RESEARCH ARTICLE

A survey of the cellular responses in *Pseudomonas putida* KT2440 growing in sterilized soil by microarray analysis

Yong Wang[1], Sho Morimoto[1], Naoto Ogawa[2] & Takeshi Fujii[1]

[1]Environmental Biofunction Division, National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan; and [2]Faculty of Agriculture, Shizuoka University, Suruga-ku, Shizuoka, Japan

Correspondence: Takeshi Fujii, Environmental Biofunction Division, National Institute for Agro-Environmental Sciences, 3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604, Japan. Tel.: +81 29 838 8262; fax: +81 29 838 8199; e-mail: ftakeshi@affrc.go.jp

Present address: Sho Morimoto, National Agricultural Research Center for Tohoku Region, Fukushima Research Station, 50 Harajukuminami Arai, Fukushima city, Fukushima 960-2156, Japan.

Received 12 April 2011; revised 2 June 2011; accepted 2 June 2011.
Final version published online 4 July 2011.

DOI:10.1111/j.1574-6941.2011.01146.x

Editor: Max Häggblom

Keywords
gene expression; RNA extraction; quantitative RT-PCR; mRNA amplification.

Abstract

Genome-wide scanning of gene expression by microarray techniques was successfully performed on RNA extracted from sterilized soil inoculated with *Pseudomonas putida* KT2440/pSL1, which contains a chloroaromatic degrading plasmid, in the presence or absence of 3-chlorobenzoic acid (3CB). The genes showing significant changes in their expression in both the triplicate-microarray analysis using amplified RNA and the single-microarray analysis using unamplified RNA were investigated. Pathway analysis revealed that the benzoate degradation pathway underwent the most significant changes following treatment with 3CB. Analysis based on categorization of differentially expressed genes against 3CB revealed new findings about the cellular responses of the bacteria to 3CB. Genes specifically involved in the transport of 3CB were upregulated, including a K^+/H^+ antiporter complex, a universal stress protein, two cytochrome P450 proteins and an efflux transporter. The downregulated expression of several genes involved in carbon metabolism and the genes belonging to a prophage in the presence of 3CB was observed. This study demonstrated the applicability of the method of soil RNA extraction for microarray analysis of gene expression in bacteria growing in sterilized soil.

Introduction

Soils contain large numbers of bacteria of numerous species (Torsvik & Øvreås, 2002; Torsvik *et al.*, 2002; Gans *et al.*, 2005; Roesch *et al.*, 2007), but > 99% of these species remain unidentified and are difficult to culture (Torsvik *et al.*, 1990). Sequencing of DNA extracted from soil can provide information on the structure and potential function of soil bacterial communities (Torsvik & Øvreås, 2002; Rajendhran & Gunasekaran, 2008). However, to acquire detailed knowledge of the function of a soil community, gene expression studies are required (Torsvik & Øvreås, 2002; Saleh-Lakha *et al.*, 2005). For example, to gain knowledge of bacterial responses to various factors in the soil environment, including nutrients, oxygen status, pH, pollutants, agro-chemicals, moisture and temperature, it is necessary to extract RNA from the soil and assess bacterial gene expression. Thus, bacterial gene expression in soils is increasingly being studied in various contexts, such as bioremediation (Bælum *et al.*, 2008; Nicolaisen *et al.*, 2008; Wang *et al.*, 2008, 2009), nitrogen cycling (Bürgmann *et al.*, 2003; Sharma *et al.*, 2005; Treusch *et al.*, 2005; Nicol *et al.*, 2008), carbon cycling (Han & Semrau, 2004; Chen *et al.*, 2007; Angel & Conrad, 2009) and bacterial community structure (Ludemann *et al.*, 2000; Urich *et al.*, 2008; Shrestha *et al.*, 2009).

Microarray techniques are powerful tools to monitor gene expression genome-wide, and have been applied extensively to many aspects of biological studies (Watson *et al.*, 1998; Epstein & Butow, 2000). Soon after this methodology became available, soil microbiologists speculated on its usefulness for the detection of bacterial gene expression in soils (Insam, 2001). However, because of the difficulties involved in RNA extraction from soil, these expectations have not yet been realized. One of the major problems in soil RNA extraction is contamination with humic acids, which

FEMS MICROBIOLOGY ECOLOGY

affect the accurate measurement of nucleic acids (Bachoon *et al.*, 2001; Zipper *et al.*, 2003), suppress enzyme activity (Tebbe & Vahjen, 1993) and inhibit hybridization (Alm *et al.*, 2000). Recently, we developed a new strategy to extract bacterial RNA from soils, enabling us to acquire high-quality RNA with very low levels of humic acid contamination, allowing for a quantitative reverse transcription-PCR (qRT-PCR) to obtain reliable gene expression data (Wang *et al.*, 2009). While our ultimate target is to detect bacterial gene expression in raw soil, we reasoned that a useful first step would be the successful microarray analysis of a sterilized soil inoculated with a bacterial strain whose genome had been fully sequenced. In this study, we used *Pseudomonas putida* KT2440 as a target strain, whose genome sequence (Nelson *et al.*, 2002) and catabolic potential against a wide range of natural aromatic compounds (Jiménez *et al.*, 2002) has been determined. We extracted RNA from sterilized soil inoculated with a *Pseudomonas* strain (*P. putida* KT2440/pSL1) containing a plasmid that carried genes for the degradation of chloroaromatic compounds (Liu *et al.*, 2001), in the presence or absence of 3-chlorobenzoic acid (3CB). Microarray analysis and subsequent validation by qRT-PCR provided us with new knowledge of the cellular responses of bacteria to 3CB, including induction of several genes involved in transport and stress response, and downregulation of the genes belonging to a prophage and several genes involved in carbon metabolism.

Materials and methods

Bacterial culture conditions

Pseudomonas putida KT2440/pSL1 (Liu *et al.*, 2001) was transferred from a glycerol stock to an LB agar plate (Sambrook & Russell, 2001) with kanamycin (50 μg mL^{-1}) and incubated at 30 °C overnight. The strain was then subcultured overnight in an LB medium (Sambrook & Russell, 2001) with kanamycin (50 μg mL^{-1}) at 28 °C. A 1-mL volume of the liquid culture ($OD_{600\,nm} = 1.0$) was centrifuged to collect cells. After washing twice with 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.8), cells were resuspended and diluted 10-fold with the same buffer. To prepare bacterial soil cultures, 0.2 mL of the diluted bacterial suspension was mixed with 1.8 g sterilized soil in 15 mL tubes (about 5×10^6 CFU g^{-1} soil), to which 3CB had already been added at a final concentration of 250 μg g^{-1} soil (the 3CB+group). Brown forest soil (FAO classification: Gleyic Cambisols) was sampled from the surface of a field at the Ehime Agricultural Experimental Station in Ehime, Japan (33°50′24″N and 132°46′12″E). Preparation of the sterilized soil samples and the properties of the soil were described previously (Wang *et al.*, 2008). To ensure a homogeneous distribution of 3CB in the soil, 3CB was first mixed with Celite® powder (Wako, Osaka, Japan) as described previously (Morimoto *et al.*, 2005). Equal amounts of Celite powder were added to the control soil samples (3CB − group). The soil cultures were incubated at 30 °C for the appropriate periods. To determine the concentration of 3CB in soil, soil samples were subjected to HPLC analysis with a reversed-phase C18 column as described previously (Morimoto *et al.*, 2008).

