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ARTICLE

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ORGANOPHOSPHATE DEGRADATION: AN ATTEMPT TOWARDS UNDERSTANDING THE MOLECULAR RESPONSE OF *ACINETOBACTER* SP. STRAIN MEMCL4 TOWARDS PARATHION BY COMPARATIVE PROTEOMICS STUDY

SANTANU PAILAN*

Acinetobacter sp. strain MemCl4, isolated from rhizosphere of a paddy cultivation filed in Burdwan, West Bengal, India, was demonstrated for parathion degradation. Although, during degradation, there was reduction in residual amount of parathion with gradual passage of time, no significant intermediates for its biodegradation could be detected even with analytical techniques such as high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analyses. In order to have clear idea of response of this strain towards parathion (during its degradation), comparative proteomic studies (2-dimensional gel electrophoresis; 2-DE) were undertaken to identify 12 proteins which were specifically expressed/over expressed in response to parathion. Several bioinformatics analyses were carried out to predict probable functions of these proteins. Presence of glutathion-S-transferase (GST) gene and its higher enzyme activity in parathion induced cells were also highlighted. Based on existing knowledge and evidence obtained from experiments, a possible working hypothesis was framed for the strain MemCl4 in context to parathion utilization as well as its stress encounter.

Introduction

In spite of ban, parathion and methyl parathion continues to be major Organophosphate (OP) insecticides for control of insects[1]. Burdwan is one of the important rice growing stations of India and farmers have been using diverse kinds of OP insecticides to control insect pests[2]. These have probably resulted in contamination of agro-ecosystems and other adjoining niches[3]. OP insecticides being inhibitors of acetylcholine esterase are potent neurotoxins[4] and are therefore threat to non-target animals such as fishes[5], bees[6], rats and silk worms[7] and humans[1]. Microbial bioremediation is considered to be cost effective, eco-friendly, greener alternative over other physico-chemical methods[8]. With this aims and objective, several OP degrading bacterial strains were isolated from agricultural niches of Burdwan[9-11]. In one such enrichment isolation attempt, using chlorpyrifos as sole source of carbon, three morphologically identical OP degrading bacterial strains (designated MemCl1, MemClNew and MemCl4) were isolated from paddy field agricultural soil of Memari village, Burdwan district (lat. 23.172798$^{\ddot{E}\%}$N and long. 88.095894$^{\ddot{E}\%}$E). All of them were affiliated to *Acinetobacter* genus by 16S rRNA gene based molecular phylogenetic approach. Out of these three, strain MemCl4 was chosen for detail OP degradation studies. The chlorpyrifos degradation property of strain MemCl4 has already been documented in Pailan et al. (2016). Information from literatures suggested that although *Acinetobacter* spp. can degrade methyl-parathion phenol, benzoate and various nitro aromatics, they do so by an unknown process that might involve ring cleavage (as

1 Assistant Professor, Department of Biotechnology, The University of Burdwan

e-mail: spailan@biotech.buruniv.ac.in; santanu.pailan@gmail.com

reported for *A. radioresistance* USB-04 by Fang-Yao et al. (2007)[12] . Moreover, no OP degrading genes has been reported from the genomes of different *Acinetobacter* spp. sequenced so far[13]. However, few years back Longkumar et al. (2014)[14] has demonstrated the role of Glutathion-S-transferase (GST) enzyme in co-metabolic detoxification of methyl-parathion in *Acinetobacter baumannii* DS002.

Since, structurally, parathion is also similar to chlorpyrifos (both being -thiol) and till date no report of parathion degrading *Acinetobacter* strain is available in literature, parathion degradation property of the strain MemCl4 was explored in this study. Additionally, in order to understand the overall molecular response of strain during parathion exposure by this strain, comparative proteomics study of parathion treated and untreated were undertaken. Based on existing knowledge from literature and the present comparative proteomic study, author presents a possible hypothesis for mineralization of parathion by the strain MemCl4. This aspect has never been presented/ reported, to the best of my knowledge.

Materials and Methods

Growth and Degradation Studies: Growth studies, inoculum preparation for parathion degradation studies and extraction process were carried out as described earlier by Pailan et al. (2016)[11] for chlorpyrifos degradation by the same strain. Determination of intermediates of parathion degradation by the strain MemCl4 was carried out by HPLC and GC-MS as described earlier by Pailan and Saha (2015)[10].

Glutathion-S-transferase (GST) Assay: For carrying out GST assay, the culture was grown under two different conditions; parathion treated condition was used as test set while the culture grown on MM-citrate was considered as control set. Cells from both the conditions were washed, suspended in PBS (pH-7.0, 50 mM) and cell lysis was carried out by sonication (5 min for 3 times with full cycle of 100% amplitude, using Hielcher UP50H, USA) followed by incubation for 20 min on ice-flex with periodic vortexing for crude enzyme extraction. The homogenate was centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatant was used as crude enzyme extract. The GST activity was determined by increment of absorbance at 340 nm due to the formation of GSH (L-Glutathione reduced) and CDNB (1-Chloro-2, 4-dinitrobenzene) conjugate in the reaction mixture. The reaction mixture was prepared according to Habig and Jakoby (1981)[15]. Crude enzyme extract samples (20 µl) were added to an assay mixture containing 970 µl of PBS, 5 µl of GSH (from 100 mM stock) and 5 µl of CDNB (from 100 mM stock). The reaction mixtures were incubated at 37 ºC. After a specific (10 min) time interval aliquot of the reaction mixtures were taken and monitored A_{340}, spectrophotometrically.

GST Gene Amplification and Analyses of Gst and Abgst Gene Sequences of Strain MemCl4: For this, first genomic DNA from strain MemCl4 was isolated following Murmur's protocol[16]. This genomic DNA was used as template for amplification of *gst* and *Abgst* genes by using specific primers [(*gst* F-primer 5' CGNTTYGGNTTYTAYGGNYTNTAGAAGAG 3' and R-primer 5' CCTTGAARACKNGAYTGTARNGCYTCNAGNGG 3'), (*Abgst* F-primer 5' & CCCTCATATGATTAGTGTACACCACCTGG 3' R-primer 5' CCCGTTCCTCTCGAGTTATATATCAGCAC 3')] as reported by Longkumar et al. (2014)[14]. The PCR (polymerase chain reaction) cycling parameters consisted of an initial denaturation at 94 ºC for 10 min followed by 29 cycles of denaturation at 94 ºC for 1 min, annealing at 53 ºC for 1 min amplification at 72 ºC for 0.50 min, and final extension at 72 ºC for 10 min. Excised the amplicon gel pieces (size for *gst* 0.511 kb and *Abgst* 0.668 kb approximately) from agarose gel and purification of amplicons were carried out as per instructions of QIAquick gel extraction kit (Qiagen, Germany), for sequence determination. The GST (*gst* and *Abgst*) gene sequences were subjected to thorough analyses and the nearly related sequences were determined using n-BLAST program of NCBI (www.ncbi.nlm.nih.gov/Blast/nblast).

Whole Cell Protein Extraction from MemCl4 for 2-DE for Comparative Proteomic Study: In order to carry out comparative proteomic studies, strain MemCl4 was grown on MM containing 250 µg ml^{-1} of parathion as sole source of carbon (test set) and on MM containing 0.5% tri sodium citrate as sole source of carbon as control set. Cells were harvested from mid-log phase by centrifugation at 6000g for 10 min followed by washing (twice with 50 mM Tris-CL; pH-7.2) and extraction according to Alam and Ghosh (2014)[17] with some modification. The dried protein pellets were resuspended in a Ready Prep rehydration buffer (BioRad) and quantified by Bradford method (using kit from BioRad).

