CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to the Genus *Burkholderia*

*Burkholderia sp*ecies is a group of closely related bacteria with vast ecological and metabolic diversity whose reputations as pathogens including their potential as biological weapons, is equalised by their value as biopesticides and remediators. *Burkholderia* species are aerobic, non-spore-forming, straight or slightly curved Gram negative rods that are 1 to 5 µm in length and 0.5 to 1.0µm wide (Holt *et al.* 1994). Most *Burkholderia* species are motile and possess one or more polar flagella (Palleroni, 1984). They are catalase positive, and either weakly or strongly oxidase positive. All appear as non-fermenters when grown on MacConkey agar. Originally identified as Gram negative *Pseudomonas* spp. belonging to the rRNA group II, the *Burkholderia* genus was reclassified in Yabuuchi *et al*. (1992). *Pseudomonas cepacia* and six other pseudomonads named *viz. P. solanacearum, P. pikettii, P. gladioli, P. mallei, P. pseudomallei* and *P. caryophylli* were the first pseudomonads transferred to the new genus *Burkholderia* to honour Walter Burkholder for his seminal work in 1940's and 1950's.

Pseudomonas cepacia was first isolated by Burkholder in 1950 from onion bulbs as the causative agent of sour skin/ slippery skin disease (Burkholder, 1950). Later, some other pseudomonads were shown to represent the same species including a group of human opportunistic pathogens originally known as "eugenic oxidizers group I" (Ballard *et al*. 1970; Samuels *et al*. 1973; Sinsabaugh and Howards, 1975; Snell *et al*. 1972). *P. cepacia* was not included in the approved list of bacterial names (Skerman *et al*. 1980) and lost its bacterial nomenclature until 1981 when

it was revived by Palleroni and Holmes (1981). Reclassification of species belong to rRNA group II by Yabuuchi *et al.* (1992) into the novel genus *Burkholderia* was based on restricted set of strains which led to additional reclassification of *Pseudomonas* rRNA group II into the *Burkholderia* genus in later studies (Zhao *et al.* 1995; Urakami *et al*. 1994; Gillis *et al*. 1995)

Burkholderia species are versatile bacteria occupying a wide array of ecological niches and include a range of interactions with their hosts which are complex and diverse. The interactions of some species seem restricted to one type of host, whereas others have a much wider host range (Vandamme *et al*. 2007). Interactions can be pathogenic but also can be beneficial to the host.

Currently there are 40 validly named *Burkholderia* species (http://bacterio.cict.fr/). Within the genus *Burkholderia*, a group of ten closely related species collectively known as the *Burkholderia cepacia* complex (Bcc) attracted particular interest as they show significant heterogeneity. The diversity among these presumed *B. cepacia* strains and the lack of reliable identification schemes led Vandamme *et al*. (1997) to define a polyphasic taxonomic study where *B. cepacia* isolated from cystic fibrosis (CF) patients , other human sources and environmental samples belong to at least five distinct genomic species or genomovars. Currently 10 species are known to belong to the Bcc. They share a high degree of 16S rRNA gene (98-100%) and *rec*A gene (94-95%) sequence similarity and moderate levels of DNA-DNA hybridization (30-50%) (Vandamme *et al.* 1997; 2000; 2002; 2003; Coenye *et al*. 2001a; Vermis *et al*. 2004).

2.2 Diversity of *Burkholderia*

Burkholderia are traditionally known as plant pathogens and include *B. caryophylli, B. plantarii, B. glumae and B. andropogonis*. Ballard *et al*. (1970) and Palleroni (1984) showed that *B. caryophylli* is pathogenic not only for carnations but also causes onion rot. Azegami *et al*. (1987) and Urakami *et al*. (1994) demonstrated that *B. plantarii* causes seedling blight of rice and forms the disease causing substance tropolone, a compound consisting a ring of seven carbon atoms with three conjugated alkene groups and a ketone group. However, there seems to be even more versatility as evidence in *B. gladioli* which has been described as a plant pathogen but was also isolated from various clinical sources (Palleroni, 1984; Christenson *et al*. 1989). Among the first named *Burkholderia sp*ecies were primary pathogens for human and animals; *B. mallei* which cause glanders in horses, mules and donkeys and used once as biological weapon during WWI (Wheelis, 1998) and *B. pseudomallei*, an etiological agent of melioidosis in humans and animals which is endemic to Southeast Asia, Northern Australia and other temperate regions (Dance, 1991).

Several *Burkholderia* species develop benign interactions with their plant hosts and colonise roots, stems and leaves. A growing number of species known as diazotrophs which fix atmospheric nitrogen by reducing it to ammonia were also identified as *Burkholderia* species which present in symbiosis with various fungal hoses. *B. fungorum* has been isolated from the white-rot fungus, *Phenerochaete chrysosporium* (Coenye *et al.* 2001b).

However, the majority of *Burkholderia* species are known as soil bacteria with no pathogenic interactions with plants. Several *Burkholderia* species isolated from xenobiotic-contaminated and polluted sites were found to be able in utilising xenobiotic compounds as carbon source.

2.3 Clinical role in humans

One of the most important and challenging aspects of *Burkholderia* is their role as opportunistic pathogens to humans and animals. The most important *Burkholderia* species in terms of pathogenic potential are *B. mallei, B. pseudomallei* and the Bcc. *B. mallei* is a well-known causative agent of glanders, a highly infectious equine disease and has no known reservoir other than horses, mules and donkeys (Niermann *et al.* 2004). *B. pseudomallei* are the causative agent of meliodosis, a potential fatal infection of humans which is endemic in tropical and subtropical region (Dance, 1991; Gan, 2005). *B pseudomallei* was studied by the United States, the former Soviet Union, and possibly Egypt as a potential biological warfare agent, but was never used in this capacity (Kortepeter *et al*. 2001; Alibek and Handelman, 1999; Shoham, 1998). Another recent clinical and epidemiological development is the emergence of *B. pseudomallei* as a life threatening pathogen in individuals with CF who encountered the organisms whilst on holiday in areas where the organism was endemic (O'Carroll *et al*. 2003). This disease associated with *B. pseudomallei* is also known as biological time bomb (Ngauy *et al*. 2005) or "sleeping with the enemy" (Gan, 2005) due to the lengthy incubation period in recrudescent melioidosis.