RNA extraction from soil

Total RNA was extracted from 2 g of soil culture using an RNA PowerSoil Total RNA Isolation Kit (Mo Bio, Carlsbad, CA) according to the manufacturer's instructions, with some modifications. Centrifugation (2500 ***g***, 10 min, room temperature) was conducted between the cell lysis and the phenol extraction steps to separate the cell lysate from the soil. Purification using a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK), DNase digestion using a TURBO DNA-free DNase (Ambion, Austin, TX) and concentration using an RNeasy Mini Kit column (Qiagen, Valencia, CA) were conducted as described previously (Wang *et al.*, 2009). The concentration of RNA was determined using a Nanodrop system (Nanodrop, Wilmington, DE). The integrity and purity of extracted soil RNA was assessed by agarose gel electrophoresis, UV spectrometry and 23S/16S ratio on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Microarray analysis – single array without RNA amplification

In this analysis, the pooled RNA extracted from 32 (3CB −) and 48 (3CB+) soil cultures was directly applied to microarray analysis. A single microarray was used for each treatment (3CB+ or 3CB −); therefore, two NimbleGen Custom Prokaryotic Gene Expression 385K Arrays were used in this analysis. Each array contained five sets of 14 sequence-specific 60-mer probes per gene corresponding to 5341 genes from the *P. putida* KT2440 genome and five genes (*tfdT*, *tfdC*, *tfdD*, *tfdE* and *tfdF*) from the pSL1 plasmid.

Five micrograms of total RNA was processed and labeled according to the standard protocols from Roche NimbleGen (http://www.nimblegen.com). Briefly, double-stranded cDNA was synthesized using a SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), then labeled with Cy3-random nonamers using a NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen) and hybridized to the microarrays for 16 h at 42 °C on a NimbleGen Hybridization System. The arrays were washed, dried and scanned at 5 μm resolutions using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA). NIMBLESCAN v2.5 (Roche NimbleGen) was used to

extract data from scanned images and to perform quantile normalization (Bolstad *et al.*, 2003) and robust multiarray average (RMA) analysis (Irizarry *et al.*, 2003) across arrays to generate gene expression values. Statistical analysis and fold change calculations were performed using NANDEMO ANALYSIS v1.0.2 (Roche NimbleGen). Student's *t*-test with Bonferroni correction for multiple testing (a total of 5346 ORFs on arrays) was applied to evaluate genes with significantly altered signal intensity.

Microarray analysis – triplicate arrays with RNA amplification

In this analysis, the total RNA extracted from three replicate soil cultures of each treatment (3CB+ or 3CB−) was amplified using a MessageAmp^{TM} II-Bacteria Kit for Prokaryotic RNA Amplification according to the manufacturer's instructions (ABI Ambion, Tokyo, Japan). For each soil culture, 100 ng total RNA was used as the starting material in the RNA amplification reaction. One array was used for each of the triplicate RNA samples in each treatment; therefore, six NimbleGen Custom Prokaryotic Gene Expression 4×72K Arrays were used in this analysis. Each array contained two sets of six sequence-specific 60-mer probes per gene corresponding to 5341 genes from the *P. putida* KT2440 genome. Both the NimbleGen 385K and the 4×72K arrays used in this study were designed and manufactured by Roche NimbleGen.

Amplified RNA (10 μg) was processed and labeled according to the standard protocols from Roche NimbleGen as described above. Data were extracted from scanned images using NIMBLESCAN v2.5 (Roche NimbleGen). The ARRAYSTAR v4.0 software (DNASTAR, Madison, WI) was used to perform quantile normalization (Bolstad *et al.*, 2003) and RMA analysis (Irizarry *et al.*, 2003) across arrays to generate gene expression values, fold change calculation and statistical analysis. Student's *t*-test with a false discovery rate (FDR) correction for multiple testing (a total of 5,341 ORFs on arrays) was applied to evaluate genes with a significantly altered signal intensity.

qRT-PCR

Expression of genes selected from the microarray screening was validated using two-step qRT-PCR; the genes for validation and the corresponding primers are listed in Supporting Information, Table S1. Total RNA (700 ng) extracted from 3CB+ or 3CB− soil cultures (three independent pools of RNA were used and each pool contained RNA recovered from two soil cultures) in either the logarithmic or the transition phase was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions for random hexamer primed reactions. qRT-PCR was performed using SYBR Premix DimerEraser (Perfect Real Time) (Takara, Shiga, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). A 2-μL sample of fourfold diluted cDNA was used as a template in a 20 μL reaction mixture. The final concentration of each primer in the PCR mixture was 300 nM. The reaction conditions were as follows: 30 s at 95 °C for the activation of DNA polymerase, 40 cycles of 5 s at 95 °C and 1 min at the temperature indicated in Table S1, followed by a melting curve stage, which generated curves with continuous fluorescence acquisition from 60 to 95 °C at a rate of 0.3 °C s^{-1}. Standards for the assays were prepared with PCR amplicons from genomic DNA or cDNA of *P. putida* KT2440 or the plasmid pSL1 with the same primers used in the qRT-PCR. A standard curve was constructed by comparing the copy numbers of 10-fold dilutions of the standard with their respective threshold cycles. The amplification efficiencies of all standard curves, which were calculated using STEPONE Software (version 2.1; Applied Biosystems), were $> 90\%$. The negative controls without a template and RT− controls (RNA without reverse transcriptase treatment) for all examined genes showed C_q values at least five cycles higher than those of samples. A recent report found that actually no gene is universally stable enough to serve as a general reference gene to normalize qRT-PCR data, and a subset of stable genes that has smaller variance than commonly used reference genes exists in each biological context (Hruz *et al.*, 2011). Thus, we searched for proper reference genes using the current gold standard method, which combines the evaluation of a set of reference genes together with a method for selecting reference genes with the most stable expression (Huggett *et al.*, 2005; Nolan *et al.*, 2006). The candidate reference genes for normalization of qRT-PCR data were selected according to the following criteria: in both the triplicate-array and the single-array analyses, fold changes were smaller than ± 1.20 and signal intensities were 20 − 60% of the maximum signal on arrays to avoid signal saturation or undetectable signals. Six genes fitted these criteria: flagellar protein FliS (PP_4375); translation initiation factor IF-3 (PP_2466); 50S ribosomal protein L1 (PP_0444); ornithine carbamoyltransferase (PP_1000); flagellar cap protein FliD (PP_4376); and tRNA [guanine-*N*(1)-]-methyltransferase (PP_1464). The best reference gene was selected by evaluation of the qRT-PCR data for these genes as well as for the 16S rRNA gene using BESTKEEPER (Pfaffl *et al.*, 2004). This analysis suggested that the gene encoding the flagellar protein FliS (PP_4375) was the more stable gene. Thus, the expression level of each gene was normalized using the qRT-PCR signal for PP_4375. Student's *t*-test (two-sided) was applied to identify genes with a significantly altered signal intensity. A *P*-value < 0.05 was considered statistically significant.

Analysis of biochemical pathways and cellular responses

To perform automatic biochemical pathway analysis, the Entrez Gene ID numbers of genes to be analyzed were submitted to the DAVID Bioinformatics Resources server (Dennis *et al.*, 2003; Huang *et al.*, 2009), followed by running the Functional Annotation Chart with default settings. The categorization of differentially expressed genes against 3CB was conducted by manual searches on the website of the Comprehensive Microbial Resource (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). The latest information for each differentially expressed gene was confirmed, or updated when necessary, by referring to the Entrez Gene database (http://www.ncbi.nlm.nih.gov/gene). Annotation of hypothetical proteins was carried out by performing a BLASTP search against the NCBI nonredundant protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a domain search against the NCBI CDD (Conserved Domain Database, http://www.ncbi.nlm.nih.gov/cdd) and the Pfam (http://pfam.sanger.ac.uk/) databases. The hypothetical proteins with assigned function by homology search were recategorized accordingly. Manual analysis of biochemical pathways was conducted, where required, by locating differentially expressed genes on the corresponding pathway maps that were downloaded from the KEGG website (http://www.genome.jp/kegg/).

Microarray data accession number

The microarray data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002; Barrett *et al.*, 2011) and are accessible through the GEO Series accession number GSE19516 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19516) and GSE28215 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28215).