Whole cell protein (~430 µg from each) were applied to 11 cm linear IPG strips of 4-7 pH range and rehydrated for 16 h at 20 °C. Isoelectric focusing (IEF) was performed on a PROTEAN IEF cell (BioRad) maintaining the conditions such as linear ramping to 250 V (volt) for 20 min, linear ramping to 8,000 V for 3 h, rapid ramping to 20,000 Vh (volt-hour), and eventual termination at 30,000 Vh. The focused IPG strips were placed in equilibrium buffer (0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% v/v

glycerol and 2% w/v SDS) containing 2% w/v DTT for 10 min and soaked again in equilibrium buffer containing 2.5% w/v iodoacetamide for 10 min as reported by Alam and Ghosh (2014)[17].

Second dimension electrophoresis of the proteins in the focused IPG strips was performed on 12% SDS-Polyacrylamide gels. The strips were placed on 12% Acrylamide: Bis-acrylamide SDS-PAGE and sealed with agarose sealing solution (0.5% w/v agarose, 25 mM Tris, 190 mM Glycine, 0.1% w/v SDS, and a trace of bromophenol blue). The second dimension SDS-PAGE was run at 80 V for the first 30 min followed by 160 V until the dye front reached the bottom of the gel. After run, gels were stained (0.1% w/v of coomassie brilliant blue R-250 dissolved in 50% v/v methanol and 10% v/v glacial acetic acid) for at least 1h and destained (45% v/v methanol and 10% glacial acetic acid) to increase clarity of spots. The gel imaging and spots scanning were carried out with a Gel Documentation system (BioRad).

Tryptic Digestion Proteins and MALDI TOF/TOF Analyses: Protein spots of interest were excised from the gel manually with a sterile scalpel, chopped into small pieces (approximately 1X1mm) and transferred into a microcentrifuge tube. De-staining, dehydration, rehydration, alkylation, washing were performed as per Shevchenko et al. (2006)[18]. Dried gel pieces were rehydrated with trypsin solution (12.5 ng μl^{-1} trypsin in 50 mM NH_4HCO_3) and incubated for overnight at 37 °C. Peptides were extracted from gel matrix by incubating the gel pieces twice with 1:1 v/v extraction buffer [3% trifluoroacetic acid (TFA), 30% acetonitrile, diluted with water] for 15 min with vigorous vortexing followed by second round of extraction (twice) with 100% acetonitrile for 15 min (with vigorous vortexing). After extraction, the gel pieces were spun down (at 8,000 rpm for 1 min) and the supernatant was collected. The samples were dried to remove acetonitrile that was 10-20% of original volume, re-acidified with 40% (v/v) with 2% v/v TFA and the left over gel pieces were spin down. Digested peptides were used in MALDI TOF/TOF analysis for generation peptide mass finger prints.

Tandem mass spectrophotometric analyses of the extracted peptides were performed using an AutoFlex II (Burker Daltonics) MALDI TOF/TOF machine equipped with a pulse N_2 laser (λ = 337 nm, 50 Hz).

Bioinformatics Analyses of Proteins: Extracted peptides were analyzed by flex analysis software (version 2.4) and the processed peaks were transferred through the MS BioTools (version 3.0) program as input to MASCOT (http:// www.matrixscience.com) search engine (version 2.2) for protein identification. The following specified parameters were applied for database search: database (NCBInr), taxonomy (Other *Proteobacteria*), proteolytic enzyme (Trypsin), peptide mass tolerance (≤ 0.5 Da) fragment mass tolerance (≤ ±1.0 Da), global modification (carbamidomethyl, Cys), variable modification (oxidation, Met), peptide charge state (1+), and maximum missed cleavage (1). Several other parameters such as source, strain name with whom the particular protein gave best hit, detail characteristics of the protein such as its pI value and NCBI accession number of these proteins were determined from the output of MASCOT search followed

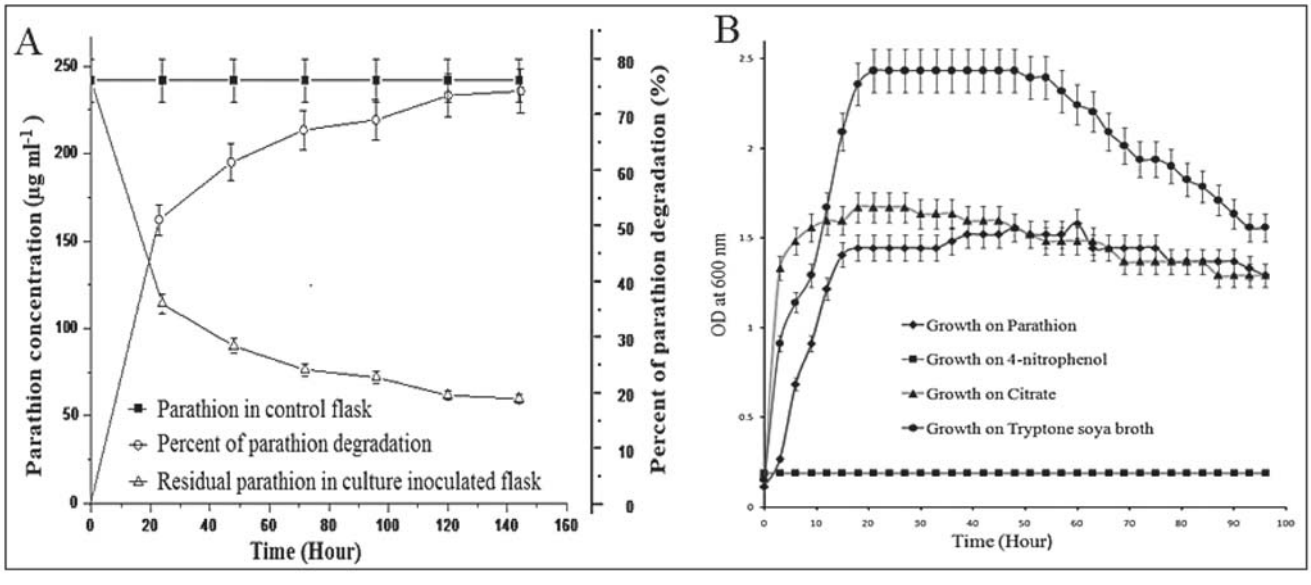


Fig. 1 (A) Parathion degradation profile by the strain MemCl4 and (B) Growth profile of strain MemCl4 on parathion, 4-NP, citrate (0.5%) and tryptone soya broth (3%).

by NCBI (https://www.ncbi.nlm.nih.gov/protein/?term=) search. The cellular localization of the identified proteins was predicted by using two web based software PSORTb, version 3.0.2 (http://www.psort.org/psortb/) and CELLO, version 2.5 (http://cello.life.nctu.edu.tw/). Attempts were made to predict the probable function of the proteins by understanding interacting components (i.e. interactome) that was determined by STRING (http://string-db.org/cgi/input.pl?UserId=VjmY5OYRC2hc&sessionId=1i89SGW21AFi&input_page_show_search=on) database and NCBI protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) search.