2.3.1 *Burkholderia cepacia* complex (Bcc)

Bcc members have only been recently recognised as human pathogen as compared to *B. pseudomallei*. Presently, the genomic sequences of 18 strains from seven Bcc species are publicly available at (http://pathema.jcvi.org/cgi-bin/Burkholderia/PathemaHomePage.cgi) (Sousa *et al*. 2011). Growing evidence raised the awareness of the infectious risk due to Bcc, especially in CF patients. Bcc disease in CF patients provides a paradigmatic example of bacterial opportunism because infection occurs only in predisposed individuals who have become heavily colonised by bacteria with multifactorial pathogenic potential, which induces an inflammatory response in the host. Bcc infection in CF patients is often secondary to *Pseudomonas aeruginosa* infection but the proportional hazard for severe clinical decline and death is substantially increased following infection with Bcc. Although all Bcc species have been isolated from lung secretions of CF patients, *B. cenocepacia* and *B. multivorans* are by far the most frequently isolated species (Mahenthiralingam *et al*. 2001; Speert *et al*. 2002). The distribution of Bcc species among isolates from CF patients is clearly disproportionate where *B. cenocepacia* and *B. multivorans* account for the vast majority of Bcc infection in CF patients from the USA (45.6% and 38.7% of patients, respectively). The prevalent species in Canada and Europe are also *B. cenocepacia* and *B. multivorans* with marked variation in local isolation rates (Speert *et al.* 2002; Brisse *et al.* 2004; Campana *et al*. 2005). *B. cenocepacia* and *B. multivorans* are associated with most cases of chronic infection and once acquired, *B. cenocepacia* is difficult to eradicate by antibiotic therapy. Even though members of Bcc are always being connected with pathogenic bacteria, they also have potential as bioremediators (Parke and Gurian-Sherman,

2001). For example *B. vietnamiensis* G4 can degrade aromatic pollutants such as toluene and chlorinated solvents such as trichloroethylene.

2.3.2 *Burkholderia cenocepacia* J2315.

Burkholderia cenocepacia J2315 is considered as the clinically important member in Bcc group as opportunistic pathogens to immunocompromised patients or those suffering from CF (Govan *et al*. 1996). *Burkholderia cenocepacia* was subdivided into four distinct subgroups by phylogenetic analysis of the *rec*A gene (Vanlaere *et al*. 2008). Among four subgroups, subgroup IIIA showed the electrophoretic type 12 (ET12) epidemic strain which is associated with "cepacia syndrome", clinical deterioration, increased mortality and has the ability to superinfect existing *Burkholderia multivorans* lung infection (Holden *et al*. 2009). It was originally designated as isolate CF5610 from a UK epidemic and was characterised by possession of the cable pilus gene (Sajjan *et al*. 1995) and *B. cepacia* Epidemic Strain Marker (BCESM) (Mahenthiralingam *et al*. 1997) Beside these pathogenic markers, numerous other virulence factors including siderophores, proteases and quorum sensing systems have also been identified and described in another ET12 lineage (Mahenthiraligam *et al*. 2005) as well as the expression of hemolytic toxin. The *Burkholderia cenocepacia* J2315 genome sequence shows three chromosomal replicons (3.87 Mbp, 3.22 Mbp and 0.88 Mbp) and a small plasmid of 92 Kbp (Holden *et al*. 2009) (Figure 2.1). Chromosomes 2 and 3 were designated as chromosomes rather than megaplasmids due to the presence of essential genes. Holden *et al*. (2009) also showed that chromosome 1 contains a higher proportion of conserved domain sequences (CDSs) which involved in core functions such as cell division, central metabolism and other house-keeping

functions. Chromosomes 2 and 3 were demonstrated by Holden and coworkers (2009) to have a greater proportion of CDSs that encode for accessory functions, such as horizontal gene transfer and protective responses, and also contain a greater proportion of CDSs with unknown functions. The *Burkholderia cenocepacia* J2315 genome contains 14 genome islands that are absent from the other *Burkholderia cenocepacia* strains including mobile genetic elements, contributing to the genome plasticity of this versatile group of organisms. The possession of genomic islands appears to have been seminal in the evolution of the ET12 lineage, introducing functions that promote survival and pathogenesis in the CF lung (Holden *et al*. 2009). Strain J2315 appears to be well equipped with functions associated with virulence in the CF lung.

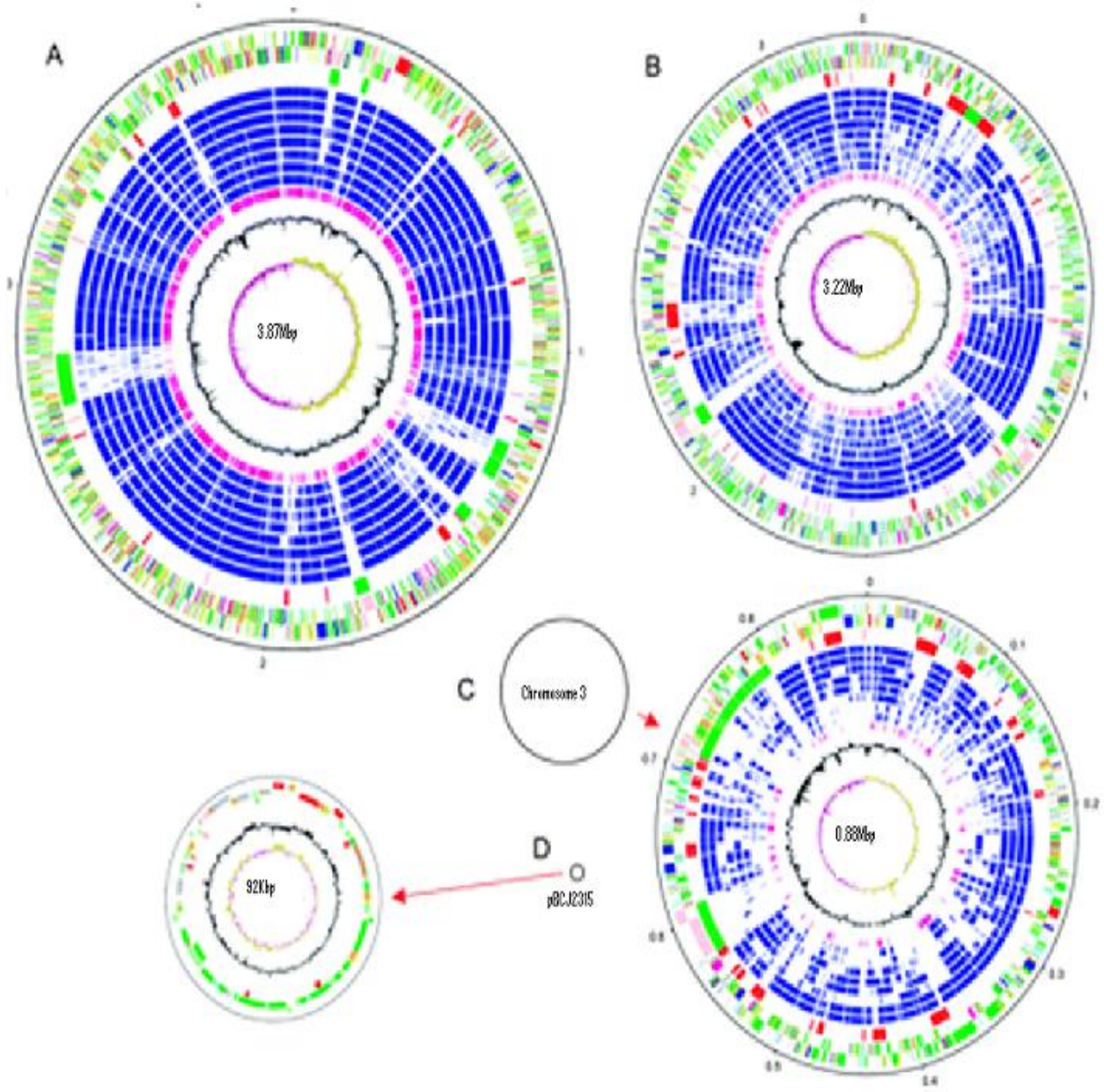

Figure 2.1: The J2315 genome sequence shows three chromosomal replicons (A) 3.87 Mbp, B) 3.22 Mbp and C) 0.88 Mbp) and D) a small plasmid of 92 Kbp (Holden *et al*. 2009).