Results

Bacterial growth and 3CB degradation in soil

In preliminary experiments, we observed that a 10-fold dilution of the *P. putida* KT2440/pSL1 liquid culture ($OD_{600\,nm} = 1.0$) for soil inoculation was appropriate to generate a growth curve that reached a maximum level within several days (Fig. 1a). Thus, we applied this dilution step to prepare all soil cultures used in this study. The 3CB is degraded completely by the *P. putida* KT2440/pSL1 strain as it possesses genomic genes for the breakdown of 3CB to 3-chlorocatechol, and an introduced plasmid, which contains 3-chlorocatechol degradation genes (Liu *et al.*, 2001). When soil was treated with $250\,\mu g\,g^{-1}$ soil 3CB, this strain degraded 3CB almost completely within 3 days of incubation (Fig. 1b). To detect the expression of 3CB degradation genes in populations at comparable growth stages by microarray analysis, bacterial cells were harvested in the transition phase, 2 days and 1 day after incubation for 3CB+ and 3CB− groups, respectively. For qRT-PCR analysis to validate the data obtained by microarray analysis, bacterial cells were harvested after 1 day (log phase of 3CB+ treatment) or 2 days (transition phase of 3CB+ treatment) and half a day (log phase of 3CB− treatment) or 1 day (transition phase of 3CB− treatment) after incubation.

Quality of RNA extracted from soil

For microarray analysis, the extracted soil RNA possessed the typical UV absorption spectrum of pure RNA, in which the ratios of $OD_{260\,nm}/OD_{280\,nm}$ were > 2.00 and $OD_{260\,nm}/OD_{230\,nm}$ were > 1.95, suggesting the successful removal of impurities such as humic acids during RNA purification. The rRNA ratio (23S/16S) examined using an Agilent 2100 Bioanalyzer was 1.0 for all samples, indicating that the integrity of the extracted RNA was appropriate for

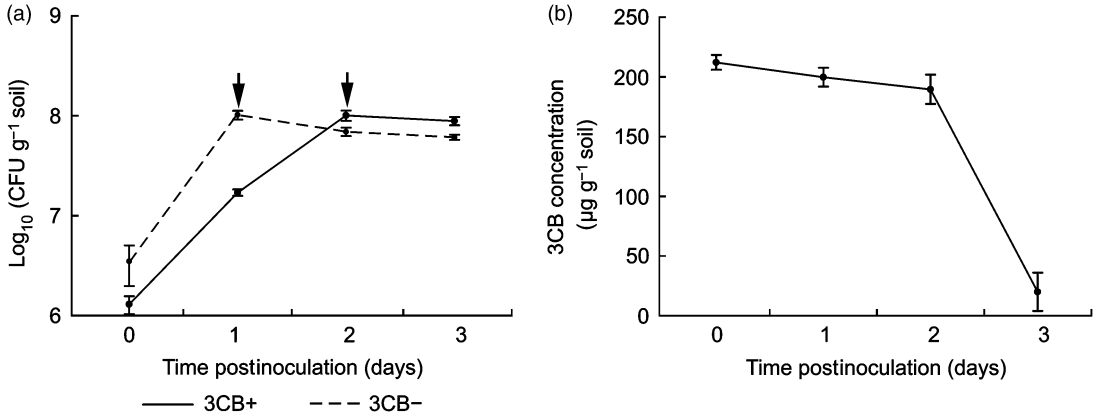

Fig. 1. Time courses of bacterial growth (a) and 3CB degradation (b) of *Pseudomonas putida* KT2440/pSL1 in soil cultures. Arrows indicate the sampling time for microarray analysis.

microarray analysis. The 23S/16S ratio suggested should be ≥ 1.0 as suggested by Roche.

Overview of microarray analysis and qRT-PCR validation

In the triplicate array analysis, 197 genes possessed fold changes higher than three and the maximum FDR *P*-value among these genes was lower than 0.005 (Table S2). In the single array analysis, 217 genes possessed fold changes higher than 3 and low Bonferroni ($P < 0.001$; Table S3). These data suggested that there were no false-positive data among these genes in both analyses. Because false-positive genes could be selected if the threshold of fold change was lower than 3, we used a threefold change as a common criterion to select significantly altered genes from both analyses. The genes showing significant changes in their expression levels in both analyses were collected for further analysis, covering 51 upregulated genes (Table S4) and 59 downregulated genes (Table S5). Among them, the genes involved in 3CB degradation and transport, and major cellular responses were validated by qRT-PCR (Tables 1 and 2).

Expression of genes involved in 3CB degradation and transport

To identify biologically significant cellular responses, we analyzed the 51 upregulated genes and 59 downregulated genes using the DAVID Bioinformatics Resources server. This analysis suggested that benzoate degradation via the hydroxylation pathway was the pathway most significantly affected by 3CB treatment in multiple tests (Bonferroni, $P < 0.001$).

For the benzoate degradation-related genes located in the chromosome (*benABCD* operon), the upregulated expression on microarrays was validated by qRT-PCR (Table 1). It was also confirmed by qRT-PCR analysis that the 3CB degradation genes located in the plasmid (*tfdC*, *tfdD*, *tfdE* and *tfdF*) exhibited strong responses to 3CB treatment in both the log and the transition phases (Table 1).

For small compound transport in Gram-negative bacteria, a porin protein in the outer membrane and transporter proteins in the inner cell membrane are required (Schirmer, 1998; Klebba, 2005). In the *P. putida* KT2440 genome, there were seven candidate genes involved in benzoate transport according to the KEGG gene annotation (http://www.genome.jp/kegg/), including three porin genes (PP_1383,

Table 1. Validation of microarray data by qRT-PCR for genes involved in 3CB degradation and transport

			Microarray (transition phase)				Fold changes in qRT-PCR (3CB+/3CB)*	
			Single-array analysis		Triplicate-array analysis			
Locus tag	Gene	Definition	Fold change†	*P*-values in a *t*-test with Bonferroni correction	Fold change†	*P*-values in a *t*-test with FDR correction	Log phase	Transition phase
3CB degradation								
PP_3161	*benA*	Benzoate dioxygenase, α subunit	6.8	1.7×10^{-8}	18.8	6.1×10^{-5}	36.7 ± 32.6	14.2 ± 2.9
PP_3162	*benB*	Benzoate dioxygenase, β subunit	5.9	1.7×10^{-8}	13.4	2.2×10^{-4}	30.6 ± 27.0	14.0 ± 4.5
PP_3163	*benC*	Benzoate 1,2-dioxygenase ferredoxin reductase subunit	7.8	1.7×10^{-8}	32.3	4.0×10^{-5}	29.2 ± 27.9	19.0 ± 5.1
PP_3164	*benD*	*Cis*-diol dehydrogenase	6.7	1.7×10^{-8}	23.2	2.4×10^{-4}	26.1 ± 16.7	19.8 ± 4.5
Plasmid	*tfdC*	Chlorocatechol 1,2-dioxygenase	1.4‡	2.3×10^{-8}	–	–	71.4 ± 12.6	37.1 ± 6.4
Plasmid	*tfdD*	Chloromuconate cycloisomerase	2.9‡	1.7×10^{-8}	–	–	15.9 ± 4.0	14.6 ± 2.9
Plasmid	*tfdE*	Dienelactone hydrolase	1.1‡	2.4×10^{-3}	–	–	7.0 ± 1.8	8.8 ± 1.8
Plasmid	*tfdF*	Maleylacetate reductase	1.2‡	4.3×10^{-6}	–	–	6.1 ± 1.4	10.8 ± 4.1
3CB transport								
PP_1383		BenF-like porin	1.9	1.7×10^{-8}	2.8	1.4×10^{-4}	6.3 ± 4.6	17.4 ± 2.9
PP_1820		Benzoate Transport protein	1.0	1.0	-1.1	0.05	NC	NC
PP_2035	*benE*-1	Benzoate transport protein	1.0	1.0	1.0	0.72	NC	NC
PP_2517		BenF-like porin	1.1	2.3×10^{-3}	1.1	0.48	NC	NC
PP_3165	*benK*	Benzoate MFS transporter BenK	8.3	1.7×10^{-8}	22.7	6.6×10^{-5}	28.4 ± 25.4	23.5 ± 8.0
PP_3167	*benE*-2	Benzoate transport protein	4.1	1.7×10^{-8}	23.3	4.1×10^{-5}	14.0 ± 11.4	25.4 ± 7.7
PP_3168	*benF*	Benzoate-specific porin	6.4	1.7×10^{-8}	19.8	9.8×10^{-5}	11.0 ± 9.1	19.2 ± 4.5

NC, no change (fold changes with $P > 0.05$ in a *t*-test were considered as no change).

