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Response of Selected Genes of *Burkholderia xenovorans* Strain LB400 to Onion Extract Using A DNA:RNA Hybrid Capture Detection System

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Abstract: *Burkholderia xenovorans* strain LB400 was exposed to *Allium cepa* (onion) extract to investigate the expression of selected putative genes including four polychlorinated biphenyls (PCBs) degrading genes (*bphA*, *bphE*, *bphF* and *bphG*). *B. xenovorans* strain LB400 is renowned for its appetite towards the most difficult to degrade compounds in soil. Onion, medicinally important as well as antibacterial and antifungal plant, was used in this study to further investigate the nutritional versatility of LB400. A cDNA microarray containing Polymerase Chain Reaction (PCR) products of 530 selected genes of LB400 was prepared. Total RNAs isolated from LB400 cells grown in minimal medium (M9) and minimal medium supplemented with 1% onion extract (M9O) were used as targets for hybridization to the LB400 array. A novel DNA-RNA detection system with indirect labeling was used to obtain the mRNA abundance under the two conditions. Approximately, 12% of the total genes included on the array were expressed significantly above the background level in M9 while more than 36% were expressed in M9O. Comparing the transcripts expression in M9O to M9 identified the difference in the expression level of about 60 genes including *bphA*, *bphE* and *bphG*. Further, it was observed that onion extract enhanced the growth of LB400. Approximately, 30% increase in the growth rate of LB400 was observed due to the addition of onion extract in M9 compared to M9 without onion extract, at both 24 and 48 h after the inoculation. Hybridization analyses using varying amounts of total RNA also revealed a linear correlation between the amount of targeted RNA (1, 5 and 10 μg of total RNA) and signal intensity of the hybridized genes. These results indicate that onion extract has the potential to support and enhance the growth of *B. xenovorans* strain LB400, as well as positively regulate the expression of PCBs degrading genes. Further, LB400 may be a virulent pathogen of onion.

Key words: *B. xenovorans,* LB400, onion, M9, M9O, PCBs

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Introduction

Burkholderia xenovorans strain LB400, formerly known as *B. fungorum*, which was first identified as a phytopathogen of onions by Burkholder (1950). The *Burkholderia* genus consists of more than 32 species belonging to very diverse ecological niches, including the contaminated soils, water, plant surfaces as well as the respiratory tract of humans (Coenye and Vandamme, 2003). However, most members of this genus are known as plant pathogen (Parke and Gurian-Sherman, 2001), saprophytes and biocontrol agents (Cartwright *et al*., 1995; King and Parke, 1993; Mao *et al*., 1998). The strain LB400 is a gram-negative bacterium, currently attaining a considerable attention due to its great significance in environmental microbiology and the degradation of polychlorinated biphenyls (PCBs), various antibiotics, herbicides and numerous other toxic compounds (Bhat *et al*., 1994; Bopp, 1986; Denef *et al*., 2004; Goris *et al*., 2004; Sangodkar *et al*., 1989; Shields and Reagin, 1992; Valvano *et al*., 2005).

It has one of the largest prokaryotic genomes (9.7Mb) consisting of 2 chromosomes and a megaplasmid (Goris *et al*., 2004; Lessie *et al*., 1996). It also has numerous insertion elements, which promote genomic rearrangements and modify the expression of neighboring genes. In addition, its genome possesses a highly adaptive capacity, in which genes are transferred laterally among *Burkholderia* and other genera. This imparts new metabolic capabilities by integration of genes or modification of existing pathways for degradation of toxic compounds (Lessie *et al*., 1996). This genomic plasticity could be the reason for its remarkable nutritional versatility and evolutionary adaptability in different environments with new selection pressures.

In order to explore further, the adaptive capacity and nutritional versatility of LB400 and because *B. xenovorans*, previously, identified as a pathogen of *Allium cepa* (onion), it was suggested to challenge LB400 with onion extract and investigate the changes in its growth rate and the expression profile of its selected genes. Onion is rich in organic sulphides, catechols, protocatechúic acid, allyl propyl disulphide and glycolic acid (Bakhru, 1994; Block, 1985). Onion is also well known for its antibacterial, antifungal and medicinal properties. Therefore, it would be of highly interest to divulge the effect of onion extract on the growth as well as gene expression profile of LB400.

Here, as a first step to reveal the interaction between LB400 and onion, we prepared a partial cDNA microarray representing 530 selected genes of LB400 covered almost all the functional categories assigned by the Joint Genome Institute (JGI) and included four PCBs degrading genes namely *bphA*, *bphE*, *bphF* and *bphG*. This cDNA array is an important source for studying the changes in the expression profile of various metabolic pathways through microarray hybridizations. In order to determine the effect of onion extract on the expression of these selected genes, total RNAs isolated from LB400 cells grown in minimal medium (M9) and minimal medium with 1% onion extract (M9O) were used as targets to the array.

Here, we present evidence for faster growth of LB400 grown in M9O as compared to M9, due to onion extract. We also present evidence for the induction of certain genes including PCBs degrading genes in response to onion extract. This is the first report describing the interactions between LB400 and onion.