Results

Degradation of Parathion by the Strain MemCl4: Parathion degradation study by *Acinetobacter* sp. strain MemCl4 revealed that the strain could degrade 54% of parathion within 24 h and 77.3% of the same within 144 h (Fig. 1A). During the course of parathion degradation by the strain MemCl4, no significant peak of any hydrolytic intermediate (other than the parent parathion) peak could be detected by HPLC analysis (HPLC elution, Fig. S1) as well as by GC-MS analysis (Fig. 2). Through quantitative analysis by HPLC, it was clear that parathion concentration was decreasing in broth culture medium with the gradual passage of time, there by indicating utilization of parathion by the strain MemCl4 (Fig. S1). Growth studies (Fig. 1B), suggested that MemCl4 utilized parathion catabolically but could not grow and utilize 4-nitrophenol (4-NP) (the major hydrolytic intermediate of parathion) catabolically.

Glutathion-S-transferase Assay from Crude Cell lsate of Strain MemCl4: Cell free extract of parathion treated cells showed significant increase in GST enzyme activity with respect to time (Fig. 3). While, the GST activity was low and remained unchanged in case of citrate grown cell free extract. The higher GST activity in parathion treated condition suggested that somehow GST enzyme or GST like enzyme system was over- expressed in parathion grown cells, which might be involved in parathion degradation by the strain MemCl4.

Gst and Abgst01gene Amplification from MemCl4 and Their Identification: The PCR amplicons of both *gst* and *Abgst01* (*A. baumannii* specific *gst* gene) were obtained successfully and were approximately 0.5 kb and 0.7 kb in size, respectively (Fig. 4A**)**. Partial nucleotide sequence information obtained by direct sequencing of PCR amplicons (Fig. 4B) indicated that *gst* gene from strain MemCl4 showed 97% of sequence identity with *gst* gene of the strain *Acinetobacter pittii* strain AP_882 [complete genome (CP014477.1) followed by 96% (to the same gene) from *Acinetobacter* sp. DUT-2 (CP014651.1) and 93% *A. pittii* strain IEC338SC (CP015145.1). With remaining strains it showed less than 90% sequence similarity. While, *Abgst01* gene from the strain MemCl4 showed closest 98% identity to *gst* gene sequence from *Acinetobacter* sp. DUT-2 (CP014651.1), followed by 95% with *A. pittii* strain AP_882 (CP014477.1) and 93% with *A. pittii* strain IEC338SC (CP015145.1), respectively (Table 1).

Comparative Proteomic Study of Strain MemCl4 in Presence and Absence of Parathion: In to order have more clear holistic idea regarding expression of proteins, whole cell protein profile was analyzed by 2-D-proteomics approach. During analyses, protein spots which were exclusively (either or) and over expressed (i.e. up-regulated)

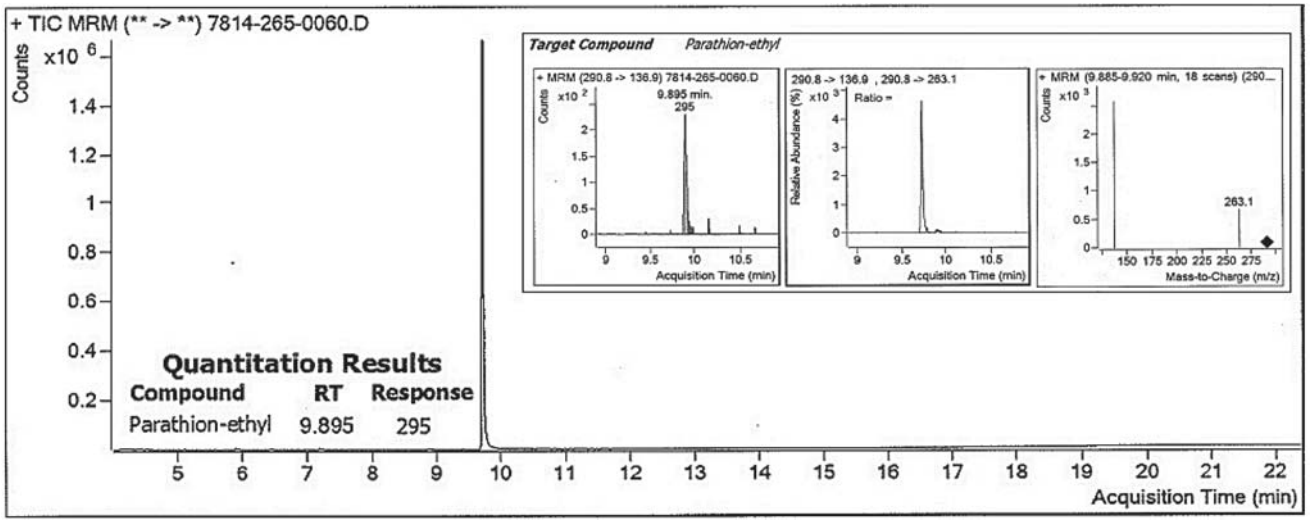
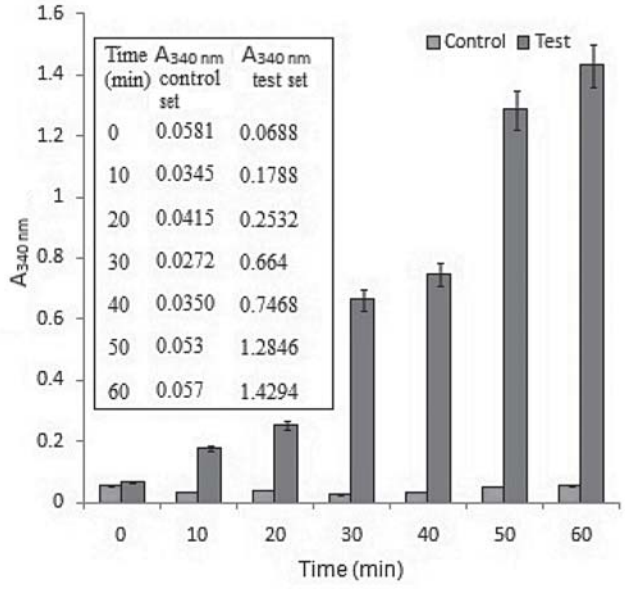


Fig. 2 Analyses of extract of parathion degradation by the strain MemCl4 by GC-MS. The GC chromatogram showing only parathion peak at RT-9.895 min. The Inset image indicates the mass-spectrum of parathion only.



Time (min)	$A_{340\ nm}$ control set	$A_{340\ nm}$ test set
0	0.0581	0.0688
10	0.0345	0.1788
20	0.0415	0.2532
30	0.0272	0.664
40	0.0350	0.7468
50	0.053	1.2846
60	0.057	1.4294

Fig. 3 The GST assay by crude enzyme of parathion induced cell of MemCl4

in the parathion treated condition (Fig. 5) were excised from the gel and subjected to peptide mass fingerprinting (PMF) analyses followed by database search. This approach was chosen since, genome sequences from quite a large number of strains belonging to *A. baumannii* cluster were available in public databases, and possibility of finding a statistically significant hit was higher.

During comparative proteomic analysis, several protein spots were observed to be specifically over expressed in the proteome of parathion treated cells. From this primary data, finally, 12 protein spots (spot numbers 1-5, 9, 11, 13, 17, 21, 22 and 23 from Fig. 5) were selected for further studies by PMF approach followed their identification by employing bioinformatics tools. For identification, MASCOT search was carried out. Identification report of these 12 proteins (spots) based on MASCOT search is summarized in Table 2.