However, orthologous matches to putative J2315 virulence factors were also found in the other *Burkholderia* genomes including environmental bacteria suggesting its wide functions, which may promote survival in challenging and complex environments such as the soil and rhizosphere but may also have utility in the CF lung (Holden *et al*. 2009). Comparative analysis has suggested that *Burkholderia cenocepacia* J2315 strain may have supplemented its core virulence determinants with accessory virulence functions to enhance its disease causing ability. Contained within this unique component of *Burkholderia cenocepacia* J2315 genome are the cable pilus locus and the 22 kDa adhesion protein AdhA. The cable pilus/AdhA complex is associated with the ability of *Burkholderia cenocepacia* to bind to CF lung explant tissue (Sajjan *et al*. 1992) and also bind and invade epithelial cells (Sajjan *et al*. 2003). Other virulence functions found within *Burkholderia cenocepacia* J2315-specific regions of difference (RDs) in comparative genomic studies include surface polysaccharide biosynthesis, BuHA family putative adhesions, chaperone-usher type fimbriae and phopholipase C (Holden *et al*. 2009)

2.4 *Burkholderia* as a potential bioremediator

The diverse genus *Burkholderia* is characterized by the wide ecological niches occupied by its different species (Coenye and Vandamme, 2003). Many *Burkholderia* isolates have been isolated for their potent capacities to biodegrade anthropogenic organochemical pollutants (O'Sullivan and Mahenthiralingam, 2005). Niche variability of the genus may be due to their high genome plasticity through their large and complex genome sequence, spread over two to four replicons as well as a large number of sequence insertions (Coenye and Vandamme, 2003; Lessie *et al*.. 1996) and are likely involved in particular biodegrading capabilities of a range of

bacterial strains in this genus. The wide substrate diversity of *Burkholderia* makes them attractive bioremediation agents.

Burkholderia have been shown not only in the laboratory but also in field trials to degrade many xenobiotic compounds including polychlorinated biphenyls (PCBs), trichloroethylene (TCE), organopesticides (2,4-D and 2,4,5-T), polycyclic aromatic hydrocarbons (PAHs) and munitions (RDX, TNT, dinitrotoluenes [DNTs]). The most well known *Burkholderia* species possess the ability to degrade hazardous contaminants are *Burkholderia xenovorans* LB400, *Burkholderia* sp. JB1 and *Burkholderia* sp. WD285 isolated from PCB contaminated landfill, *Burkholderia* sp. N3P2, N2P5, N2P6, CRE7, VUN10013, RP007, NAH1, S49 and A6.2 isolated from phenanthrene-enriched soil, *Burkholderia* sp. TFD6 isolated from 2, 4-D enriched soil and *Burkholderia* sp. KN-B3 and KN-B9 isolated from 3-chlorobenzoate-enriched soil. Several isolated *Burkholderia* strains have been identified capable of biodegrade anthropogenic aromatic pollutants (Table 2.1)

Table 2.1: *Burkholderia* strains that able to degrade aromatic compounds

Species/strain	**Substrate**	**Reference**
Burkholderia sp 2A-12	Phenanthrene, naphthalene, pyrene	Kim *et al*. 2003
Burkholderia sp 8	benzoate, 4-fluorobenzoate, 4-hydroxybenzoate	Schlomann *et al*. 1990
Burkholderia phenoliruptrix AC1100	2,3,4,6-tetrachlorophenol; 2,4,5-trichlorophenoxyacetate; pentachlorophenol	Kilbane *et al*. 1982; Coenye *et al*. 2004
Burkholderia sp AK-5	4-Aminophenol	Takenaka *et al*. 2003
Burkholderia sp BL	RDX (1,3,4-trinitro-1,3,5-triazine)	Lee and Brodman, 2004
Burkholderia sp BRI6001	2,4-dichlorophenoxyacetate (2,4-D)	Greer *et al*. 1990
Burkholderia sp BS3702	Phenanthrene, naphthalene	Balashova *et al*. 1998
Burkholderia sp BS3770	phenanthrene, naphthalene	Balashova *et al*. 1998
Burkholderia sp CBS3	4-chlorobenzoate; 2-nitrobenzoate; 3-nitrobezoate; 4-nitrobenzoate; 3-chloronitrobenzoate; 2-nitrophenol; 3-nitrophenol;4-nitropheno; 2,4,6trinitrotoluene	Loffler *et al*. 1995
Burkholderia sp Ch1-1	phenanthrene	Vacca *et al*. 2005
Burkholderia sp Ch3-5	phenanthrene	Vacca *et al*. 2005
Burkholderia sp CRE7	PAH	Mueller *et al*. 1997
Burkholderia sp Cs1-4	Phenanthrene	Vacca *et al*. 2005
Burkholderia sp CSV90	2,4-dichlorophenoxyacetate (2,4-D)	Bhat *et al*. 1994
Burkholderia sp DBT	dibenzothiophene	Di Gregorio *et al*. 2004
Burkholderia sp DNT	dinitrotoluene	Spanggord *et al*. 1991
Burkholderia sp Eh1-1	phenanthrene	Vacca *et al*. 2005
Burkholderia sp EML1549	2,4-dichlorophenoxyacetate (2,4-D)	McGowan *et al*. 1998
Burkholderia vietnamensis G4	benzene; m/o/p-cresol; phenol; toluene; trichloroethylene (TCE)	Nelson *et al*. 1986
Burkholderia sp JR7B3	2,4,5-trichlorophenxyacetate, 2,4-dichlorophenixyacetate (2,4-D)	Rice *et al*. 2005
Burkholderia sp JRB1	2,4-dichlorophenoxyacetate (2,4-D)	Rice *et al*. 2005
Burkholderia sp JS150	benzene, benzoate; 1,2-dichlorobenzene; 1,4-dichlorobenzene; 2,5-dichlorophenol; 2-chlorophenol; 3-chlorophenol, m/o-cresol; ethylbenzene; naphthalene; nitrobenzene; 2-nitrotoluene, 3nitrotoluene; 4-nitrotoluene; phenol; salicylate; toluene; trichloroethylene	Spain and Nishino, 1987