*qRT-PCR was conducted using different pooled samples of RNA.

†A fold-change of 1.0 is the same as no change.

‡The fluorescent signals of genes *tfdC*, *tfdD*, *tfdE* and *tfdF* were saturated on the microarray.

Table 2. Validation of microarray data by qRT-PCR for genes involved in major cellular responses

			Microarray (transition phase)				Fold changes in qRT-PCR (3CB+/3CB −)	
			Single-array analysis		Triplicate-array analysis			
Locus tag	Gene	Definition	Fold change	*P*-values in *t*-test with Bonferroni correction	Fold change	*P*-values in *t*-test with FDR correction	Log phase	Transition phase
Transporter								
PP_2225		Putative monovalent cation/H^+ antiporter subunit G	5.6	1.7×10^{-8}	5.3	1.1×10^{-4}	7.9 ± 1.8	9.3 ± 2.4
PP_2226 (PP_2225 operon)		Putative monovalent cation/H^+ antiporter subunit F	4.9	1.7×10^{-8}	5.1	1.2×10^{-4}	–	–
PP_2227 (PP_2225 operon)		Putative monovalent cation/H^+ antiporter subunit E	4.4	1.7×10^{-8}	5.4	1.4×10^{-4}	–	–
PP_2228 (PP_2225 operon)		Putative monovalent cation/H^+ antiporter subunit D	3.9	1.7×10^{-8}	4.1	1.8×10^{-4}	–	–
PP_1271		Multidrug efflux MFS transporter, putative	3.1	1.7×10^{-8}	4.7	2.8×10^{-4}	11.1 ± 2.0	9.0 ± 2.9
Carbon metabolism								
PP_0552	*adh*	2,3-Butanediol dehydrogenase	−8.4	1.7×10^{-8}	−5.9	1.4×10^{-4}	-3.4 ± 1.1	-1.7 ± 0.8
PP_0553 (adh operon)	*acoC*	Dihydrolipoamide acetyltransferase	−7.4	1.7×10^{-8}	−6.1	6.0×10^{-5}	–	–
PP_0554 (adh operon)	*acoB*	Acetoin dehydrogenase, β subunit	−12.5	1.7×10^{-8}	−6.8	6.2×10^{-5}	–	–
PP_0555 (adh operon)	*acoA*	Acetoin dehydrogenase, α subunit	−8.4	1.7×10^{-8}	−6.2	1.4×10^{-4}	–	–
PP_0557 (adh operon)	*acoR*	Acetoin catabolism regulatory protein	−6.8	1.7×10^{-8}	−4.7	1.4×10^{-4}	–	–
Prophage								
PP_1567		Phage major capsid protein, HK97 family	−8.1	1.7×10^{-8}	−5.2	1.1×10^{-4}	NC	-4.1 ± 0.8
PP_1573		Major tail protein, putative	−11.7	1.7×10^{-8}	−7.8	8.9×10^{-5}	NC	-4.0 ± 0.9
Others								
PP_1269		Universal stress protein family	10.4	1.7×10^{-8}	6.6	1.4×10^{-4}	15.4 ± 4.1	11.6 ± 2.7
PP_1950		Cytochrome P450 CYP199	72.6	1.7×10^{-8}	46.0	9.7×10^{-5}	53.1 ± 23.6	84.5 ± 26.7
PP_1943	*purU*	Formyltetrahydrofolate deformylase	310	1.7×10^{-8}	66.1	6.7×10^{-5}	430 ± 99	662 ± 159

NC, no change (fold-changes with $P > 0.05$ in a *t*-test were considered as no change).

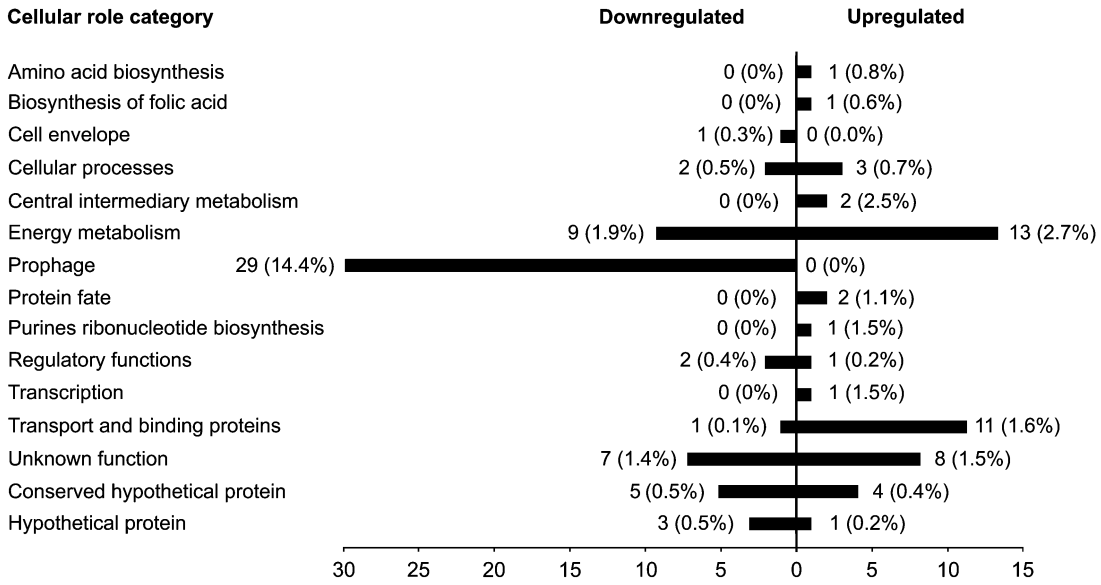

Fig. 2. Number of differentially expressed genes in different functional categories. Percentages in parentheses indicate the ratio of the number of differentially expressed genes to the total number of genes in each category.

PP_2517, *benF*) and four transporter genes (PP_1820, *benK*, *benE*-1, *benE*-2). However, according to our microarray and qRT-PCR data (Table 1), only two transporter genes (*benE*-2 and *benK*) and two porin genes (*benF* and PP_1383) responded to 3CB treatment.

Survey of cellular responses to 3CB

To characterize the cellular responses of *P. putida* KT2440/pSL1 to 3CB, 110 differentially expressed genes (51 upregulated and 59 downregulated genes), which had been used for pathway analysis, were categorized according to their cellular roles as annotated by the Comprehensive Microbial Resource database (Fig. 2, Tables S4 and S5). The categories 'energy metabolism' and 'transport and binding proteins' included 47% of the upregulated genes and the categories 'prophage' and 'energy metabolism' included 64% of the downregulated genes. These suggested that major cellular responses to 3CB occurred in these categories. In terms of the ratio of the number of differentially expressed genes to the total gene number in each category, 'central intermediary metabolism' (2.5%) and 'energy metabolism' (2.7%) were the categories affected most by 3CB exposure, suggesting that important cellular responses also occurred in 'central intermediary metabolism'.