MASCOT Search Result: Several Proteins with Possible Function: Database search revealed that these twelve proteins can be categorized into several groups whose possible function(s) are as follows

a. Possible Function of Hypothetical Proteins: Four protein spots (1, 2, 3 and 5 from Fig 5B) were identified to be belonging to this group, as they showed closest hit following systematic search using peptide mass profile data. Out of these four, spots1 and 2 are identical so only 1 was considered.

1. Hypothetical protein J610_1036 (NCBI accession no. EXI18152).
3. Hypothetical protein BDGL_000762 (YP_004995030) and
5. Hypothetical protein J518_2320 (EXB31333).

All these belonged to the *A. baumannii* (but different strains). Through bioinformatics, hypothetical protein spot 1, from above was predicted to be outer membrane proteins while 3 and 5 were predicted to be cytoplasmic protein, in terms of cellular localization. Through interactome analyses (carried out at STRING database), hypothetical protein J610_1036 (spot 1 and 2) seems to interact with Na+/Proline symport system in *A. baumannii* with highest bit score while in *A. radioresistens* it interacted with o-methyltransferase. From these, it appeared that this hypothetical protein may be part of a membrane bound transport system (which might participate in transport at outer membrane and or inner cytoplasmic membrane) involved in transport. It was not possible to predict the function of Hypothetical protein BDGL_000762 (spot 3).

Study of interacting partners for the Hypothetical protein J518_2320 (spot 5), predicted few interesting proteins such as LysR family transcriptional regulator (which are reported to be involved

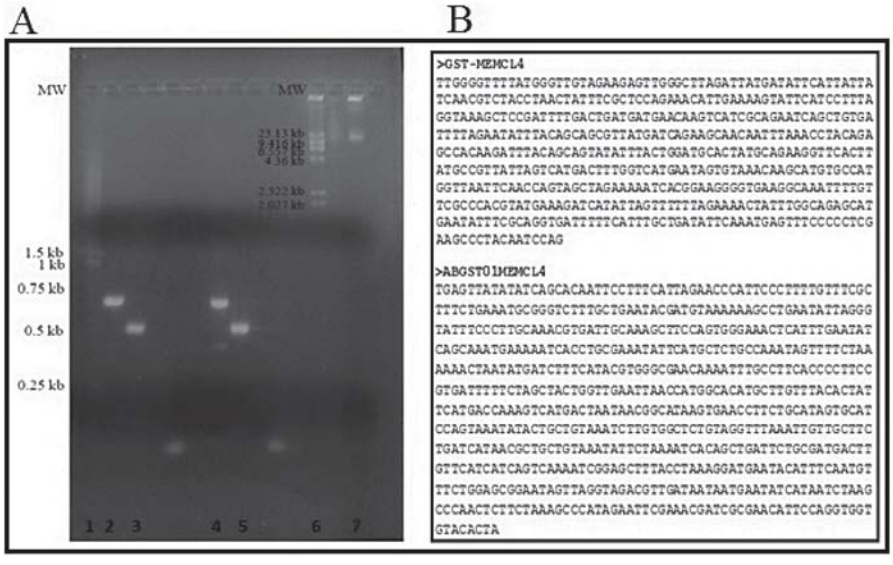


Fig. 4 (A) Agarose gel showing PCR amplicons for *gst* (lane 2) and *Abgst01* (lane 3) genes amplified from genomic DNA of strain MemCl4 and lane 1 and lane 6 indicate 1kb DNA ladder and lambda DNA Hind III digest respectively and (B) The partial nucleotide sequence of *gst* and *Abgst01* genes from the strain MemCl4.

Table 1 Results of BLAST Analysis for *Gst* and *Abgst01* Genes from the Strain MemC14

Name of gene in MemCl4	Closest match (accession no. of complete genome sequence)	Range (bp) of nucleotide sequence that matched with genome sequence in close relatives	Sequence identity
gst	*Acinetobacter pittii* strain AP_882 (CP014477.1)	1233175-1233655	97%
	Acinetobacter sp. DUT-2 (CP014651.1)	3615365-3615863	96%
	Acinetobacter pittii strain IEC338SC (CP015145.1)	640949-641441	93%
	Acinetobacter baumannii strain XH856 (CP014541.1)	3755633-37566154	86%
Abgst01	*Acinetobacter* sp. DUT-2 (CP014651.1)	3615241-3615904	98%
	Acinetobacter pittii strain AP_882 (CP014477.1)	1233137-1233800	95%
	Acinetobacter pittii strain IEC338SC (CP015145.1)	640911-641574	93%
	Acinetobacter baumannii strain XH856 **(**CP014541.1)	3755529-3756192	86%

in various cellular functions like quorum sensing, cell survival and regulation in utilization of aromatic compounds), Pyrimidine utilization flavin reductase F (involved in utilization of pyrimidines) and Methionine synthase II (known to be involved in methionine regeneration from homocysteine). However, its distinct role is not clear; one speculation is, it might help the cell to survive under nutrient stress condition by enhancing aromatic compound utilization.

b. Possible Functions of Putative N-acetyltransferase (spot 9): Previously, N-acetyltransferase from microbial origin was reported for various xenobiotics detoxification and degradation. N-acetyltransferase has been reported to occur in soil bacteria such as *Pseudomonas* sp[19]. Identification and sequencing of two putative N-acetyltransferase enzymes from the genome of *Mesorhizobium loti* (MAFF 303099 strain), a rhizobial nitrogen-fixing bacterium that lives in symbiosis with several species of the genus *Lotus*, including *Lotus corniculatus* was also carried out by Rodrigues-Lima and Dupret (2002)[20]. These two enzymes were shown to catalyze the N acetylation reaction of several known NAT substrates including aniline-derived pesticide residues (3, 4-DCA, 4-BA and 4-iodoaniline)[20]. Martins et al. (2010)[21] suggested that detoxifying activity of N-acetyltransferase and presence of N-acetyltransferase genes in many fungi and the fungal biomass in fungi-bearing soils is a promising fungal bioremediation strategy for pesticide-derived aromatic amines.

c. Possible Function of Antibiotic Biosynthesis Monooxygenase (spot 11): The antibiotic biosynthesis monooxygenase (ABM) domain is found in proteins involved in a diverse range of biological processes, including metabolism, transcription, translation and biosynthesis of secondary metabolites (https://www.ebi.ac.uk/interpro/entry/IPR007138).