Table 2.1: continue

Burkholderia sp JS850	Dinitrotoluene	Nishino *et al*. 2000
Burkholderia sp JT1500	2-Naphthoate	Morawski *et al*. 1997
Burkholderia sp K712	2,4-dichlorophenoxyacetate (2,4-D)	Ka *et al*. 1994; McGowan *et al*. 1998
Burkholderia kururiensis KP23	Trichloroethylene (TCE)	Zhang *et al*. 2000
Burkholderia sp KZ2	2-chlorobenzoate	Zaitsev *et al*. 1991
Burkholderia xenovorans LB400	Biphenyl; polychlorinated biphenyls (PCBs)	Bopp, 1985; Goris *et al*. 2004
Burkholderia sp MB2	2-methylbenzoate; 3-chloro-3-methylbenzoate	Higson and Focht, 1992
Burkholderia sp NF100	fenitrothion	Hayatsu *et al*. 2000
Burkholderia sp PB4	4-Aminobenzoate, 4-nitrobenzoate	Peres *et al*. 1999
Burkholderia sp PS12	1,2,4-trichlorobenzene; 1,2,4,5-tetrachlorobenzene	Sander *et al*. 1991
Burkholderia sp PS14	1,2,4-trichlorobenzene; 1,2,4,5-tetrachlorobenzene	Sander *et al*. 1991
Burkholderia sp PW3	phenol	El-Sayed *et al*. 2003
Burkholderia sp RASC	2,4-dichlorophenoxyacetate (2,4-D)	McGowan *et al.* 1998
Burkholderia sp RKJ200	p-Nitrophenol	Chauhan *et al*. 2000
Burkholderia sp RP007	Benzoate;naphtahlene; phenanthrene; anthracene	Laurie and Lloyd-Jones, 1999
Burkholderia sp TFD2	2,4-dichlorophenoxyacetate (2,4-D)	McGowan *et al.* 1998
Burkholderia sp TFD39	2,4-dichlorophenoxyacetate (2,4-D)	McGowan *et al.* 1998
Burkholderia sp TFD6	2,4-dichlorophenoxyacetate (2,4-D)	McGowan *et al.* 1998
Burkholderia sp TNFYE-5	phenanthrene	Kang *et al.* 2003
Burkholderia sp TSN101	Biphenyl; polychlorinated biphenyls (PCBs)	Mukerjee-Dhar *et al.* 1997
Burkholderia sp VUN10001	(high molecular weight) PAH	Juhasz *et al*. 1997
Burkholderia sp VUN10002	(high molecular weight) PAH	Juhasz *et al*. 1997
Burkholderia sp VUN10003	(high molecular weight) PAH	Juhasz *et al*.1997
Burkholderia sp WZI	Quinlorac (herbicide), naphthalene, 2,4,5-T; phenol	Lu *et al*. 2003

2.4.1 *Burkholderia xenovorans* LB400

Burkholderia xenovorans LB400 has been the focus of many studies over the past two decades due to its exceptional ability to transform polychlorinated biphenyls (PCBs) even though this ability is not limited to PCB-degrading only. The degradation pathways of PCB in *B. xenovorans* LB400 have been extensively characterised at the genetic and molecular level and has become a model system for bacterial degradation of PCB. Bedard *et al.* (1986) reported that *B. xenovorans* LB400 can oxidize more than 20 congeners including some with 4, 5 and 6 chlorine substitutions on the biphenyl rings.

Burkholderia xenovorans LB400 (=LMG 21463 = CCUG 46959 =NRRL B-18064) was originally described as *Pseudomonas cepacia* LB400 (Bopp, 1986; 1989) and later, in succession, *Burkholderia* sp. LB400 and *B. fungorum* LB400. The original strain was isolated from PCB contaminated soil and deposited in the USDA ARS Culture Collection in Peoria III, under its patent collection, Accession no NRRL B-18064. *B. xenovorans* LB400 has one of the two largest known bacterial genomes and was the first non-pathogenic *Burkholderia* species isolated to be sequenced (Chain *et al*. 2006).

In terms of genomic size, *Burkholderia xenovorans* LB400 has a size of 9.7 Mbp, harbouring ~9000 coding sequences distributed over three circular replicons that have been designated chromosome 1 (4.90 Mbp), chromosome 2 (3.36 Mbp) and megaplasmid (1.47 Mbp) (Figure 2.2). The large chromosome (4.90 Mbp) carries the core cellular function such as translation machinery, DNA replication, cell division and nucleotide metabolism and can be considered as

the core chromosome. The small chromosome and the megaplasmid have a functional bias towards energy metabolism and amino acid metabolism and transport (COG E) (Chain *et al*. 2006). The 3.36 Mbp was defined as a chromosome and not a megaplasmid based on the presence of three ribosomal operons and several tRNAs as well as the unique occurrence of core cellular function involved in DNA replication [DNA primase (*dna*G), DNA polymerase I (*pol*A) and associated elongation factor (*pol*B) and amino acid metabolism. Besides that, this replicon also harbours several functions crucial for adaptation of this organism to its niche. The protein content of chromosome 2 is biased toward transcription, carbohydrate transport and metabolism and signal transduction mechanism which can be defined as "lifestyle determining replicon" (Chain *et al*. 2006). The megaplasmid has a bias toward lipid metabolism which can be explained by the presence of a large number of CoA ligases as well as other enzymes similar to fatty acid metabolic pathways and encodes highly specialized strain-specific functionality. Gene and functional redundancy seems to be very important in the *Burkholderia xenovorans* LB400 genome and other large bacterial genomes. Analysis by Chain *et al*. (2006) shows 1,581 coding sequence (17.6% of all genes) were found to have a better match within the *Burkholderia xenovorans* LB400 genome than in the database of 260 completed genomes which make it the highest extent of gene and potential functional redundancy among genomes of free-living bacteria.