Transport and binding proteins

Among the 3CB responsive genes, 12 were related to transport (11 upregulated and one downregulated; Fig. 2, Fig. 3, Tables S4 and S5). The upregulated genes included transporter genes for benzoate and its analogues and for other compounds (Table 2). The genes coding for predicted subunits of a K^+/H^+ antiporter complex (PP_2225–PP_2228) were upregulated in both the log and the transition phases. As a response to 3CB, the expression of a drug resistance efflux transporter gene (PP_1271) increased in both the log and the transition phases. This suggested that this gene was involved in the extrusion of excess 3CB molecules or its toxic metabolites from *P. putida* KT2440/pSL1 cells, although further study is required to confirm this.

Cytochrome P450 and stress response proteins

The two upregulated genes in the 'central intermediary metabolism' category, PP_1950 and PP_1955, encode cytochrome P450 family proteins, which are involved in the oxidative degradation of various compounds, especially environmental toxins and mutagens (Werck-Reichhart & Feyereisen, 2000). PP_1950 showed a dramatic fold change by the 3CB treatment (72- and 46-fold in the single array and triplicate array analyses, respectively), suggesting a high response to the treatment. In the 'cellular processes' category, PP_1269 encodes a UspA-like universal stress protein. The UspA protein is a small cytoplasmic protein whose expression is enhanced when the cell is exposed to stress agents (Nystrom & Neidhardt, 1994). It is likely that the universal stress protein in strain KT2440 was responsive to the 3CB treatment to protect cells from 3CB molecules or the intermediate product molecules generated during 3CB degradation (Fig. 3).

Carbon metabolism

Genes involved in carbon metabolism were mostly found in the 'energy metabolism' category and included the benzoate

degradation genes. As shown in Table 2, the genes encoding 2,3-butanediol dehydrogenase and the components of the acetoin-cleaving system (PP_0552 to PP_0557), which are required for the conversion of 2,3-butanediol to central metabolites (Huang *et al.*, 1994), were downregulated following 3CB treatment in both the log and the transition phases (Fig. 3), suggesting a reduced consumption of carbon sources other than 3CB in the soil. The utilization of carbon sources was switched from acetoin-related compounds to 3CB in the cells exposed to 3CB in a soil environment.

Prophage genes

There are four prophages in the *P. putida* KT2440 genome (Canchaya *et al.*, 2003). Among 53 genes (from PP_1532 to PP_1584) belonging to phage 04, 29 genes were downregulated in the cells exposed to 3CB (Table S5 and Fig. 3). The qRT-PCR data of the genes encoding a capsid protein (PP_1567) and a major tail protein (PP_1573) confirmed the microarray data (Table 2). In the 3CB − samples, the expression levels of these two prophage genes were increased at the transition phase (1.8 ± 0.1-fold for PP_1567 and 2.3 ± 0.3-fold for PP_1573, $P < 0.05$, Student's *t*-test) compared with the log phase, but such a tendency was not observed in the 3CB+ samples.

Discussion

For successful microarray analysis, a large quantity of high-quality RNA is required. It is easy to obtain sufficient quantities of pure RNA (tens of micrograms) from a liquid culture. Conversely, the RNA yield from soil is low, ranging from tens to hundreds of nanograms per gram of soil (Wang *et al.*, 2008). For this reason, large amounts of source soil and concentration of the extracted RNA are required before microarray analysis. Furthermore, humic acids coextracted with nucleic acids from the soil interfere with microarray analysis. Because humic acids can be concentrated together with nucleic acids during ethanol precipitation (Torsvik, 1980), the coextracted humic acids need to be reduced to an extremely low level before the final concentration procedure. These problems make it difficult to analyze gene expression of microorganisms living in soils using microarray methods. Recently, we reported a method to overcome these problems, in which approximately 99.9% of the humic acids could be removed (Wang *et al.*, 2008, 2009). To render the method more feasible for routine laboratory use so that the microarray data can be compared with those obtained in future studies, we used a combination of commercial kits (PowerSoil Total RNA Isolation Kit plus MicroSpin S-400 HR spin column) to purify soil RNA, which proved to be highly successful in removing humic acids from soil RNA (Wang *et al.*, 2009). To establish the reliability of the method, we conducted microarray analyses using RNA extracted from sterilized soil inoculated with a single bacterial strain.

In the microarray analysis using a single array per treatment, we collected 30 μg RNA from 48 independent soil cultures treated with 3CB and an equal quantity of RNA from 32 soil cultures untreated with 3CB. Because it is difficult to prepare such large amounts of RNA for triplicate array analysis, we adopted an RNA amplification strategy to obtain a sufficient amount of RNA for triplicate microarray analysis, in which around 100 μg amplified RNA was generated from as little as 100 ng total RNA. This made microarray analysis using a small amount of soil (e.g. 2 g of soil) available so that it saved much effort in RNA extraction from soil. The high reproducibility of microarray data using RNA amplified by the MessageAmp II-Bacteria Kit has been verified by the manufacturer (http://www.ambion.com/) and other researchers (Frias-Lopez *et al.*, 2008). This technique has been used previously by researchers successfully (Frias-Lopez *et al.*, 2008; Shi *et al.*, 2009; Stewart *et al.*, 2010). However, the correlation between gene expression data acquired using amplified RNA and those acquired using unamplified RNA ($r^2 = 0.85$–0.92) was slightly lower than that for biological replicates ($r^2 = 0.94$–0.99) (Frias-Lopez *et al.*, 2008), suggesting that a small bias was generated during RNA amplification. Although this small bias might be ignored in some cases, to obtain reliable microarray data, we selected the significantly differentially expressed genes in both the single array analysis using unamplified RNA and the triplicate array analysis using amplified RNA. Because there was no replicate sample and the unit of observation in the statistical test was a probe (70 probes per gene), Student's *t*-test with Bonferroni correction was applied to select differentially expressed genes in the single array analysis. In the triplicate array analysis, there were three replicate samples per treatment and the unit of observation in the statistical test was a sample. In such cases, Bonferroni correction would be too stringent to select significantly differentially expressed genes and FDR correction was more appropriate as discussed previously (Leung & Cavalieri, 2003; Reimers, 2010). Thus, Student's *t*-test with FDR correction was applied to this analysis.

Pseudomonas putida KT2440 can metabolize many xenobiotic compounds such as benzoate, but cannot convert 2-, 3- and 4-chlorobenzoate to the central intermediate compounds, which can then enter the citrate cycle (Jiménez *et al.*, 2002; Ogawa *et al.*, 2003). To confer *P. putida* KT2440 with the ability to degrade 3CB completely, a plasmid containing the *tfd* operon was introduced into *P. putida* KT2440 (Liu *et al.*, 2001). In the single array analysis, the 3CB degradation genes located in the plasmid (*tfd* operon) showed almost no significant change of expression in the microarray (Table 1). The signal intensities of these genes on the microarray were very high, indicating that the