Upon BLAST search, the peptide was found to contain YgiN domain as well. This domain is usually present in widely distributed enzymes that carry out oxidation-reduction reactions. They are also called monooxygenases (since they incorporate one atom of O_2 into organic substrate) or hydroxylase (http://www.reference.md/files/C498/mC498878.html). Study of crystal structure predicted that YgiN (from *E. coli*) as quinol monooxygenase. It oxidizes menadiol to menadione[22]. In *Staphylococcus* it participates in signaling and in

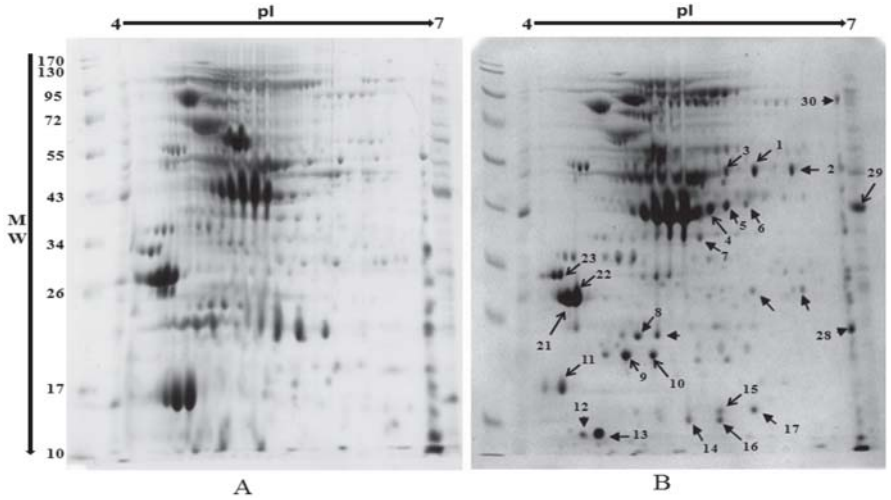


Fig. 5 Comparative proteome analysis of strain MemCl4 by 2-DE. **A**, the proteome of citrate grown cell and **B**, the proteome of parathion grown cell. The spots indicated by arrow were up-regulated in presence of parathion.

Table 2 Details Features/properties of the Proteins Identified During the Study

Spot number[a]	Protein name	Source organism	gi/ accession no.	NCBI accession no.	Score/ coverage (%)[b]	Matched/ searched[c]	pI(Theoretical/ practical)	Cellular localization (score)[d]
1	Hypothetical protein J610_1036	*Acinetobacter baumannii* 723929	gi/589538515	EXI18152	110/41	15/56	6.86/6.2	Outer membrane protein (9.52/2.920)
2	Hypothetical protein J610_1036	*Acinetobacter baumannii* 723929	gi/589538515	EXI18152	170/49	19/53	6.86/6.4	Outer membrane protein (9.52/2.875)
3	Hypothetical protein BDGL_000762	*Acinetobacter calcoaceticus* PHEA-2	gi/375134380	YP_004995030	76/77	6/53	4.70/5.8	Cytoplasmic protein (2.00/1.988)
4	Putative membrane protein	*Acinetobacter baumannii* 15827	gi/630468740	KCX81343	41/94	1/1	5.69/5.65	Cytoplasmic protein (2.00/2.131)
5	Hypothetical protein J518_2320	*Acinetobacter baumannii* 1419130	gi/587840710	EXB31333	112/32	8/36	5.89/5.8	Cytoplasmic protein (8.96/1.679)
9	putative N-acetyl transferase	*Variovorax paradoxus*	gi|540273498	WP_021012635	59/44	7/18	5.36/4.9	Cytoplasmic protein (2.00/ 3.437)
11	Antibiotic biosynthesis monooxygenase	*Massilia* sp. LC238	gi|668946759	KFC65568	47/24	2/2	5.36/4.3	Cytoplasmic protein (2.00/4.110)
13	Putative surface antigen	*Acinetobacter baumannii* 846928	gi/589560768	EXI39753	88/81	7/53	4.7/4.7	Cytoplasmic protein (2.00/2.065)
17	Tyrosyl-tRNA synthetase	*Labrenzia aggregata*	gi/493992029	WP_006934748	62/28	15/95	5.64/6.2	Cytoplasmic protein (9.97/4.454)
21	pyridoxamine 5'-phosphate oxidase	*Pseudomonas fluorescens*	gi|515908301	WP_017338884	61/33	4/21	6.92/4.5	Cytoplasmic protein (9.26/2.339)
22	7,8- dihydro-8-oxoguanine-triphosphatase	*Desulfobulbus mediterraneus*	gi/655137701	WP_028584621	49/30	3/9	4.78/4.5	Cytoplasmic protein (8.96/3.806)
23	Metal-dependent hydrolase	*Pseudomonas syringae* pv. japonica str. M301072	gi/330899949	EGH31368	66/23	4/12	9.18/4.3	Cytoplasmic protein (8.96/4.232)

a) The spot numbers are same as shown in Fig. 5.

b) MASCOT score and coverage (%) of the total sequence of the corresponding protein by the matched peptides obtained during peptide mass fingerprint.

c) Number of mass values matched out of total number of mass values searched.

d) Cellular localization of the proteins predicted by the web-based software utilities PSORTb, version 3.0.2 (http://www.psort.org/psortb/) and CELLO, version 2.5 (http://cello.life.nctu.edu.tw/). Probability scores (PSORTb/CELLO) are given in brackets.

Mycobacterium it helps in degradation of heme[23]. This is suggested to be adaptive cellular response to protect the cells from oxidative damage of menaquinones and other quinonone compounds. The ABM domain is reported from various monooxygenases (from *Streptomyces* spp.) which are involved in the synthesis of antibiotics (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?ascbin=8&maxaln=10&seltype=2&uid= pfam03992), aromatic polyketides by oxidation of phenolic groups to quinones.

This spot appears to possess a monooxygenase enzyme that might be involved in initiating ring cleavage. Exact location of this protein is not known. It appears to be cytosolic however, may have membrane bound component(s) as well.

d. Possible Function of Putative Surface Antigen (Spot 13): No conserved or putative functional domain was detected upon BLAST search, for this peptide. However, all the hits were from *Acinetobacter* spp. Hypothetical/multispecies hypothetical protein. One match [from *Acinetobacter baumannii*, with 50%, identity; 6e-19, e value & 86.7 bits (213)] with BLAST and PsI-BLAST was glycine zipper which gives hunches for probable DNA binding activities. But it is difficult to speculate any function right now.

e. Possible Function of Tyrosyl-tRNA Synthetase (TtRS, Spot 17): In general, TtRS is involved in protein synthesis where they participate in incorporation of tyrosine amino acid in a growing polypeptide as per instruction through codon-anticodon interaction and this enzyme is suggested to have promiscuous activity[24]. In *Neurospora* the mitochondrial TtRS is reported to be involved in splicing[25]. It may be concluded to be involved in translation process but how this might be involved with parathion degradation is difficult to speculate.

f. Possible Function of Pyridoxamine 5'-phosphate Oxidase (PPO, Spot 21): PPO catalyzes several reactions; the two most important are the deamination of pyridoxamine-5'-phosphate and the deamination of pyridoxine 5-phosphate, both of which are key intermediates in the metabolism of vitamin B_6. It also plays a role in nitrogen metabolism, converting amines to aldehydes NH_3 (https://en.wikipedia.org/wiki/