Materials and Methods

Strain, Media and Growth Conditions

Burkholderia xenovorans strain LB400 was obtained from the Center for Microbial Ecology (CME) at Michigan State University (MSU), East Lansing, USA. Two media were used to grow *B. xenovorans* strain LB400:

- M9 medium supplemented with 0.05% glucose and amino acids (1 mM thiamine HCl and 0.002% proline) as described (Sambrook *et al*., 1989).
- M9 medium as mentioned above and additionally supplemented with 1% (w/v) onion extract (named-M9O).

Onion extract was prepared fresh before use. Yellow onion bulbs (20 g) were cut and blended for 10 min in 100 mL of sterilized distilled water, the extract was passed through mirra cloth first and then filter sterilized using 0.45 μm filter units (Amicon, Beverly, MA). The filter sterilized 20% onion extract was diluted to 1% final concentration in M9 medium.

In the beginning of each experiment, LB400 strain maintained in Luria-Bertani (LB) (Sambrook *et al*., 1989) agar plates was cultured on agar plates containing M9 and M9O medium for two days. A freshly grown single colony was inoculated into 5 mL broth media (M9 and M9O) and grown until the stationary phase at 28°C with shaking at 200 rpm. The 5 mL cultures were then diluted to 100 times in M9 and M9O broth media and grown further till harvested at 48 h (optimum Log phase time in the normal M9 medium) after the inoculation for RNA isolation. Samples were taken from the growing cultures over time and cell density was recorded at OD_{600}. Measurements were made from two biological replicate samples for M9 and M9O.

DNA Microarray Design, Development and Preparation

The information available on the genome sequence of *B. xenovorans* LB400 from the JGI (www.jgi.doe.gov) was transferred and processed by primer design software (Primer 3) to produce primers for a set of 530 genes (Table S1) from 20 different functional categories according to Clusters of Orthologous Groups (COGs) at JGI. A detailed description about each group and the corresponding total number of genes present among the selected LB400 genes is presented in Fig. 1. In addition, 4 genes from PCBs degrading pathways i.e., *bphA*, *bphE*, *bphF* and *bphG* (M86348; Genbank) and 6 housekeeping genes namely, *rpoA*, *rpoD*, *rpoS*, *rpmA*, *recA* and *plcC* were also included. 16S rRNAs of *B. xenovorans* strains LB400 and *B. cepacia* strain G4 (a trichloroethylene degrading strain of

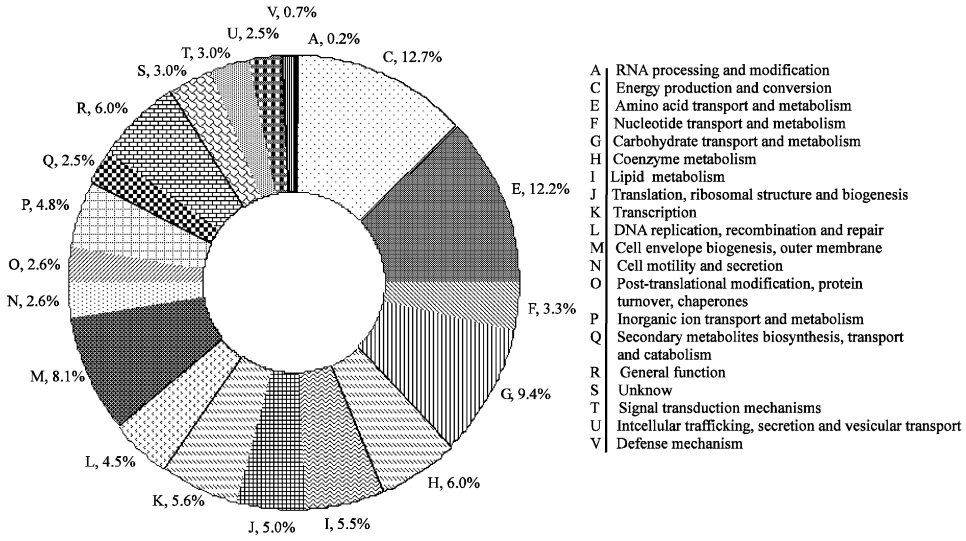

Fig. 1: Functional classification of the selected *B. xenovorans* strain LB400 genes according to Clusters of Orthologous Groups (COGs) at Joint Genome Institute (JGI) and a schematic representation of the percentage of genes in each group

Burkholderia) (Luu *et al*., 1995) were used as positive controls, whereas ten different human DNA sequences were used as negative controls. These were B-cell receptor protein, Myosin heavy chain, Myosin regulated light chain 2, Insulin-like growth factor, FLJ10917fis, HSPC120, Beta 2 microglobulin, phosphoglycerate kinase, Tyrosine phosphatase and G10 homolog.

A selected region of each Open Reading Frame (ORF) was amplified as 400 to 600 bp PCR product using gene specific primers (synthesized by the Macromolecular Structure, Sequencing and Synthesis facility at MSU). PCR reactions of 50 μL were prepared in 0.2 mL microfuge tubes using each primer pair (1 μM) and RedTaq Ready Mix (Sigma, St. Louis, MO). PCR reactions were performed in Thermolyne Amplitron II (Barnstead, Dubuque, Iowa) with an initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec and extension at 72°C for 1 min. The final extension was carried out at 72°C for 1 min. Amplified products were verified by gel electrophoresis and purified using Qiagen purification kit (Qiagen, Valencia, CA), eluted using 50 μL of molecular grade water (Sigma), lyophilized and resuspended in 10 μL of 3×SSC for microarray printing.