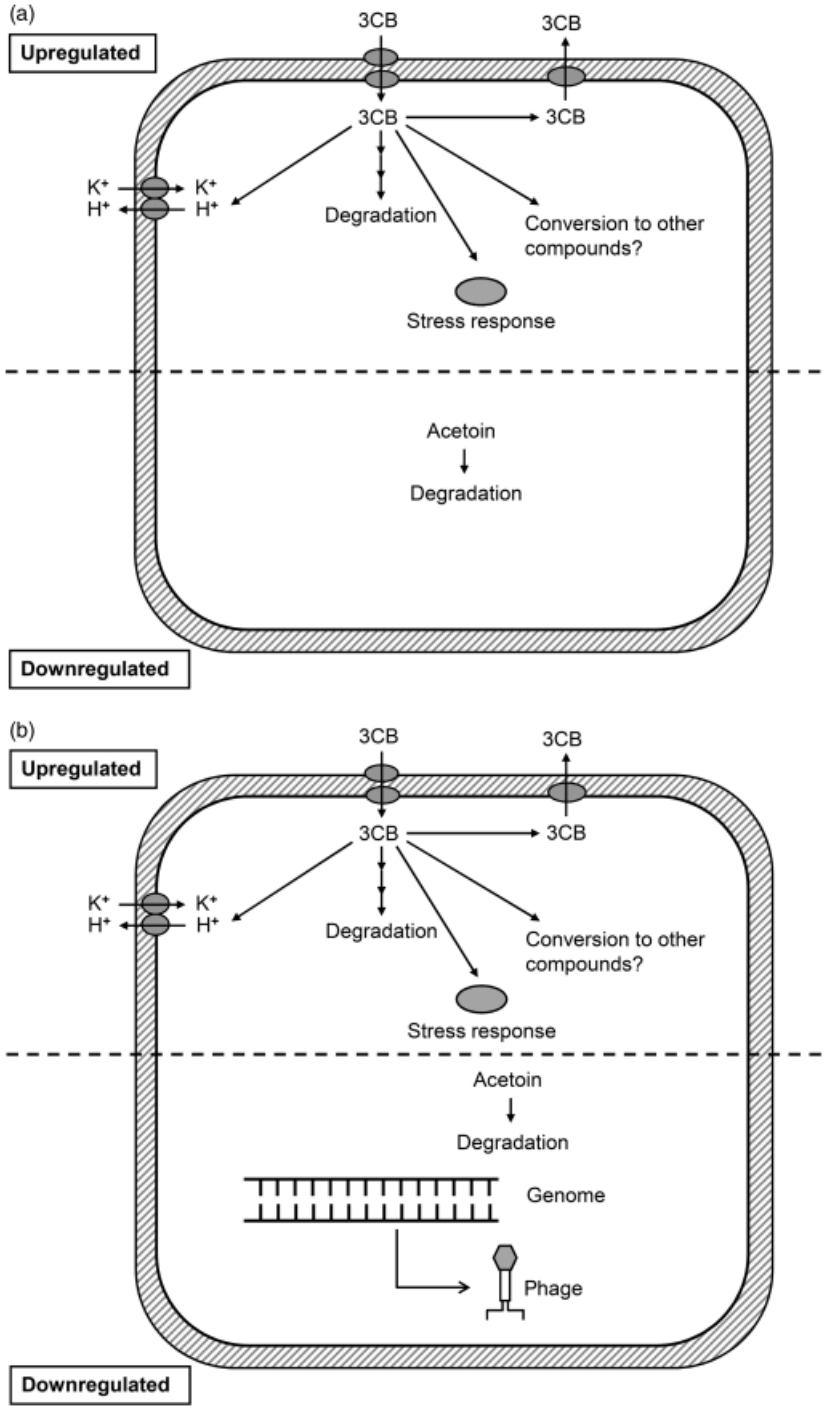

Fig. 3. Summary of biochemical pathways and major cellular responses in *Pseudomonas putida* KT2440/pSL1 cells exposed to 3CB in the log phase (a) and the transition phase (b). The horizontal broken line serves as a boundary between the upregulated (upper part) and the downregulated (lower part) biochemical pathways and cellular responses.

expressions of these genes were saturated even in 3CB − samples, possibly because of multiple copy numbers. The qRT-PCR analysis confirmed that four enzyme genes (*tfdC*, *tfdD*, *tfdE* and *tfdF*) in the plasmid exhibited strong responses to 3CB (Table 1). For the benzoate degradation-related genes located in the chromosome (*benABCD* operon), the upregulated expression on the microarray was validated by qRT-PCR (Table 1). The gene expression data of the *tfd* operon together with the *benABCD* operon are consistent with the HPLC data, which indicated the complete degradation of 3CB after a 3-day incubation (Fig. 1b).

In biodegradation studies, most efforts have been directed towards identifying or characterizing enzymes that can degrade a certain compound. Therefore, other cellular responses to the treatment of the compound have been rarely analyzed. Although it has been known that *benK* and

benF are involved in the transport of benzoate and its analogous molecules (Cowles *et al.*, 2000), there are five other genes in the *P. putida* KT2440 genome putatively involved in this process. In this study, besides *benK* and *benF*, we confirmed that a BenF-like porin (PP_1383) and a transporter gene (*benE*-2) were also induced by 3CB treatment in both the log and the transition phases, suggesting that these genes are involved in 3CB transport. Recent experimental evidence suggests that *benE*-1, *benE*-2 and *benK* are benzoate transporter genes in the KT2440 strain (Nishikawa *et al.*, 2008). However, *benE*-1 and two other genes (PP_1820 and PP_2517) were not induced by 3CB, an analogue of benzoate, suggesting that these three genes might be expressed in response to exposure to substrates more specific than *benK*, *benE-2* and *benF*.

In this study, the microarray analyses detected the induction of an efflux transporter protein gene, a universal stress protein gene and a K^+/H^+ antiporter gene operon under the existence of 3CB in soil. These results were subsequently validated by qRT-PCR (Table 2). All of these genes were first reported to be affected by 3CB treatment. The universal stress protein and the multidrug efflux transporter probably represented two strategies adopted by the bacteria to deal with excess 3CB molecules. The multidrug efflux transporter pumped out excess 3CB molecules; meanwhile, the universal stress protein helped the cells to survive in the presence of excess 3CB. Although it seemed that these genes might be involved in resistance against 3CB, we still cannot exclude the possibility that it might also be involved in resistance against the intermediate products during 3CB degradation. On the other hand, the K^+/H^+ antiporter complex shuttles K^+ into cells while extruding H^+ to maintain the intracellular pH at an appropriate level when bacterial cells are exposed to a low pH environment (Moat *et al.*, 2002). The pH of both 3CB+ and 3CB − soil samples increased slightly during incubation (from 6.04 to 6.26 in the 3CB+ samples and from 6.13 to 6.37 in the 3CB − samples), and at each time point, the pH of the 3CB+samples was lower than that of the 3CB − samples. The difference of pH between the 3CB+ and the 3CB − samples in the transition phase was 0.08. Because the addition of 3CB acidifies the soil, the upregulation of the K^+/H^+ antiporter genes could be helpful in maintaining cytoplasmic pH in bacteria taking up 3CB; therefore, these genes were induced in both the log and the transition phases. It remains to be elucidated whether any other gene is affected by a change of pH in KT2440 containing pSL1.

It has been known that several members of the cytochrome P450 protein family are involved in the oxidation of benzoic acid (Matsuzaki & Wariishi, 2005) and its derivatives, such as 3-chloroperoxybenzoic acid (Spolitak *et al.*, 2005), 4-methoxybenzoic acid and 4-ethylbenzoic acid (Bell *et al.*, 2008). In a recent study, it was found that 3CB induced the expression of a fungus P450 protein, which was demonstrated to be involved in the degradation of benzoic acid (Ning *et al.*, 2010). Thus, we speculate that the two P450 proteins (PP_1950 and PP_1955) in the *P. putida* KT2440 strain might play a role in 3CB degradation or its conversion to nontoxic compounds; however, further investigation is required.

Because the P450 proteins mainly catalyze the oxidation reaction in compound degradation, there should be some other related genes encoding corresponding enzymes either to convert a compound to the substrate of P450 proteins or to convert the product generated by the P450 proteins to a downstream product. When we survey the list of differentially expressed genes in the microarray analysis, we noticed that a large gene cluster showed high fold changes in both microarray analyses and contained the genes encoding the two cytochrome P450 proteins mentioned previously. This gene cluster possesses 15 genes ranging from PP_1943 to PP_1957 with the same orientation of transcription, suggesting that coexpression may occur among these genes. More than half of the genes in this cluster showed several tens to more than 100-fold changes in both microarray analyses (Table S4) and expressions of two genes, PP_1943 and PP_1950, were validated by qRT-PCR (Table 2). This suggested that these genes are highly responsive to 3CB treatment.