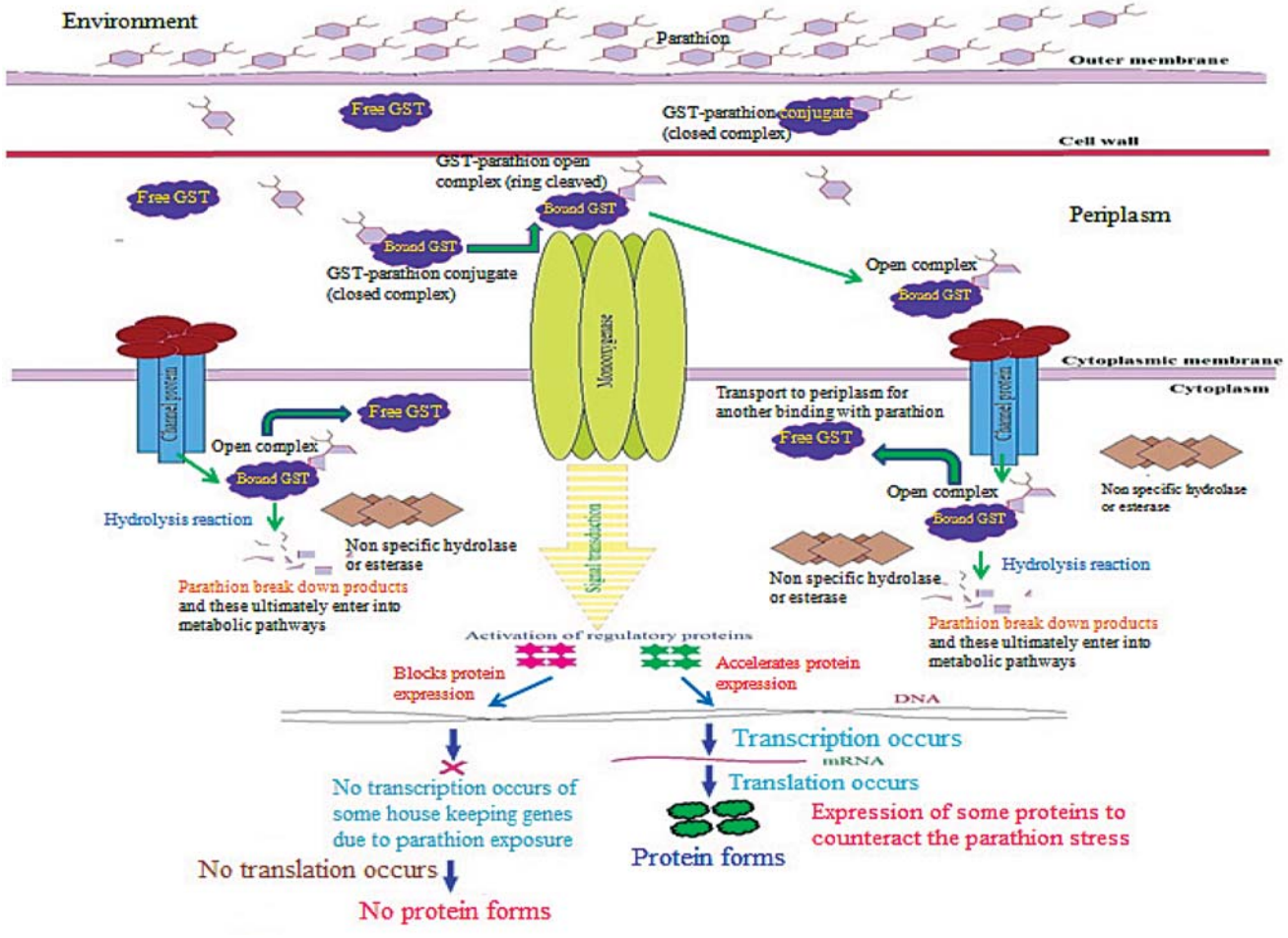


Fig. 6 Pictorial diagram of possible hypothesis for overall molecular response towards parathion by MemCl4

Pyridoxine_5%27-phosphate_oxidase). Since, Vitamin B_6 participates as coenzyme for proper functioning of enzymes involved in synthesis amino acids, PPO might influence amino acid biosynthesis, during parathion degradation.

g. Possible Function of 7, 8-dihydro-8-oxoguanine-triphosphatase (Spot 22): This triphosphatase is known to hydrolyse 8-oxo-dGTP into monophosphates thereby preventing the incorporation of mutagenic substrate 8-oxo-dGTP, which creates mutation in DNA upon incorporation. The enzyme has some similarity to MutT domain of MutT protein that controls excessive spontaneous mutation due to guanine nucleotide in *E. coli*. This enzyme has wide occurrence and with respect to *E. coli* system, MutT works in association with MutM and MutY proteins to carry out prevention and correction work[26].

h. Function of Metal-dependent Hydrolase (Spot 23): Based on BLAST search, the protein was found to be part of DUF 45 super family. This super family includes proteins whose function is unknown (i.e. Domain of unknown function)[27]. From CDD (Conserved Domain Database) search, DUF 45 (pfam01863) was reported to have no function. Its representative members are found in some archaebacteria, as well as *Helicobacter pylori*. The proteins are 190-240 amino acids long, with the C-terminus being the most conserved region, containing three conserved histidines. This motif is similar to that found in Zinc proteases, suggesting that this family may also be proteases.