PCR amplified probes were printed onto super amine coated glass slides (TeleChem, Sunny-vale, CA) with 350 μm spacing between the centers of each spot using TeleChem micro-spotting pins (SMP-4) and an arrayer robot (http://cmgm.stanford.edu/pbrown/mguide/index. html). Printed DNA was rehydrated and cross-linked to the glass slide by UV irradiation at 200 mJ using UV Stratalinker 1800 (Stratagene, La Jolla, CA). Half of the printed slides were blocked with a solution containing 5.5 g succinic anhydride, 325 mL 1-methyl-2-pyrrolidinone and 30 mL sodium borate and the other half slides were kept as unblocked. The blocking reagent react with residual covalent binding sites in both, the printed and unprinted areas of the slide surface to prevent non-specific binding of labeled target to the slide surface. Hybridizations were performed using blocked and unblocked slides.

RNA Extraction and Purification

LB400 cells grown in M9 and M9O media were harvested by pipeting 10 mL of cultures into a 15 mL conical tube containing 1.25 mL of ice-cold ethanol and centrifuged at 8,000 rpm for 2 min at 4°C. Cells were resuspended in Tris-EDTA solution (10 mM Tris pH 8.0, I mM EDTA) containing 500 μg mL^{-1} lysozyme (Sigma) and RNA was isolated using RNeasy kit (Qiagen). RNA was visualized in 1% agarose gel, quantified by absorbance at 260 nm and stored at -20°C until used.

Hybridization of Total RNA to the Array Using HC Express Array Kit

Isolated total RNA from M9 and M9O cultures were used directly for hybridization on blocked and unblocked arrays using a DNA:RNA hybrid capture detection system (HC Express Array kit; Digene, Inc., CA) following the manufacturers instructions. Briefly, the slide was placed in a hybridization chamber (TeleChem), spotted side up and covered with a lifterslip (22×30; Erie Scientific Co, Portsmouth, NH) over the spotted area. Hybridization mixture was prepared by mixing 25 μL of total RNA (1, 5 or 10 μg total) with 25 μL of hybridization buffer (Digene), vortexed for 10 sec, denatured at 95°C for 2 min, spun briefly and pipetted immediately onto the glass slide at the edge of the lifterslip and allowed to spread by capillary action. Total of 40 μL of distilled water was added into the hybridization cassette at the four corners to provide humidity and the chamber was sealed immediately.

The slide was hybridized at 75°C for 5 h, washed with 1×phosphate buffer saline and 0.05% Tween 20 (PBST) and air-dried for approximately 1 min. The slide was then hybridized with primary antibody solution (5 μL primary antibody and 45 μL antibody dilution buffer; included in the HC Express Array kit) at 37°C for 1 h, washed with PBST and air dried for 1 min. Hybridization with secondary antibody solution (2 μL Cy3 labeled secondary antibody included in the HC Express Array kit, 43 μL PBST and 5 μL of 10% normal goat serum included in the HC Express Array kit) was then

performed at 37°C for 1 h. The hybridization chamber was covered with aluminum foil to protect fluorescent conjugates from direct light. The secondary antibody treated slide was washed first with wash buffer and kept for about 10 sec in pre-warmed enhance buffer and washed again in wash buffer with vertical shaking for 1 min. The slide was dried in a centrifuge at 600 rpm for 1 min and scanned with GenePix 4000A (Axon™ Instruments, Union City, CA) with an excitation wavelength of 532 nm suitable for Cy3 detection.

Preparation of Fluorescent Probes and Hybridization

One hundred micrograms of total RNA isolated from M9O was reverse transcribed in the presence of Cy3 dUTP (Amersham Pharmacia) as described (Eisen and Brown, 1999). The labeled sample was concentrated and purified using Microcon 30 microconcentrator (Amicon) with TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The quantitation of Cy3 labeled cDNA target was done by spectrophotometry (Eisen and Brown, 1999). The Cy3 labeled cDNA target was mixed with 20 μg of yeast tRNA, 3.4 μL of 20×SSC and 0.6 μL of 10% SDS in a total volume of 20 μL. The target sample was denatured by placing them in a 100°C water bath for 1 min, cooled on ice and then used for hybridization. The target sample was hybridized with LB400 array at 65°C for 16 h. The hybridized slide was washed and scanned with GenePix 4000A (Axon™ Instruments) for detection of Cy3 fluorescence.

Data Analyses

Following a quantitative scan of each array, the data was analyzed using GenePix Pro 4.0 software. The average fluorescence intensity for Cy3 at each printed spot was recorded. Mean signal intensity after background subtraction and background intensity for each spot were used for all data analysis. Detection limit was calculated for each slide as the average background signal intensity plus three standard deviations (B532+3SD) for all spots. This value is then used as cut-off value to discard signals having signal strength less than the detection limit and also to evaluate the strength of the signal above the cut-off value. Blocked and unblocked arrays were normalized using total signal intensity for each array. Further data analyses such as average of two experimental replicates, significance of induction and suppression, sorting and counting and graphical representation, all were performed using data analysis software Acuity 4 (Axon) and Microsoft Excel. For the final analysis, data points were averaged from the two unblocked and two blocked replicate arrays. Data points without replicate values or replicated values with percentage standard error more than 30% of the mean were not included in the final lists. The normalized values for each signal were then divided by the average of normalized cut-off value (B532+3SD). These values were then used to determine the difference in the expression level of LB400 genes when grown in M9 and M9O. Statistical analysis was performed using Student's t-test.