In this gene cluster, three genes, PP_1943 to PP_1945, are involved in one carbon pool (the pathway of conversion of different types of tetrahydrofolate), suggesting that the transfer of the methyl group may become more active in the cells exposed to 3CB. If some enzymes catalyzed demethylation reactions, it is reasonable to observe this phenomenon. Two genes coding for demethylase were found in the list of upregulated genes: PP_1957 and PP_3736 (Table S4). Both of them are vanillate demethylase. We did not add vanillate into the soil for bacteria culture; therefore, the mechanism by which 3CB induced the expression of these genes is unclear. There are five genes encoding oxidoreductases in the gene cluster (PP_1946, PP_1949, PP_1951, PP_1953 and PP_1957). Except for PP_1957 (a vanillate demethylase), all of them have unknown function(s). It is possible that these oxidoreductases play a role in 3CB conversion or degradation as they seem to be coexpressed with the two cytochrome P450 genes.

In a BLASTP search against the NCBI database using the amino acid sequence of each gene in this gene cluster, we found that the genes from PP_1943 to PP_1955 showed high similarity to the genes belonging to bacteria that were not pseudomonads. A comparative genomic analysis of 19 genomes in the *Pseudomonadaceae* family using the RECOG server (http://mbgd.nibb.ac.jp/RECOG/) revealed that the organization of this gene cluster is only present in *P. putida* KT2440 (Fig. S1). The integrated microbial genomes (IMG) database (Markowitz *et al.*, 2010) predicted these genes to be

putative horizontally transferred genes (Table S6). Among them, most genes (PP_1943, PP_1944, PP_1945, PP_1948, PP_1951 and PP_1952) were possibly transferred from *Alphaproteobacteria* and others were possibly transferred from *Betaproteobacteria* (PP_1949 and PP_1956), *Actinobacteria* (PP_1950, PP_1953, PP_1954 and PP_1955), *Chloroflexi* (PP_1946) and *Firmicutes* (PP_1947). It is likely that *P. putida* KT2440 acquired these genes from different donors so that a complete set of genes for the conversion or the degradation of a certain compound (probably 3CB-like molecules) was organized as the current status.

Among the downregulated genes, the genes belonging to the phage 04 outnumbered the genes in all other categories, suggesting that the response of this prophage was one of the major responses to 3CB (Fig. 3). The phage 04 is a putative lysogenic bacteriophage with high similarity to the bacteriophage D3 (Canchaya *et al.*, 2003). The bacteriophage D3 belongs to the unclassified *Siphoviridae* of double-stranded DNA viruses. The organization of the phage 04 genome (Fig. S2) is similar to that of the lambda phage. However, the mechanisms by which 3CB or its intermediate products affected the behavior of this prophage during degradation remain unclear. We also found that the expression of the prophage capsid and tail genes in the transition phase was higher than that in the log phase in the 3CB − samples. This suggested that the lytic activity of the prophage was more active in the transition phase than in the log phase, which was consistent with previous reports (Webb *et al.*, 2003; Clark *et al.*, 2006). The mechanism for this phenomenon, as suggested by recent studies, is involved in quorum-sensing signaling, which is RecA-independent and does not involve an SOS response (Ghosh *et al.*, 2009; Oinuma & Greenberg, 2011). It has been known that about 30% of the cultivable soil bacteria may contain inducible prophages (Williamson *et al.*, 2008). Probably, the phage 04 in *P. putida* KT2440, which was downregulated by 3CB treatment in sterilized soil, could be used as a model system to investigate the transition of prophage between the lytic and the lysogenic life cycles in a soil-like environment.

One of the major contributions of this work is that it is the first successful genome-wide microarray analysis using RNA extracted from a bacterial strain growing in sterilized soil. The new knowledge obtained from this analysis regarding the genes involved in transport allows us to gain a more precise understanding regarding the 3CB degradation process, which might also be helpful in understanding the degradation process of other compounds.

Acknowledgements

We thank Dr Takanori Oomori for supplying us with soil for the experiments conducted in this research. This work was supported by a grant-in-aid (eDNA-11-102-2, Soil eDNA) from the Ministry of Agriculture, Forestry, and Fisheries of Japan and in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology.

References

Alm EW, Zheng D & Raskin L (2000) The presence of humic substances and DNA in RNA extracts affects hybridization results. *Appl Environ Microb* **66**: 4547–4554.

Angel R & Conrad R (2009) *In situ* measurement of methane fluxes and analysis of transcribed particulate methane monooxygenase in desert soils. *Environ Microbiol* **11**: 2598–2610.

Bachoon DS, Otero E & Hodson RE (2001) Effects of humic substances on fluorometric DNA quantification and DNA hybridization. *J Microbiol Meth* **47**: 73–82.

Bælum J, Nicolaisen MH, Holben WE, Strobel BW, Sørensen J & Jacobsen CS (2008) Direct analysis of *tfdA* gene expression by indigenous bacteria in phenoxy acid amended agricultural soil. *ISME J* **2**: 677–687.

Barrett T, Troup DB, Wilhite SE *et al.* (2011) NCBI GEO: archive for functional genomics data sets–10 years on. *Nucleic Acids Res* **39**: D1005–D1010.

Bell SG, Xu F, Forward I, Bartlam M, Rao Z & Wong LL (2008) Crystal structure of CYP199A2, a para-substituted benzoic acid oxidizing cytochrome P450 from *Rhodopseudomonas palustris*. *J Mol Biol* **383**: 561–574.

Bolstad BM, Irizarry RA, Astrand M & Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**: 185–193.

Bürgmann H, Widmer F, Sigler WV & Zeyer J (2003) mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. *Appl Environ Microb* **69**: 1928–1935.

Canchaya C, Proux C, Fournous G, Bruttin A & Brussow H (2003) Prophage genomics. *Microbiol Mol Biol R* **67**: 238–276.

Chen Y, Dumont MG, Cebron A & Murrell JC (2007) Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. *Environ Microbiol* **9**: 2855–2869.

Clark ME, He Q, He Z *et al.* (2006) Temporal transcriptomic analysis as *Desulfovibrio vulgaris* Hildenborough transitions into stationary phase during electron donor depletion. *Appl Environ Microb* **72**: 5578–5588.

Cowles CE, Nichols NN & Harwood CS (2000) BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. *J Bacteriol* **182**: 6339–6346.

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC & Lempicki RA (2003) DAVID: Database for annotation, visualization, and integrated discovery. *Genome Biol* **4**: R60.

Edgar R, Domrachev M & Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**: 207–210.

Epstein CB & Butow RA (2000) Microarray technology – enhanced versatility, persistent challenge. *Curr Opin Biotechnol* **11**: 36–41.

Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW & Delong EF (2008) Microbial community gene expression in ocean surface waters. *P Natl Acad Sci USA* **105**: 3805–3810.

Gans J, Wolinsky M & Dunbar J (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* **309**: 1387–1390.

Ghosh D, Roy K, Williamson KE, Srinivasiah S, Wommack KE & Radosevich M (2009) Acyl-homoserine lactones can induce virus production in lysogenic bacteria: an alternative paradigm for prophage induction. *Appl Environ Microb* **75**: 7142–7152.

Han JI & Semrau JD (2004) Quantification of gene expression in methanotrophs by competitive reverse transcription-polymerase chain reaction. *Environ Microbiol* **6**: 388–399.

Hruz T, Wyss M, Docquier M *et al.* (2011) RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. *BMC Genomics* **12**: 156.

Huang DW, Sherman BT & Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**: 44–57.

Huang M, Oppermann FB & Steinbüchel A (1994) Molecular characterization of the *Pseudomonas putida* 2,3-butanediol catabolic pathway. *FEMS Microbiol Lett* **124**: 141–150.

Huggett J, Dheda K, Bustin S & Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* **6**: 279–284.

Insam H (2001) Developments in soil microbiology since the mid 1960s. *Geoderma* **100**: 389–402.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U & Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jiménez JI, Miñambres B, García JL & Díaz E (2002) Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ Microbiol* **4**: 824–841.

