pathways in *Pseudomonas* sp. PAMC 26793. Although our results are promising, to date, we remain unable to clarify the precise microbial HA degradation mechanisms. Further studies are needed to address this knowledge gap. However, the assays and corresponding bioinformatics analyses may help researchers identify additional enzymes and catabolic pathways for HS (primarily HA and FA) degradation in soil bacteria and enhance our understanding of carbon cycling mechanisms in natural environments, including those in the Arctic and Antarctic.

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