Results and Discussion

Effect of Onion Extract on the Growth Rate of LB400

It was observed that LB400 grows faster in the presence of onion extract in addition to normal growth medium M9. By measuring the cell density the difference in the growth rate of LB400 grown in M9O to that grown in M9 was 30% (Fig. 2). The cell density at 0-6 h (Lag phase) confirmed that both LB400 cultures (M9 and M9O) had the similar amount of bacterial cells. However, slowly over the time, when bacteria reached in the Log phase (12-24 h), the difference in the growth rate of LB400 between two cultures was evident. This difference reached to maximum at 48 h ($p<0.05$). However, after approximately 70 h this difference was not statistically significant ($p>0.05$).

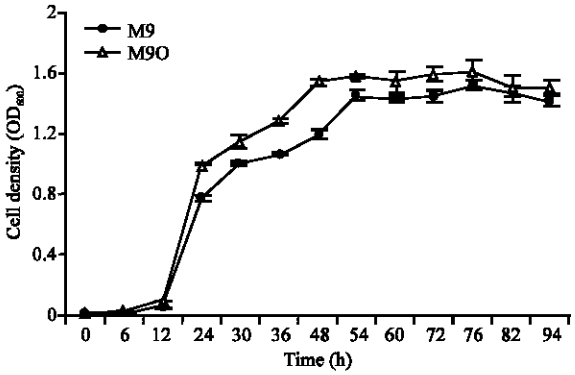

Fig. 2: Growth curve of LB400 grown in M9 and M9O

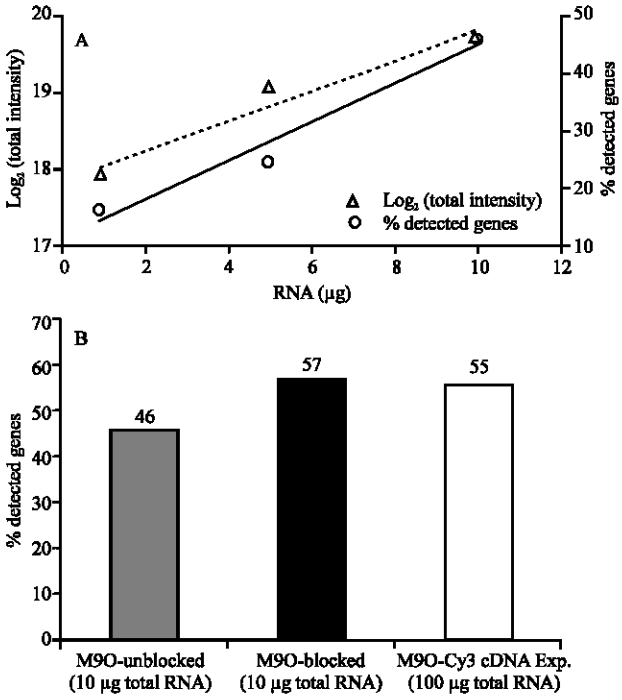

Fig. 3: A: A correlation between total intensity of hybridized signals/total detected genes above the cut-off value in percentage and the amount of target RNA used; B: A comparison between hybridization experiments using total number of detected genes obtained following total RNA hybridization on blocked and unblocked arrays as well as in Cy3 labeled cDNA as target to custom cDNA array of LB400

A fresh onion bulb contains about 88% water, 11% carbohydrates, 4% sugars and 1% protein (Bakhru, 1994). The faster growth of LB400 in M9O may be mostly attributable to the extra carbohydrates due to onion extract. The increase in the growth rate of LB400 in the presence of onion extract reflects the nutritional versatility of this bacterium. Further experiments using specific components of onion may reveal a more conclusive evidence for faster growth of LB400.

Optimization of DNA:RNA HC Express Array Hybridization Protocol

To explore the use of a smaller amount of total RNA as needed in many environmental applications of microarrays, this study used a DNA:RNA hybrid capture detection system to evaluate the expression response. Sensitivity of HC Express Array kit (Digene Corp. Gaithersburg, MD 20878, USA) was evaluated using 1, 5 and 10 μg of total RNA, isolated from M9O. A linear correlation was found between the amount of total RNA used to hybridize and the intensity of the hybridized signals as well as the total number of detected genes above the cut-off value for LB400 (Fig. 3A). Experiments using 20 μg of total RNA yielded saturated signals for a significant large number of spots (data not shown). Because the percentage of genes detected with 10 μg of total RNA was similar to that obtained with traditional labeling protocol (Fig. 3B), a higher amount of total RNA was not evaluated. All the remaining experiments reported in this study used 10 μg of total RNA.