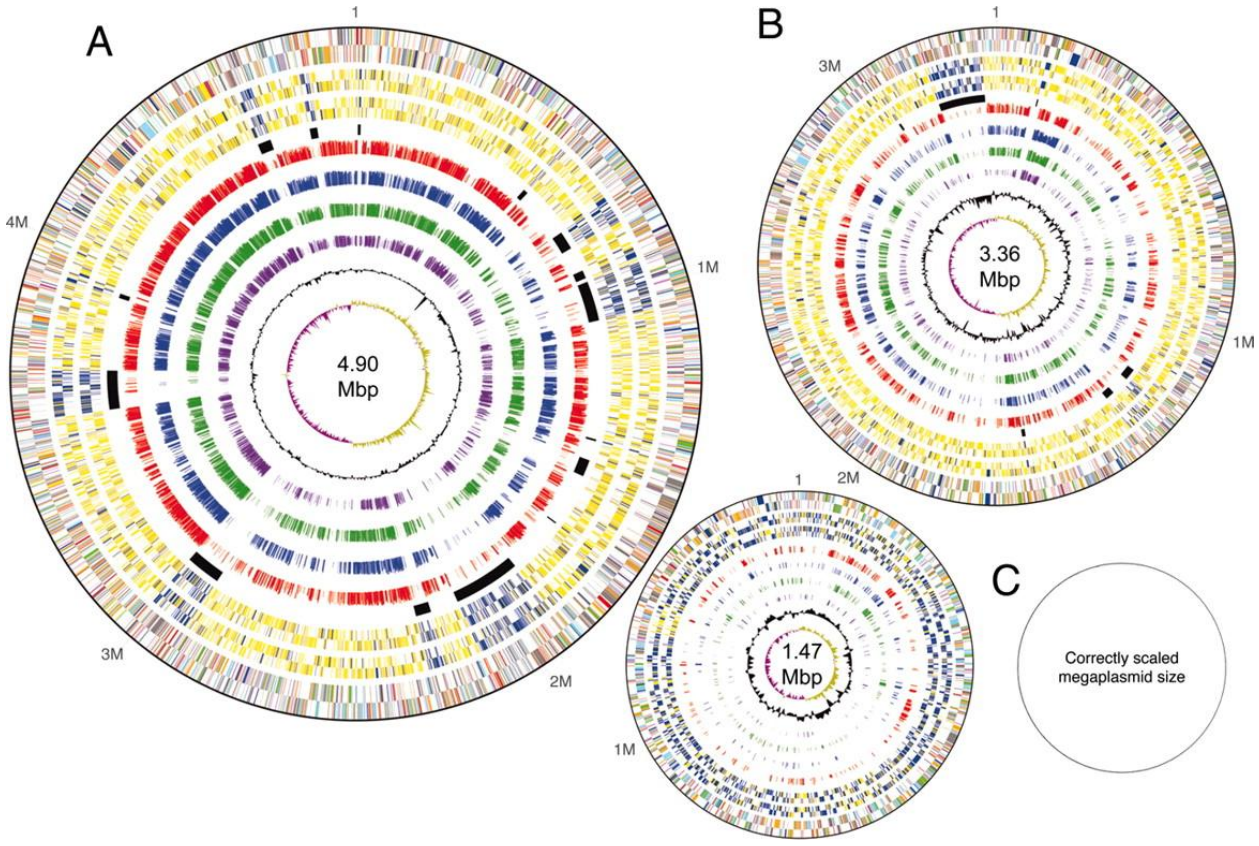

Figure 2.2: LB400 genome which has a size of 9.7 Mbp, harboring ~9000 coding sequences distributed over three circular replicons that have been designated A) chromosome 1 (4.90 Mbp), B) chromosome 2 (3.36 Mbp) and C) megaplasmid (1.47 Mbp) (Chain *et al*. 2006)

The list of paralogs in the LB400 genome is enriched in genes related to transport (230 paralogs), signal transduction (164 paralogs), mobile elements (112 paralogs), membrane protein (66 paralogs) and secondary metabolism, including 120 dehydrogenases, 32 di-oxygenases and 13 mono-oxygenases. For most paralogs, divergent evolution can lead to changes in substrate range, kinetic properties or even function, thus further extending LB400's great metabolic versatility. The gene and functional redundancy for organisms with large genomes such as LB400 is not entirely unexpected as these organisms presumably have to contend with varying conditions in nutrient source types and concentration (Chain *et al*. 2006).

Chain *et al*. (2006) found seven complete copies of the 3,372 bp ISP*pu*12 insertion sequence in *Burkholderia xenovorans* LB400 which is completely absent in other *Burkholderia xenovorans* strains. This sequence was firstly described in the toluene-xylene TOL catabolic plasmid pWWO of *P. putida* mt-2 (Williams *et al*. 2002) and also occurs in several other bacteria, generally associated with plasmids and xenobiotic degradation genes (Weightman *et al*. 2002).

In silico analysis demonstrated that the *Burkholderia xenovorans* LB400 genome has eleven central aromatic catabolic pathways indicating an unusually high metabolic versatility. In addition, the *Burkholderia xenovorans* LB400 genome contains twenty "peripheral aromatic" catabolic pathways. The genes of the central aromatic pathways are generally organized in operon-like structures and genes encoding transcriptional regulators are adjacent to 10 of 11 central aromatic pathway operons (Chain *et al*. 2006) (Figure 2.3).

Virulence traits such as resistance to cationic antimicrobial agents such as polymixin B, presence of a flagellum and iron acquisition by siderophores can also be found in LB400 and it also important in soil habitat. Although *Burkholderia xenovorans* LB400 encodes three phospholipase homologs and a *Las*A-type protease, its repertoire of phospholipases, proteases and collagenases, which have been implicated in *Burkholderia* as pathogenicity traits is limited. *Burkholderia xenovorans* LB400 also lacks a homolog of the *Burkholderia cenocepacia* cable pilus, a defining pathogenic feature of one of the most problematic strains in CF infections (Mahenthiralingam *et al*. 2005).

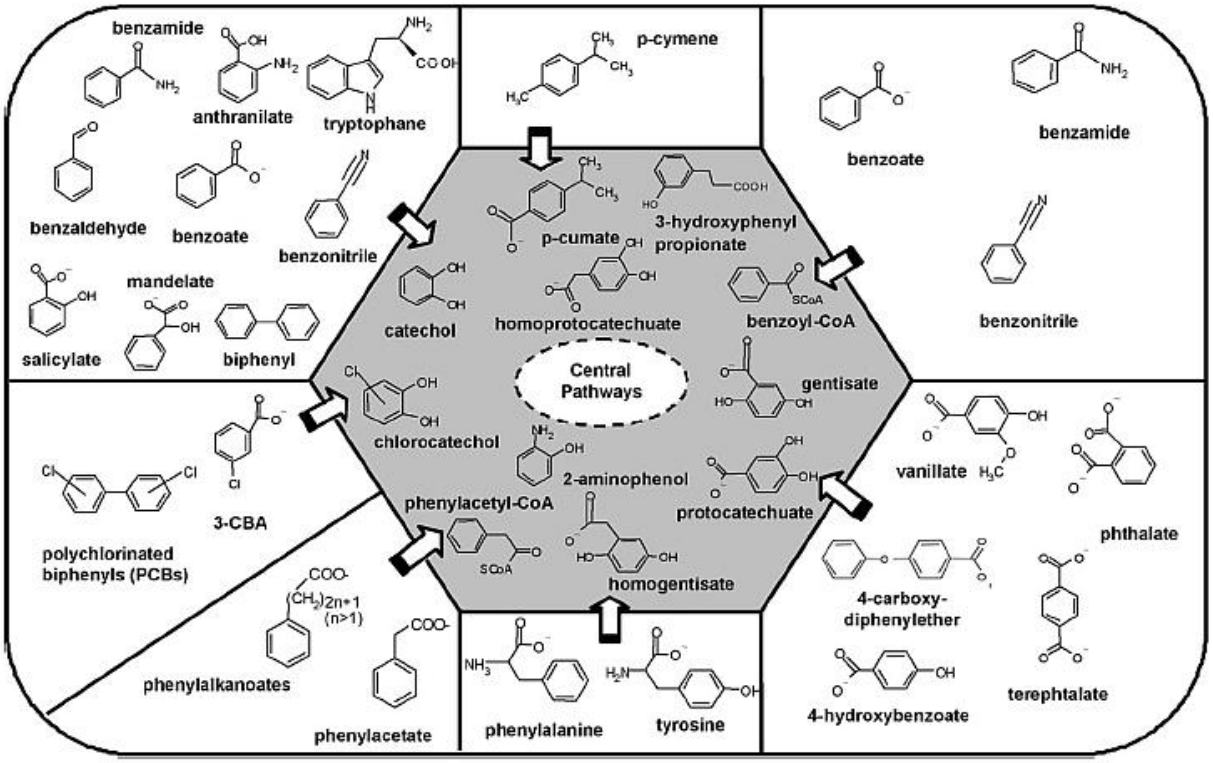

Figure 2.3: Schematic representation of all central aromatic pathways (gray background), based on their main substrate, present in LB400. Peripheral aromatic pathways are indicated in the outer sectors (Chain *et al*. 2006).