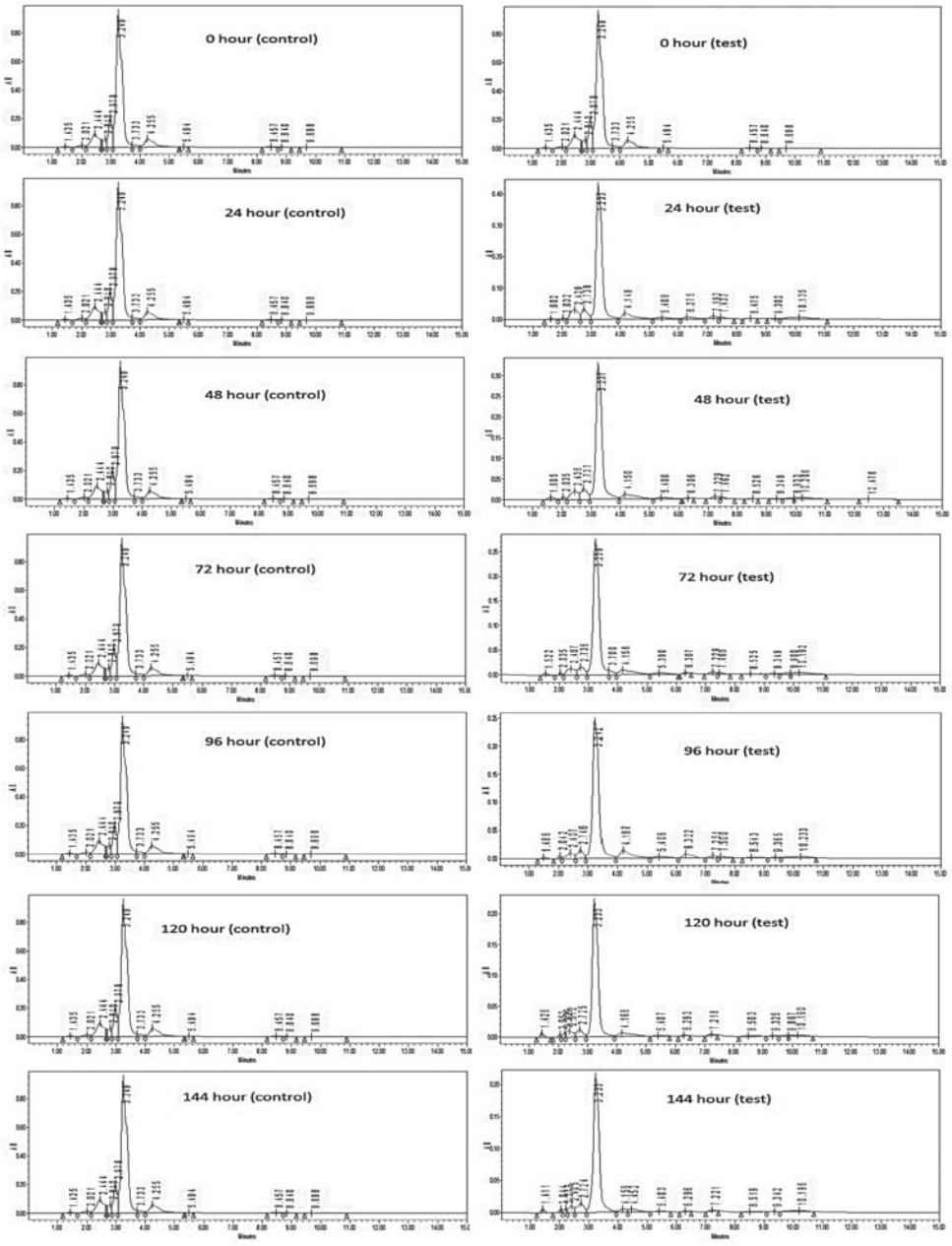


Fig. S1 Time course studies for parathion degradation by the strain MemCl4 using HPLC. The elution profile of each sample is shown as individual HPLC chromatograms.

Analysis carried out at STRING database revealed Phosphoriboisomerase A, Type I restriction enzyme, Type I restriction-modification system (M subunit), Restriction endonuclease (S subunit), Threonine ammonia-lyase (biosynthetic) (this enzyme has been considered to serve as an excellent example of the regulatory strategies used in amino acid homeostasis; https://en.wikipedia.org/wiki/Threonine_ammonia-lyase) and TrkA-N domain-containing protein (probably helps in transport) to be its predicted functional partners.

Right now it is difficult to predict the function of this metal hydrolase, but it might be involved in amino acid homeostasis process in the cell.

Discussion

Members of the genus *Acinetobacter* are wide

spread in nature (soil, water, living organisms), reported to be metabolically diverse[28] and are known to mineralize (and degrade) diverse toxic, xenobiotic compounds such as phenol[29], polychlorinated biphenyls, monohalogented biphenyls[30], toluene[31], 4-clorobenzoate[32] etc. There has been strong growing interest among microbiologists to pursue *Acinetobacter* for bioremediation purpose.

Till now, the role of *Acinetobacter* in OP compounds biodegradation and its detail pathway information are lacking in literature. Although, *A. radioresistens* USTB-04 was reported to involve in degradation of methyl parathion[12], neither any strain information, nor any sequence information exists in public databases and another plant growth promoting *A. calcoaceticus* strain D10 was reported for chlorpyrifos degradation but detail degradation pathways and molecular studies were lacking[33]. Survey of literature also suggested that till date no OP degrading genes have been identified from the publicly available genome sequences of any *Acinetobacter* strains. This strongly indicated that probably some novel enzyme system (other than product(s) of *opd*, *opdA*, *mpd* gene) might be involved in degradation of parathion by the strain MemCl4. *Acinetobacter* had been reported to grow on nitro aromatic compounds as sole source of carbon and energy by inducing two, novel catechol 1, 2 dioxygenase[12]. As already reported by Fang-Yao et al. (2007)[12], it is also strongly believe that when *Acinetobacter* sp. strain MemCl4 grows on parathion compound in minimal medium, as sole source of carbon, it does so, by degrading it through ring cleavage mechanism. During HPLC as well as GC-MS analysis, absence of any peak of hydrolysis intermediates of parathion [such as 4-NP, hydroquinone (HQ), 4-nitrocatechol (4-NC)] strongly suggests that P-O bond (ester bond) was not cleaved, otherwise it would have got signal for intermediate compounds. Probably, cleavage occurred directly at aromatic ring through some as yet unknown pathway. Recently, oxyR-dependent novel glutathione-s-transferase (GST) mediated biotransformation of Me-parathion by *A. baumannii* DS002 was reported by Longkumar et al. (2014)[14]. Many of the GST enzymes are reported and it may have promiscuous activity by virtue of which they can simultaneously recognizing and catalyze hydrolysis of diverse class of unrelated compounds[34] and have been reported from many Gram negative bacteria and evidence suggests their role in xenobiotic and antibiotic degradation[34]. The presence of GST gene in the strain MemCl4 and its significant increased expression in parathion treated condition suggested the possible existence of such promiscuous GST like enzyme system might be involved in parathion degradation in absence of ever known parathion degrading gene(s).

However, as per our previous report Pailan et al. (2016)[11] chlorpyrifos degradation results by esterase positive strain MemCl4 strongly suggested the presence of a possible OP hydrolyzing enzyme highly specific towards chlorpyrifos and not acting on parathion. Parathion bio-degradation in this strain is probably operating by some other mechanism. This might involve enzyme system(s) directly acting upon the ring structural system to carry out cleavage of C-C bonds and their further hydrolyses by processes and mechanisms that are unknown till date. For this, to have a better understanding for overall molecular response towards parathion, comparative proteomic studies were undertaken and a probable hypothesis for overall molecular response towards parathion stress has been speculated on the basis of already existing knowledge from literature and what I got from present experimental data.

The overall general response process from the cells, when exposed to parathion, can be hypothetically categorized into several major events. These possible events are discussed below:

1. Events that lead to either degradation or detoxification of toxic parathion compound.
2. Every cell tries to maintain certain degree of homeostasis, characteristic of its own type. During exposure to parathion, there might be perturbations to other housekeeping biochemical processes within the cells. Thus to maintain overall cellular homeostasis, cells must express certain genes (for proteins) that will try to counteract this perturbations and maintain these housekeeping processes to be carried on normally for the proper survival of the cells. To do so, cell must do the following:
    a. Cell will stop transport of unnecessary metabolites. Under expression of several membrane transporter might result. Similarly, expression of some selective transporters might be increased by several folds, as well.
    b. Cells at this stage will also shut down processes (and expression of genes whose products) are crucial for cell division and multiplication.
    c. It is difficult to hypothesize to what extent DNA replication is blocked. Possibly, regulators that sense threshold copy number of genomes might be activated and over expressed (eventually leading to a halt in

DNA replication process).

d. In such a stage, chances of DNA damage might rise. So, also there will be over expression of those proteins that minimizes these damaging effects.

3. Cell must also express genes that codes for stress relieving proteins. It is difficult to speculate what kinds of stresses may be faced by the cell, when it encounters parathion. The most common possible one, is oxidative stress.

4. Parathion is toxic. Cells must prepare to counteract the adverse toxic effects of parathion.

Now, for a normal bacterial culture, the genetic information is to carry out and sustain the above mentioned processes may not be present and it does not survive (for some endowed with capacity to produce endospore, enters into a dormant stage). The strain MemCl4 was isolated by enrichment culture technique on a MM (minimal medium) that only had chlorpyrifos as sole source of carbon. So, in this culture, the cells are enriched with genetic information to face the challenge imposed by parathion exposure.