As reported previously with glass slide arrays (Taylor *et al*., 2003), blocking with succinic anhydride increased the background signal intensity significantly (2.5 to 3.5 fold) compared to unblocked array. Normalized results, however, could be compared between blocked and unblocked slides for most genes that were significantly expressed as per the cut-off definition (Fig. 4A). As documented by Taylor *et al*. (2003) that signal intensity values for cDNA samples are not surface chemistry dependent, it is the background signals that make principal difference. Therefore, data from both blocked and unblocked hybridizations were used to compute changes in fold-expression. The positive controls i.e., LB400 16S rRNA and G4 rRNA were always present in large excess in all the samples. No cross hybridization was detected with negative controls. Fold change in expression of genes that varied considerably (i.e., greater than 2-fold or $p<0.9$) between blocked and unblocked slides were excluded from further analysis. The fraction of detected genes in our experiment was 12% in M9 and 36% in M9O.

Further, the stability of total RNA was evaluated by conducting comparative hybridization experiments with total RNA extracted from M9O medium and stored at -20°C for 2 and 120 days (Fig. 4B). Although the percentage of genes expressed after 120 days of storage (33%) was comparable to the percentage of genes after 2 days of storage (36%), both hybridizations yielded 80% of the mRNA with replicate expression pattern. This finding is important in comparative mRNA expression studies using environmental samples, where RNA is routinely extracted at different times and stored at -20 to -80°C for further analysis.

Gene Expression Response of LB400 With and Without Onion Extract

Transcript analysis of LB400 cells grown in M9 and M9O identified a small set of genes induced in response to onion extract (Table 1 and Fig. 5). Among them, 10 genes were from energy production and conversion metabolism; 9 were from translation, ribosomal structure and biogenesis group; 7 were from amino acid transport and metabolism and 4 from transcription and secondary metabolites biosynthesis, transport and catabolism etc.

As reported in the previous section that the addition of onion extract, in the nutritional medium, enhanced the growth of LB400 (Fig. 2). Besides, being aware of the total composition of onion extract, LB400's faster growth could be due to the extra carbon source (carbohydrate) available in the onion extract. In order to provide additional evidence to support this notion, an extensive analysis and comparison, with previous reports, for differentially expressed genes in M9O verses M9 was executed. Many genes, with well known characteristics, were identified as up-regulated in LB400 due to feeding on onion extract. For example, the expression level of *sfc*A was 5-fold induced in M9O (Table 1). *sfc*A belongs to the energy production and conversion metabolism, encode malic enzyme. Malic enzyme is required for the transition of carbon source and is involved in regulating the metabolic pathways from malate to phosphoenolpyruvate. Previously, the induction of *sfc*A has been reported during acetate metabolism (Oh *et al*., 2002), exhibiting the physiological role of *sfc*A on the transition of acetate.

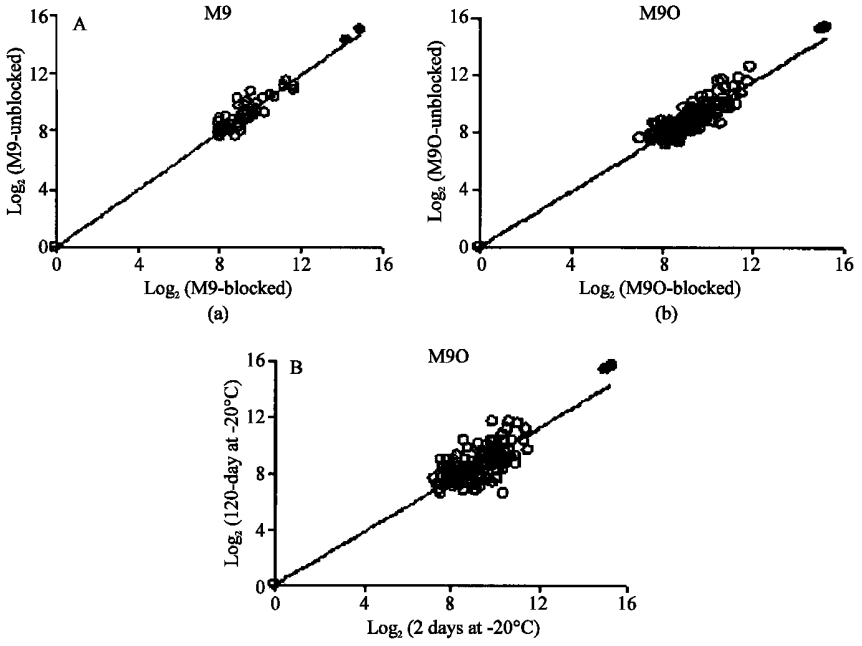

Fig. 4: A: A scatter plots showing the replication between signal intensities obtained after total RNA hybridization on blocked and unblocked custom cDNA array of LB400; B: A scatter plot showing the replication between signal intensities obtained after the hybridization of total RNA sample isolated from M9O and stored at -20°C for 2 days against the signal intensities obtained after the hybridization performed using the same RNA sample but stored for 120 days at -20°C. Hybridization was performed with blocked custom cDNA array of LB400 printed at different times. Average mean signal intensity from two arrays on the same slide was plotted on a logarithmic scale 2. The solid circles (on the top right corner of the graph) represent strain G4 and strain LB400 16S rRNAs

However, the involvement of the malic enzyme in mechanisms other than carbon transition has also been suggested (Kao *et al*., 2005). The up-regulation of *sfc*A confirmed the presence of excess carbon source in M9O due to onion extract.