2.5 Differences between health hazardous and environmentally important *Burkholderia*

Due to the fact that some *Burkholderia* species isolates can be opportunistic or obligate pathogens causing human, animal or plant diseases, any development of agricultural and/or biotechnological applications using *Burkholderia* species needs to include a stringent assessment of the potential risks (Coenye and Vandamme, 2003). Richardson *et al.* (2002) using Rep-PCR illustrated that a number of clinical *Burkholderia* isolates are distinct from rhizospheric and environmental isolates. Mapping of *Burkholderia cenocepacia* niche response *via* high-

throughput sequencing (Yoder-Himes *et al*. 2009) revealed that different dominant functional classes were expressed under soil conditions as compared to CF-like conditions. The two *Burkholderia cenocepacia* strains (clinical isolate AU1054 and environmental isolate HI2424) showed a marked difference in their regulated gene response to the CF and soil environment conditions, suggesting that adaptations that have occurred since these two strains diverged and play a role in survival in their natural environments. Under CF conditions, Yoder-Himes *et al.* (2009) observed many genes whose products display similarity to virulence factors including molecular chaperones, iron acquisition proteins, proteins expressed in macrophages and immunogenic proteins. Although both *Burkholderia cenocepacia* strains possess significant nucleotide identity conversation in homologous genes (99.8% ANI), 179 and 120 homologous genes had significantly different expression between strains in CF or environmental conditions, respectively. It has been determined that clinical isolate AU1054 contains 50 genes which were absent from environmental isolate HI2424 while 470 genes carried by HI2424 were absent from isolate AU1054 (Yoder-Himes *et al*. 2009).

2.6 Sigma subunits in bacteria

Sigma subunits of RNA polymerase (RNAP) are important as determinants of differential gene expression since they are responsible for specific binding to DNA (Helmann and Chamberlin, 1988; Wosten, 1998). The σ subunit contributes to core RNAP ability to specifically recognise and initiate transcription from promoters. The complex formed by σ factor and polymerase core subunits constitutes the bacterial holoenzyme. Many gene expression patterns rely on the formation of RNAPs that contains σ factors differing in their promoter recognition specificity.

Bacterial species differ significantly in the number of σ factors they express, reflecting the different environmental conditions to which they must respond for example *Escherichia coli* has only seven σ factors (Reitzer and Schneider, 2001) but *Bacillus subtilis* encodes 17 σ factors and *Streptomyces coelicolor* has 65 σ factors. A broader array is possible with a large number of σ factors thus allowing these organisms to meet variable conditions and undergo major morphological changes.

Generally σ factors can be divided into two major families; the σ subunit 70-like family and the σ subunit-54 like family (Wigneshweraraj *et. al.* 2003). In the primary σ subunit 70 (σ^{70}), core RNAP associated with the σ subunit to form holoenzyme complex ($E\sigma^{70}$) and initiate the transcription process of housekeeping genes (Helmann and Chamberlain, 1988). $E\sigma^{70}$ also can initiate the transcription of nonessential genes that are induced in specialized environment (Reitzer and Schneider, 2001). Another member of σ subunit-70 is σ^{S} which is known as second primary σ factor form a complex with core RNAP ($E\sigma^{S}$) binds the same sequence as $E\sigma^{70}$ but is considered as a general stress factor since it is associated with a variety of growth-impairing stresses; nutrient depletion, oxidative stress, high temperature, high osmolarity, acidic pH or exposure to ethanol (Hengge-Aronis, 2000). Some other σ factors such as σ^{32} and σ^{E} are also associated with stress while σ^{FeeI} and σ^{28} are required for synthesis of the ferric citrate transporter and flagella respectively (Angerer *et al*. 1995; Mytelka and Chamberlain, 1996).

The second family of σ subunits has only one member which is σ subunit 54. Studies have confirmed its role in nitrogen assimilation but it also has shown the involvement of σ subunit-54 in a variety of unrelated functions, such as carbon source utilization, certain fermentation

pathways, flagella synthesis and bacterial virulence (Kobayashi *et al*. 1998; Studholme and Buck, 2000).

2.6.1 Sigma subunit-54 (σ^{54})

Several alternative σ factors including σ^{54} have been observed to enable the cells to transcribe specific sets of genes in response to environmental stimuli. The σ^{54} transcription factor is quite distinct from other bacterial σ factors and displays no apparent homology to σ^{70} (Merrick, 1993). Gene regulation by RNAP containing σ factors is often dominated by recruitment of RNAP to promoter sites enabled by activator-RNAP contacts. These promoter elements can be considered as functional analogues of the -24/-12 consensus promoter elements recognizes by $E\sigma^{54}$ class holoenzymes unlike $E\sigma^{70}$ which recognizes and binds promoters containing conserved consensus elements centered around -35 and -10 nucleotides upstream of the transcription start site (Burrows *et al.* 2003).

Different from σ^{70} which can initiate transcription process spontaneously without additional factors, σ^{54} forms a RNAP holoenzyme that recognise promoters but require additional protein factors, denominated enhancer binding proteins (EBPs) and source of energy in the form of ATP and GTP hydrolysis for formation of transcriptionally competent promoters complexes. $E\sigma^{54}$ catalyses strand separation only with the help of a distinct class of transcriptional activators. The σ^{54}-dependent activators bind to sites that are effective regardless of distance and orientation (Buck *et al*. 1986; Calvo *et al.* 1994; Buck *et al.* 2000).

Two well characterised motifs were observed in *Klebsiella pneumoniae*, a helix-turn-helix and a highly conserved sequence of 10 amino acids known as RpoN box that involved in the

recognition of the -24 and -12 promoter elements, respectively (Coppard and Merrick, 1991; Merrick and Chambers, 1992; Taylor *et al*. 1996;). The level of σ^{54} could have regulatory implications where different σ^{54}-dependent operons might compete for limiting σ^{54} and it became apparently reasonable if there is some physiological relationship between the σ^{54}-dependent genes (Reitzer and Schneider, 2001).