When transferred to parathion supplemented medium, possibly cells could not resist the inward diffusion of parathion (due to its small size and lipophilic nature). But since, there exists a threshold limit for tolerance (and an optimum concentration for best utilization, for growth), there is possibly a sensor in the cell that decides, how much of parathion must be allowed inside the cell. Two possible types of sensors (either cytoplasm based or inner cytoplasmic membrane based) are:

A. Parathion may be allowed to pass through inner cytoplasmic membrane to be sensed by a cytoplasm based sensor. This sensor might be connected (through some kind of activation/ deactivation; i.e. kinase dependent phosphorylation/dephosphorylation events) with a membrane channel (may be multipurpose transporter that might be involved in transport of normal cellular analytes/metabolites) to pump out (energy requiring process) excess parathion to periplasmic space where further conversions takes place.

B. There may be another possibility; parathion may not be allowed to pass through inner cytoplasmic membrane. In the periplasmic space parathion might get bound by glutathione and GST might contribute to this conjugate formation, then possibly, an inner cytoplasmic membrane bound monooxygenase kind of enzyme might carry out catalysis of glutathione-parathion complex (close complex) by ring cleavage to produce an open molecule (open complex).

It seems process '*A*' is more energy demanding than '*B*'. So, possibility of occurrence of '*B*' is much higher. Then the inner cytoplasmic membrane bound monooxygenase would seem to serve duel role of sensor as well as catalysis. Moreover, cell cytoplasm is reductive environment and oxidative processes are only thermodynamically feasible in periplasmic space. So, process '*B*' seems to be more plausible. However, it is difficult to accept, how such small lipophilic parathion molecule is not allowed to pass through inner cytoplasmic membrane. To logically rule out this possibility, one must argue that rate at which parathion enters and reaches periplasmic space (from outside after crossing outer membrane and cell wall) must be equal to rate at which it must be converted to intermediate hydrolysis product.

The GST may participate in degradation process, probably in two ways:

1. First to detoxify the toxic effects of parathion by forming intermediate conjugate which might be made more amenable to biodegradation by the enzyme systems, switched on according to hypothesis mentioned above. Glutathione might form complex with the parathion molecule in the periplasmic space. This glutathione conjugate complex in some way might have favored the ring cleavage process to be carried out by some kind of monooxygenase enzyme, as discussed above.

2. Secondly, they might also assist in minimizing the overall stress in the cells that might have been induced due to generation of toxic free radicals.

Both the above processes are possible. Presence diverse kinds of GST/GST like enzymes in members of *Acinetobacter* genus as shown by Longkumar et al. (2014)[14] is possible clue towards execution of both the functions. However, it is difficult to speculate to what extent and at what level (temporal with respect to growth) these different GST enzymes might act.

Thus to sum up, for the strain, MemCl4, it seems, probably, first, parathion in the periplasmic space (of the bacterial cell) forms complex with glutathione by GST enzyme. This glutathione-parathion, hypothetical conjugate might undergo some change in overall structural conformation, allowing them to be sensed by a possible monooxygenase that catalyses ring cleavage reaction to

open up the ring. Once open up, this entire complex (I call it open, since there is no ring) either undergoes further degradations by nonspecific enzyme systems (like esterases and other hydrolases), thereby producing intermediates which might have ultimately entered into the Krebs cycle for complete mineralization (Fig. 6). Since, from 2-DE analyses data several possible hydrolases that are possibly localized in the cytoplasm were predicted, chance of the final non-specific degradation at periplasm might not be ruled out.

With respect to hypothesis framed above and from the results that obtained from comparative proteomics studies, on the whole, three broad groups of proteins could be featured:

i. Proteins that might be involved in degradation event directly

At first, parathion is bound by glutathione, in periplasmic space by GST enzyme. Next, this complex (closed complex) is subjected to monooxygenase attack.

Antibiotic biosynthesis monooxygenase (spot 11) could be a possible candidate involved in ring cleavage process along with events of signal transduction (i.e. dual function) from periplasmic space to cytoplasmic side. From the latter the signal might have been carried on to eventually activate some specific sigma factor for temporal expression of genes. These signal transmitters are kinases and during this study, the presence of any kinase protein in proteomic analyses did not recorded. Although, Hypothetical protein J518_2320 (spot 5) may be involved in regulation of genes expression involved in catalysis. This monooxygenase might be involved in carrying out ring cleavage in glutathione-parathion hypothetical conjugate (since ring is present so called close complex).

There was a hypothetical protein (spots 1 and 2), which was tentatively identified to have interactional function related to transport of amino acids. This might be one of the subunit of the inner cytoplasmic membrane based channel, which may be involved in transport of open complex structure (represented by glutathione conjugated ring cleaved hypothetical intermediate generated following monooxygenase attack on Glutathione-parathion closed complex).

Within the cytoplasm, the glutathione conjugated open complex might be subject to first cleavage that might have released glutathione (from the open complex) to be further transported to periplasmic space for forming the closed complex with parathion. In this way glutathione might have been recycled and participated in the degradation process. While the other part might have gone through catalytic breakdown process by non-specific hydrolases (of which one may be metal hydrolase, what I got in the proteomic analysis).

ii. Over Expression of Proteins to Maintain Cellular Homeostasis During Physiological Assault Imposed by Parathion Exposure

While degradation occurred to minimize toxic effect and derive carbon and energy, to keep the cell under normal condition (what I have mentioned as homeostasis), several other accessory proteins might have been over expressed. Some proteins that were obtained through proteomics analyses, seems to represent sub-population of accessory proteins. These might not have any direct role in degradation. They might have functioned in maintaining/ protecting homeostasis of the cell, cellular processes and the cellular environment as such. Some of these are:

7, 8- dihydro-8-oxoguanine-triphosphatase7, 8-dihydro-8-oxoguanine-triphosphatase (Spot 22), which might have been involved in protecting possible error/ mutation in DNA during parathion induced stress condition in *Acinetobacter* sp. strain MemCl4.

Putative N-acetyltransferase (Spot 9), which might be involved in biochemical transformation reaction on the hypothetical substrate that resulted after separation of glutathione part from Glutathione open complex (that was carried to cytoplasm). It is difficult to speculate what kind of transformation it might have undergone. However, it may be concluded that it was most probably involved in detoxification process.

iii. Protein Possibly Involved in Signaling Event

Hypothetical protein J518_2320 (spot 5): This protein although has been listed as hypothetical, through analyses at STRING database, it was assumed that it might interact with transcriptional regulators (LysR family of transcriptional regulator) that are reported to involved in utilization of aromatic compounds and other cellular functions.

This protein might be involved in/component of a regulatory protein that might control expression of gene(s) whose products are functionally involved/ related to transport and further degradation/metabolism of hypothetical open complex (discussed above).

Apart from these, in the result, functions of two protein hits namely**,** Pyridoxamine 5'-phosphate oxidase (PPO, Spot 21) and Tyrosyl-tRNA synthetase (spot 17).

The PPO is reported to be required for catalyzing the terminal rate limiting step in the biosynthesis of vitamin B_6 (https://en.wikipedia.org/wiki/Pyridoxine_5%27-

phosphate_oxidase). The vitamin B6 is very important and participates as cofactors for various enzymes involved in the biosynthesis of amino acids, fatty acids and carbohydrates. An over expression of PPO would indicate its requirements in higher amounts in the cells. This is possible when amino acids (specifically phenylalanine), fatty acids are being synthesized in higher amount for some as yet unknown function during parathion exposure.

Tyrosine tRNA synthetase apart from its normal function, are reported to carry out several other functions and is considered to be a moonlight enzyme[35]. These two enzymes may have some role with respect to parathion exposure in *Acinetobacter* sp. However, with current extent of study, it not possible to predict their role.

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