Klebba PE (2005) The porinologist. *J Bacteriol* **187**: 8232–8236.

Leung YF & Cavalieri D (2003) Fundamentals of cDNA microarray data analysis. *Trends Genet* **19**: 649–659.

Liu S, Ogawa N & Miyashita K (2001) The chlorocatechol degradative genes, *tfdT-CDEF*, of *Burkholderia* sp. strain NK8 are involved in chlorobenzoate degradation and induced by chlorobenzoates and chlorocatechols. *Gene* **268**: 207–214.

Ludemann H, Arth I & Liesack W (2000) Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl Environ Microb* **66**: 754–762.

Markowitz VM, Chen IM, Palaniappan K *et al.* (2010) The integrated microbial genomes system: an expanding comparative analysis resource. *Nucleic Acids Res* **38**: D382–D390.

Matsuzaki F & Wariishi H (2005) Molecular characterization of cytochrome P450 catalyzing hydroxylation of benzoates from the white-rot fungus *Phanerochaete chrysosporium*. *Biochem Bioph Res Co* **334**: 1184–1190.

Moat AG, Foster JW & Spector MP (2002) Energy production and metabolite transport. *Microbial Physiology* (Moat AG, Foster JW & Spector MP, eds), pp. 382–383. Wiley-Liss Inc., New York.

Morimoto S, Togami K, Ogawa N, Hasebe A & Fujii T (2005) Analysis of a bacterial community in 3-chlorobenzoate-contaminated soil by PCR-DGGE targeting the 16S rRNA gene and benzoate 1,2-dioxygenase gene (*ben A*). *Microbes Environ* **20**: 151–159.

Morimoto S, Ogawa N, Hasebe A & Fujii T (2008) Isolation of effective 3-chlorobenzoate-degraders in soil using community analyses by PCR-DGGE. *Microbes Environ* **23**: 285–292.

Nelson KE, Weinel C, Paulsen IT *et al.* (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**: 799–808.

Nicol GW, Leininger S, Schleper C & Prosser JI (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ Microbiol* **10**: 2966–2978.

Nicolaisen MH, Bælum J, Jacobsen CS & Sørensen J (2008) Transcription dynamics of the functional *tfdA* gene during MCPA herbicide degradation by *Cupriavidus necator* AEO106 (pRO101) in agricultural soil. *Environ Microbiol* **10**: 571–579.

Ning D, Wang H & Zhuang Y (2010) Induction of functional cytochrome P450 and its involvement in degradation of benzoic acid by *Phanerochaete chrysosporium*. *Biodegradation* **21**: 297–308.

Nishikawa Y, Yasumi Y, Noguchi S, Sakamoto H & Nikawa J (2008) Functional analyses of *Pseudomonas putida* benzoate transporters expressed in the yeast *Saccharomyces cerevisiae*. *Biosci Biotech Bioch* **72**: 2034–2038.

Nolan T, Hands RE & Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. *Nat Protoc*. **1**: 1559–1582.

Nystrom T & Neidhardt FC (1994) Expression and role of the universal stress protein, UspA, of Escherichia coli during growth arrest. *Mol Microbiol* **11**: 537–544.

Ogawa N, Miyashita K & Chakrabarty AM (2003) Microbial genes and enzymes in the degradation of chlorinated compounds. *Chem Rec* **3**: 158–171.

Oinuma K & Greenberg EP (2011) Acyl-homoserine lactone binding to and stability of the orphan *Pseudomonas aeruginosa* quorum-sensing signal receptor QscR. *J Bacteriol* **193**: 421–428.

Pfaffl MW, Tichopad A, Prgomet C & Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnol Lett* **26**: 509–515.

Rajendhran J & Gunasekaran P (2008) Strategies for accessing soil metagenome for desired applications. *Biotechnol Adv* **26**: 576–590.

Reimers M (2010) Making informed choices about microarray data analysis. *PLoS Comput Biol* **6**: e1000786.

Roesch LF, Fulthorpe RR, Riva A *et al.* (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J* **1**: 283–290.

Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM & Trevors JT (2005) Microbial gene expression in soil: methods, applications and challenges. *J Microbiol Meth* **63**: 1–19.

Sambrook J & Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schirmer T (1998) General and specific porins from bacterial outer membranes. *J Struct Biol* **121**: 101–109.

Sharma S, Aneja MK, Mayer J, Munch JC & Schloter M (2005) Diversity of transcripts of nitrite reductase genes (*nirK* and *nirS*) in rhizospheres of grain legumes. *Appl Environ Microb* **71**: 2001–2007.

Shi Y, Tyson GW & DeLong EF (2009) Metatranscriptomics reveals unique microbial small RNAs in the ocean's water column. *Nature* **459**: 266–269.

Shrestha PM, Kube M, Reinhardt R & Liesack W (2009) Transcriptional activity of paddy soil bacterial communities. *Environ Microbiol* **11**: 960–970.

Spolitak T, Dawson JH & Ballou DP (2005) Reaction of ferric cytochrome P450cam with peracids: kinetic characterization of intermediates on the reaction pathway. *J Biol Chem* **280**: 20300–20309.

Stewart FJ, Ottesen EA & DeLong EF (2010) Development and quantitative analyses of a universal rRNA-subtraction protocol for microbial metatranscriptomics. *ISME J* **4**: 896–907.

Tebbe CC & Vahjen W (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl Environ Microb* **59**: 2657–2665.

Torsvik V (1980) Isolation of bacterial DNA from soil. *Soil Biol Biochem* **12**: 15–21.

Torsvik V & Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. *Curr Opin Microbiol* **5**: 240–245.

Torsvik V, Goksøyr J & Daae FL (1990) High diversity in DNA of soil bacteria. *Appl Environ Microb* **56**: 782–787.

Torsvik V, Øvreås L & Thingstad TF (2002) Prokaryotic diversity–magnitude, dynamics, and controlling factors. *Science* **296**: 1064–1066.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP & Schleper C (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* **7**: 1985–1995.

Urich T, Lanzén A, Qi J, Huson DH, Schleper C & Schuster SC (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS One* **3**: e2527.

Wang Y, Shimodaira J, Miyasaka T *et al.* (2008) Detection of *bphAa* gene expression of *Rhodococcus* sp. strain RHA1 in soil using a new method of RNA preparation from soil. *Biosci Biotech Bioch* **72**: 694–701.

Wang Y, Morimoto S, Ogawa N, Oomori T & Fujii T (2009) An improved method to extract RNA from soil with efficient removal of humic acids. *J Appl Microbiol* **107**: 1168–1177.

Watson A, Mazumder A, Stewart M & Balasubramanian S (1998) Technology for microarray analysis of gene expression. *Curr Opin Biotechnol* **9**: 609–614.

Webb JS, Thompson LS, James S *et al.* (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **185**: 4585–4592.

Werck-Reichhart D & Feyereisen R (2000) Cytochromes P450: a success story. *Genome Biol* **1**: REVIEWS3003.

Williamson KE, Schnitker JB, Radosevich M, Smith DW & Wommack KE (2008) Cultivation-based assessment of lysogeny among soil bacteria. *Microb Ecol* **56**: 437–447.

Zipper H, Buta C, Lämmle K, Brunner H, Bernhagen J & Vitzthum F (2003) Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments. *Nucleic Acids Res* **31**: e39.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotides used in qRT-PCR to evaluate the expression of genes selected according to the microarray analyses.
Table S2. The significantly changed genes in the triplicate array analysis.
Table S3. The significantly changed genes in the single array analysis.
Table S4. The upregulated genes in both microarray analyses.
Table S5. The downregulated genes in both microarray analyses.
Table S6. Summary of the putative horizontally transferred genes.
Fig. S1. Comparative genomic analysis of the *Pseudomonadaceae* family using the RECOG (Research Environment for Comparative Genomics) server.
Fig. S2. The organization of the phage 04 genome in *Pseudomonas putida* KT2440.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.