2.6.2 Sigma subunit-54 (σ54) -dependent genes for nitrogen metabolism

Many of the characterized σ^{54} dependent promoters such as in *Escherichia coli* are involved in nitrogen metabolism and some other genes are induced by nitrogen limitation (Zimmer *et al.* 2000) and have potential σ^{54} dependent promoters. Ammonia is considered the preferred nitrogen source for many bacteria since it supports the fastest growth. The presence of ammonia will prevent the synthesis of several proteins concerned with nitrogen metabolism (Reitzer, 1996a; 1996b). Besides ammonia, bacteria can also utilise a small number of other nitrogen sources such as amino acids, nucleosides, nucleobases and a few inorganic nitrogen sources (nitrite and nitrate) which can be reduced to ammonia. Steady state growth on alternate nitrogen sources is slower and said to be nitrogen limited. Catabolism of alternate nitrogen sources that cannot be transferred to glutamine directly requires that it must be converted to ammonia for the synthesis of glutamine, the intracellular amide group donor.

For nitrogen limited growth conditions in *Escherichia coli*, nitrogen-regulated response (Ntr) encoded by *ntrC* and *ntrB* genes coordinate the response to nitrogen limitation (Reitzer and Schneider, 2000). The major function of the Ntr response is scavenging (Zimmer *et al.* 2000)

however Ntr protein does not scavenge all amino acids and bacteria generally have Ntr-dependent transport systems only for amino acids that can readily provide nitrogen for glutamate and glutamine synthesis (Reitzer and Schneider, 2000).

2.6.3 Functional roles of *rpoN* genes.

RpoN is a key regulator in the acclimation of *Pseudomonas* to complex natural environments (Jones *et. al*. 2007). Using chemosensitivity assays, Jones *et al.* (2007) illustrated that RpoN-regulated genes contribute to acid tolerance and resistance to some antibiotics including tetracycline and aminoglycosides. In *P. syringae*, RpoN is a key virulence factor for plant pathogenicity and is required for the expression of the *hrp* gene which encodes a component of a type III secretary system. Besides the activity of σ^{54} in plants, σ^{54} is also involved in the regulation of virulence related factors in animal pathogens (Jones *et al*. 2007).

In Gram-negative bacteria, σ^{54}, working in concert with a transcriptional activator that belongs to the NtrC superfamily, activates a variety of genes that are regulated in response to external stimuli (Albright *et al*. 1989). RpoN binds to RNA polymerase to form a closed holoenzyme complex that requires further activation by members of the NtrC class of EBPs (Studholme and Buck, 2000; Cases and de Lorenzo, 2001; Studholme and Dixon, 2003). Many studies clearly demonstrate that the -24/-12 promoter of σ^{54} is not only confined to nitrogen controlled genes but present in a large number of other genes within Gram-negative bacteria (Kustu *et. al*. 1989; Thőny and Hennecke, 1989). Transcription from these promoters depends on a specific activator protein whose activity was modulated by physiological signals. $E\sigma^{54}$ controls several other

ancillary processes including the degradation of xylene and toluene (Ramos *et al*. 1997), transport of dicarboxylic acids, pilin synthesis, nitrogen fixation, hydrogen uptake (Kustu *et. al*. 1989; Fischer, 1994), flagellar assembly (Arora *et al*. 1997), arginine catabolism (Gardan *et al*. 1995), alginate production (Zielinski *et al*. 1992), rhamnolipid production (Pearson *et al*. 1997), acetoin catabolism (Priefert *et al*. 1992), mannose uptake (Martin-Verstraete *et al*. 1995) and proline iminopeptidase activity (Albertson and Koomey, 1993). Besides that, *rpoN* was also reported to affect pH tolerance and osmotic stress (Jones *et al*. 2007).

2.6.4 Xenobiotic degradation mediated by sigma subunit 54.

The ability of bacteria to adapt to various stress conditions including nutrient limitation by triggering a general and transcriptionally coordinated stress response has been known for many years. Sigma 54 has been illustrated mediating several xenobiotic degradation pathways in bacteria as shown in toluene and m-xylene degradation pathway by the TOL plasmid of pWW0 *P. putida* mt-2 (Figure 2.4) and (methyl) phenol degradation encoded by plasmid pV1150 of *P. putida* CF600 (Powlowski and Shigler, 1994). The regulation systems for both XylR and DmpR are well studied and known as XylR/DmpR family of aromatic regulators. Other examples of XylR/DmpR family are TbuT and TbmR, which control toluene monooxygenase gene expression in two strains of *Burkholderia* (Byrne and Olsen, 1996; Leahy *et al*. 1997).

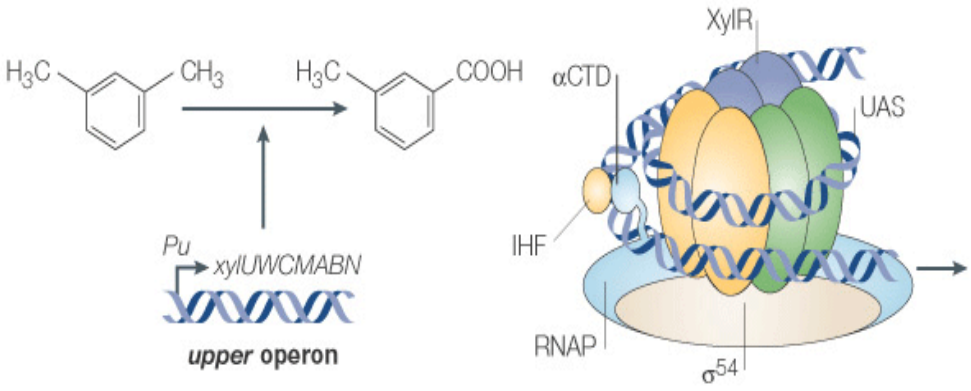

Figure 2.4: The involvement of *RpoN* (Sigma-54) in toluene and m-xylene catabolic pathway in *Pseudomonas putida* mt-2 (Adapted from Cases and de Lorenzo, 2005)

The XylR/DmpR-type regulators activate transcriptions mediated by the RNAP containing the alternative sigma factor σ^{54}. The first characterised member of this family is NtrC, a global regulator involved in nitrogen metabolism.

XylR is the regulator of the *Pu* promoter of the TOL upper operon from plasmid pWW0 of *P. putida* mt-2 for degradation of toluene, m-xylene and p-xylene (Ramos *et al*. 1997). The *Pu* promoter drives the gene cluster *xyl*UWCMABN and mediates bioconversion of toluene and xylene into corresponding carboxylic acids. *Pu* which belongs to the class of promoters that depend on the alternative σ factor, σ^{54} is activated at the distance by a toluene-responsive activator (XylR). This involves the binding of the regulator to upstream activator sequences (UAS) and the looping-out of the complex, which draws it into close proximity to the σ^{54}-containing form of RNA polymerase (σ^{54}-RNAP) that binds to the -12/-24 region of the promoter. This event is assisted by the presence of an integration host factor (IHF)-binding site in the intervening region between the UAS-XylR complex and the σ^{54}-RNAP-attachement site.