Additionally, the induction of *md*H and *gal*M in M9O also represents the presence of excess carbon source due to onion extract (Table 1). *md*H also belongs to the energy production and conversion metabolism, encode malate dehydrogenase. *md*H is required for the tricarboxylic acid and glyoxylate shunt pathways (Michaela and Kathleen, 2005). The transcripts level of *md*H was 5-fold above the cut-off value in M9O and it was not detected in M9. Whereas, *gal*M belongs to the carbohydrate transport and metabolism, encode galactose mutarotase. *gal*M is closely associated with sugar metabolism and fermentation (Bartolini *et al*., 2006). The transcripts level of *gal*M was >3-fold induced in M9O.

Extensive analysis of the LB400 expression profile grown in M9O verses M9 also revealed that amino acid levels were limited in the M9O at the time of harvest. This was evident because of the suppression of *glt*1 in M9O. *glt*1 belongs to amino acid transport and metabolism, encode glutamate synthase and is required for the net conversion of ammonium and 2-oxoglutarate to glutamate in many microorganisms as well as higher plants (Valenzuela *et al*., 1998). Glutamate is the source of 80% of cellular nitrogen (Magasanik, 2003). The suppression of *glt*1 and the elevated levels of *gltA* transcripts

Table 1: Differentially expressed genes of *B. xenovorans* strain LB400 in response to onion extract

Gene name/ID	Gene product [Functional Category]	Fold induction[a]
glt1	Glutamate synthase domain 2 [E]	-2.3[b]
hcaB	Cis-2,3-dihydrobiphenyl-2,3-diol dehydrogenase [Q/R]	2.3
LeuA	Isopropylmalate/homocitrate/citramalate synthases [E]	2
gyrA	DNA gyrase/(topo II topoisomerase IV) A subunit [L]	-3[b]
hisC2	histidinol-phosphate aminotransferase [E]	2.5[c]
afuA	ABC-type Fe3+ transport system periplasmic component [P]	2.4
sdhB	Succinate dehydrogenase/fumarate reductase Fe-S protein subunit [C]	2.7
gltA	Citrate synthase [C]	2
mdH	Malate/lactate dehydrogenases [C]	5[c]
tufA	Elongation factor TU [J]	3
infB	Translation initiation factor 2 [J]	2
BxeB0961	Acetyl-CoA carboxylase carboxyltransferase component [I]	2.3
SfcA	Malic enzyme [C]	5
pyrE	orotate phosphoribosyltransferas [F]	2[c]
paaH2	3-hydroxyacyl-CoA dehydrogenase [I]	2[c]
ileS	Isoleucyl-tRNA synthetase [J]	3
BxeA2382	Aspartate/tyrosine/aromatic aminotransferase [E]	2[c]
hom	homoserine dehydrogenase [E]	2[c]
adhP	Zn-dependent alcohol dehydrogenases [R]	2.5
ppsA	Phosphoenolpyruvate synthase [G]	2
gloB	Zn-dependent hydrolases including glyoxylases [R]	2[c]
dnaB	Replicative DNA helicase [L]	3.7
glk	Glucokinase [G]	2
serS	seryl-tRNA synthetase [J]	2
atpA	ATP synthase F1, alpha subunit [C]	-2[b]
atpG	ATP synthase gamma chain [C]	-3[b]
galM	Galactose mutarotase and related enzymes [G]	3.3
aceA	isocitrate lyase [C]	2.2[c]
relA	GTP pyrophosphokinase [T/K]	4.2
thrS	Threonyl-tRNA synthetase [J]	2.6
putA	NAD-dependent aldehyde dehydrogenases [C]	3[c]
gabT	4-aminobutyrate aminotransferase and related aminotransferases [E]	2.4[c]
trpE	anthranilate synthase component I [E/H]	2[c]
trpD	Anthranilate phosphoribosyltransferase [E]	3[c]
ahpC	Peroxiredoxin [O]	2.5[c]
lysU	Lysyl-tRNA synthetase (class II) [J]	3.4[c]
ppa	inorganic pyrophosphatase [C]	2
dus	tRNA-dihydrouridine synthase [J]	3[c]
sucA	2-oxoglutarate dehydrogenase E1 component [C]	2.2[c]
rpoN	RNA polymerase sigma-54 factor [K]	5.6
yfiA	Ribosome-associated protein Y [J]	2.7
pulK	Type II secretory pathway component PulK [U]	3.6[c]
hupA	DNA-binding protein HU [A]	2
phbB	Dehydrogenases with different specificities [Q/R]	3.7[c]
lpxC	UDP-3-O-acyl-N-acetylglucosamine deacetylase [M]	2.6
lucD	Lysine/ornithine N-monooxygenase [Q]	8.2[c]
fadR	Transcriptional regulators [K]	2.1
spoU	rRNA methylases [J]	2.2[c]
rpoD	RNA polymerase sigma factor [K]	-2[a]
opcP	porin, major outer membrane protein [M]	2.4
acrB	Cation/multidrug efflux pump [V]	2.2[c]
cheW	Chemotaxis signal transduction protein [N/T]	3.2[c]
flgL	Flagellin and related hook-associated proteins [N]	3.6[c]
recA	recombination protein RecA [L]	4.7[c]
groEL	Chaperonin GroEL (HSP60 family) [O]	7.4
BxeB2711	transposase, IS4 [S]	2.3
emrB	Arabinose efflux permease [G]	3[c]
cyoB	Heme/copper-type cytochrome/quinol oxidases subunit 1 [C]	2.5[c]
bphA	Biphenyl 2,3-dioxygenase-BPHA [P/R]	3
bphE	biphenyl dioxygenase beta subunit [Q]	2.5[c]
bphG	Biphenyl dioxygenase system ferredoxin--NAD(+) reductase component [C]	2.2

[a]Difference in gene expression of M9O to M9 (fold above the average background intensity plus three standard deviations); [b]not detected in M9O; [c]not detected in M9

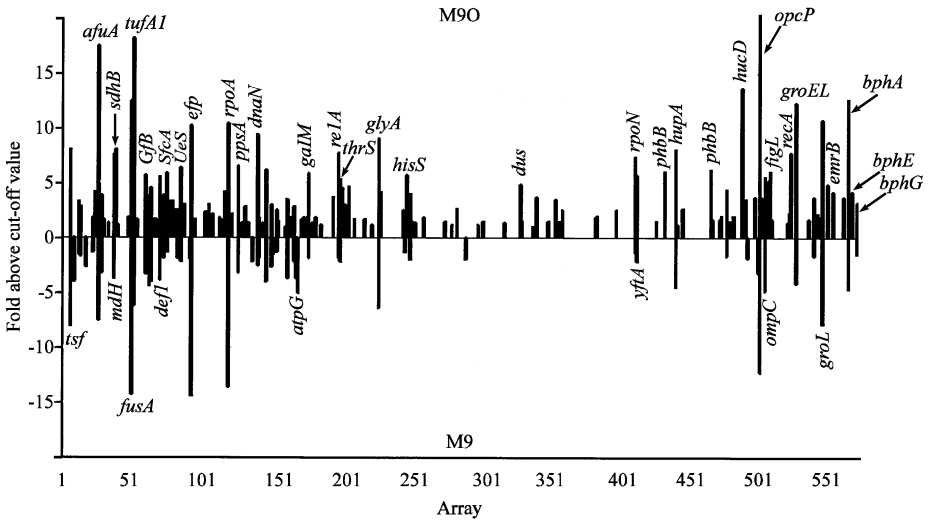

Fig. 5: A comparison between the expression profile of selected 530 LB400 genes in response to M9 (lower half) and M9O (upper half) using fold intensity above the cut-off value. The cut-off value for M9 and M9O was 318 and 311, respectively

in M9O (Table 1) reflected limited glutamate conditions (Valenzuela *et al*., 1998). *glt*A belongs to the energy production and conversion metabolism. *glt*A also encode glutamate synthase, which is required for ammonium assimilation or nitrogen source metabolism in the absence of glutamate dehydrogenase (Macheda *et al*., 1999). When bacteria is growing with an excess of carbohydrate as sole source of carbon and energy and ammonia as the sole source of nitrogen, the use of cellular glutamate dehydrogenase to assimilate ammonia determined the rate of growth (Magasanik, 2003). However, the use of the glutamate synthase, instead of the glutamate dehydrogenase, also results in a more efficient utilization of resources.

Another example of amino acid starvation in M9O is the induction of *rel*A (Table 1 and Fig. 5). *rel*A belongs to the signal transduction and transport mechanism, encode a GTP pyrophosphate kinase. This protein is ribosomal dependent and become activated during amino acid starvation. More importantly, the role of *rel*A in the virulence of *P. aeruginosa* has also been verified (Erickson *et al*., 2004). It was determined that the affect of *rel*A in the virulence of *P. aeruginosa* is dependent on the growth conditions and it acts as a stringent response. The stringent response is a phenomenon in which bacteria adapt to nutritional deficiencies. In this situation, the bacteria produce highly phosphorylated guanine nucleotides such as ppGpp and pppGpp, by the RelA enzyme. The implications of ppGpp and pppGpp in the growth rate control of gene expression and in the regulation of stationaryphase gene expression in *E. coli* has been reported (Chakraburtty and Bibb, 1997). Further, a correlation between ppGpp synthesis and the onset of antibiotic production has also been revealed. It was suggested that ppGpp plays a central role in triggering antibiotic biosynthesis. Because, ppGpp is synthesized by RelA, which is activated under conditions of nitrogen limitation and following amino acid starvation during exponential growth (Chakraburtty and Bibb, 1997). It determines that *rel*A is required for antibiotic production during amino acid starvation. In this study, *rel*A was found 4-fold induced in M9O (Table 1), indicating that LB400 may has a virulence affect towards onion. Of course, this is only a hypothesis, which can be confirmed by performing *in vivo* experiments in the future.

This hypothesis is further strengthening with the evidence of up-regulation (5-fold) of *rpoN* in M9O verses M9 (Table 1). Because, RNA polymerase sigma factor 54 (*rpoN*) is not essential for

survival and growth in most bacteria (Buck *et al*., 2000; Grossman *et al*., 1984). However, *rpoN* belongs to the transcription mechanisms and is important in the regulation of nitrogen, carbon and energy metabolism, signal transduction pathways, RNA modification, chemotaxis, development, flagellation, electron transport, response to heat and phage shock, expression of alternative sigma factors and in many other biological activities (Buck *et al*., 2000). It has also been reported that *rpoN* are involved in regulation of antibiotic production and biocontrol activity in *P. fluorescens* (Pechy-Tarr *et al*., 2005). Previously, Denef *et al*. (2004) has reported the induction of *rpoN* in only biphenyl grown cell cultures of LB400. Here, the induction of *rpoN* due to onion extract in the nutritional medium of LB400 is being reported.

The presence of phenolic compounds such as flavonoids, catechol and protocatechuic acid in the onion extract (Bakhru, 1994; Block, 1985), could be the reason for the high induction of heat shock related genes such as *groEL*, *rpoN*, *cheW* and *flgL* (Table 1 and Fig. 5). *groEL* belongs to the post-translational modification, protein turnover and chaperones mechanisms, encode chaperonin GroEL (HSp60 Family) protein. *groEL* is required for biologically significant chaperonin function in protein folding (Weber *et al*., 1998).

Moreover, *cheW* belongs to the signal transduction, cell motility and secretion pathways. CheW is an adaptor protein, which couple CheA, an autophosphorylating protein histidine kinase, to receptors (Boukhvalova *et al*., 2002). Receptors at the cell surface phosphorylate CheY, a response regulator protein, which modulates flagellar motility and thus affects swimming behavior of the cell. It has previously been described that flagella and flagellum-mediated motility contribute to the virulence of a number of pathogenic bacterial species (Tomich *et al*., 2002). *flgL* also belongs to the cell motility and secretion pathway like *cheW*.

The increase in the expression level of multidrug-efflux transporter protein *acrB* and other conserved hypothetical protein *pulK* support the above statement. *pulK* belong to the type II secretion system that permits the energy-dependent secretion of a limited number of specific proteins from the periplasm and are involved in the virulence determinants in pathogenic bacterium (Francetic *et al*., 2000; Pugsley, 1993). It has been found that the resistance of microorganisms to multiple antibiotics and other drugs is due to the activity of multidrug efflux proteins (Poole, 2002). Onion is well known for its antibacterial and antifungal properties (Block, 1985; Ghahfarokhi *et al*., 2003). Moreover, the increase in the expression level of chemotaxis protein (CheW) and flagellin (FLG) may also be related to the stress caused by the antibacterial compounds in onion extract (Spiro *et al*., 1997).

Interestingly, two to three-fold inductions of PCBs degrading genes *bphA*, *bphE* and *bphG* was also observed with onion extract (Table 1 and Fig. 5). A point to be noted, that these biphenyl degrading enzymes were not responded to benzoate or succinate (Denef *et al*., 2004). Aerobic biodegradation of PCBs requires biphenyl dioxygenase, a multicomponent enzyme consisting of four subunits, a terminal dioxygenase (large subunit encoded by *bphA* and small subunit encoded by *bphE*), ferredoxin encoded by *bphF* and ferredoxin reductase encoded by *bphG* (Bruhlmann and Chenn, 1999; Denef *et al*., 2004). Previously, it has been shown that LB400 strain grown on plant phenolic compounds catechin, chrysin, maclurin and myricetin (Donnelly *et al*., 1994) and glucose, or glycerol (Billingsley *et al*., 1997) was able to degrade certain PCB congeners. It is well known that catechols are produced as central intermediates during PCBs degradation pathways by microorganisms (Reineke and Knackmuss, 1998). It is possible that catechol and catechuic acid present in onion extract promoted the expression of *bph* genes in LB400.

Conclusions

To our knowledge this report presents the first evidence that onion extract enhance the growth of *B. xenovorans* strain LB400. However, it raises many questions regarding the potentially complex

nature of LB400 and its interaction with onion This study evaluated the use of a DNA:RNA hybrid capture detection system and established that 10 μg of total RNA is sufficient to obtain microarray expression patterns. Using this system, it was found that onion extract enhances the expression of many genes including the *bph* genes. Changes in the expression profile of differentially expressed genes illustrated the nutritional versatility of LB400 as well as determined, to some extent, the nature of the molecular interactions between LB400 and onion. The expression profile analysis revealed that the addition of onion extract in the M9 medium increased the available nutritional resources for transition for LB400. LB400 exhibited fast growth rate in M9O, indicating its ability to consume highly organic and sulphide containing compounds, as well as to resist the antibacterial nature of onion. Further, the up-regulation of *rel*A, *rpo*N, *cheW*, *flg*L and *pul*K suggested that the LB400 has a potential to be a virulent pathogen of onion. Moreover, the positive affect of onion on the induction of PCBs degrading genes also suggest that onion may also be used, instead of biphenyls, to grow LB400 for PCBs degradations.

Additional work is needed, to determine the virulence of LB400 to onion, as well as to reveal the full extent of LB400 metabolic pathways. As it is likely, the genes identified in this study represent just a small portion of LB400's genome. Availability of the gene sequence of LB400 at JGI will facilitate its genetic analysis. The available genetic resources and information for LB400 will make possible to identify genes involved in its virulence as well as the characteristic properties to survive and thrive in various ecological niche.

Supplementary Material

The following material is available as supplement:

Table S1: A list of all 530 LB400 genes with gene name/ID, short description and COGs classification

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