IHF binding to *Pu* sharply bends the target DNA sequence and facilitates contact between distant proteins and assists in the recruitment of σ^{54}-RNAP to -12/-24 through the α-carboxyl-terminal domain (α-CTD) subunit of the enzyme (Cases and de Lorenzo, 2005).

The *Po* promoter of the *dmp* operon is also a σ^{54}-dependent promoter that is activated by the phenol responsive protein DmpR. Although the *Pu* and *Po* promoter sequences are different, the UAS are similar enough to be recognised by both proteins as binding sites (Fernandez *et al*. 1994).

2.7 Bioremediation

Strict environmental regulations and legislation and the increasing public awareness for a better environment have driven the growth of the remediation sector. Physico-chemical treatment is usually costly and often the contaminant is not fully degraded. Alternatively, microorganisms can be used to degrade these xenobiotic compounds which have evolved as a cost-effective *in-situ* strategy for the remediation of contaminated sites.

Although microorganisms play a major role in the breakdown and mineralisation of man-made organic compounds the kinetics of the biodegradation process may be much slower than desired from public health or environmental considerations. The slow biodegradation of xenobiotic compounds in the natural environment may be caused by unfavourable physicochemical conditions, availability of the nutrient, predation or the low enzymatic activity due to its gene expression. Bioremediation technology is focusing on enhancing this natural process either by

altering the condition to the favourable state that organisms are able to degrade and mineralise the compound efficiently or by introduction of specifically engineered organism to clean up xenobiotic compounds particularly problematic compounds such as dioxins, PAHs and PCBs. Furthermore, additional elements that able to increase bacterial tolerance towards environmental stress might increase bioremediation efficiency. Ponce *et al.* (2011) demonstrated that antioxidant compounds such as α-tocopherol able to increase *Burkholderia xenovorans* LB400 tolerance towards oxidative stress and reduced inhibition effect by reactive oxygen species (ROS generated during PCB degradation.

2.7.1 Dioxins and Furans.

In general, dioxin is a group of chloroaromatic compounds which includes Polychlorinated dibenzo-p-dioxin (PCDDs) and Polychlorinated dibenzofuran (PCDFs) or generally known as dioxins and furans respectively. Another compound which considered as dioxin is co-planar polychlorinated biphenyl (PCBs). Both dioxins and furans consist of two benzene rings linked by two oxygen atoms at *para* position in dioxin or one oxygen atom that has a direct link between two carbon atoms within the benzene rings in furan. General chemical formula of dioxins is $C_{12}H_7Cl_xO_2$ while general chemical formula for furans is $C_{12}H_8Cl_xO$ where x is chlorine number which can be up to 8 atoms. The number of chlorine atoms and its position will distinguish the toxicity of the dioxins and furans. Figure 2.5 shows the general structure of dibenzo-p-dioxin and dibenzofuran where the numbers indicates the position for chlorine substitution. Dioxins and dioxin-like compounds comprise of 210 congeners where 75 congeners are known as PCDDs (dioxins) while 135 are known as PCDFs (furans). Out of 210 congeners, 17 of these congeners

are considered health hazard. These chloroaromatic compounds are toxic and carcinogenic due to their greater activity of the molecules especially 2,3,7,8-TCDD which was considered the most toxic congener. Dioxins are lipophilic and have strong affinity for non-polar substrate such as soils, organic matters and carbohydrates and bind strongly to biological tissue thus very bioaccumulated. PCDD/Fs can strongly attach to solid surfaces and do not easily leach from soil or sediment (ATSDR, 1998; 1994) thus very persistent in the environment and are among the most problematic environmental pollutant.

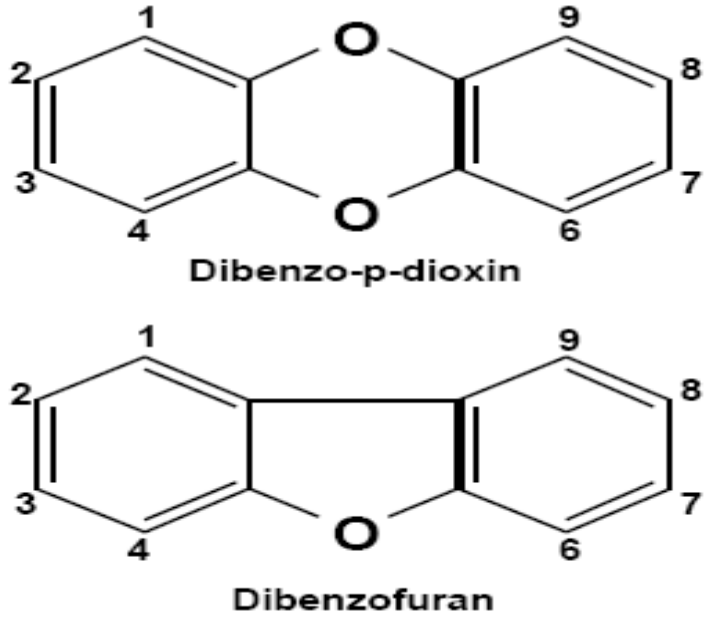

Figure 2.5: General structure of dibenzo-p-dioxin and dibenzofuran.

2.7.2 Source of dioxins and furans

Dioxins and furans can be produced naturally and occurs at trace level in the environment. However it can also be produced artificially by industries directly or indirectly by the means of by-products of some processes within some chemical industry such as pesticide manufacturing, organochemical industries and combustion system such as Municipal solid waste (MSW) incinerators. Emission sources are mainly produced from combustion processes (Kjeller and Rappe, 1995; DEFRA, 2000). The management of dioxins and furans emission has been the subject of interest for regulators not only in USA and EU but also across the globe. With the implementation of new regulation regarding the dioxins release to environment, in UK alone, the total emission of dioxins and furans itself was reduced by over 80% between 1990 to 1999 which due to the closure of several inefficient incinerators (DEFRA, 2000). Unfortunately there is no available data regarding the release of dioxins in Malaysia to date, for the researchers and regulators of the current situation in Malaysia.

2.7.3 Formation and destruction of dioxins and furans.

Extensive research on formation and suppression of dioxins and furans has so far focussed on MSW incineration with some interest on iron ore sintering and scant investigation into other forms of combustion thus the exact mechanism of PCDD/Fs remains unclear, although the suppressing of emissions into the environment have been developed successfully (McKay, 2002). However general consensus has been achieved among researchers that for the mechanisms of PCDD/Fs formation, the process must have an adequate supply of appropriate

quantities of carbon, oxygen, hydrogen and chlorine atoms to begin with and the process must also fulfil certain combustion conditions, which produce Products of Incomplete Combustion (PICs) that consist of the necessary aromatic (benzene ring-based) compounds which then will react to form dioxins and furans (Stanmore, 2004).

It has also been hypothesized that pre-existing PCDD/Fs contained in fuel and oil waste entering an incinerator may pass through the furnace and release as flue gas emissions. However, this hypothesis is doubted since most combustion-based processes employ furnace conditions capable of destroying dioxins and furans upon entry (McKay, 2002). The routes of PCDD/Fs formation has been illustrated in Figure 2.6.

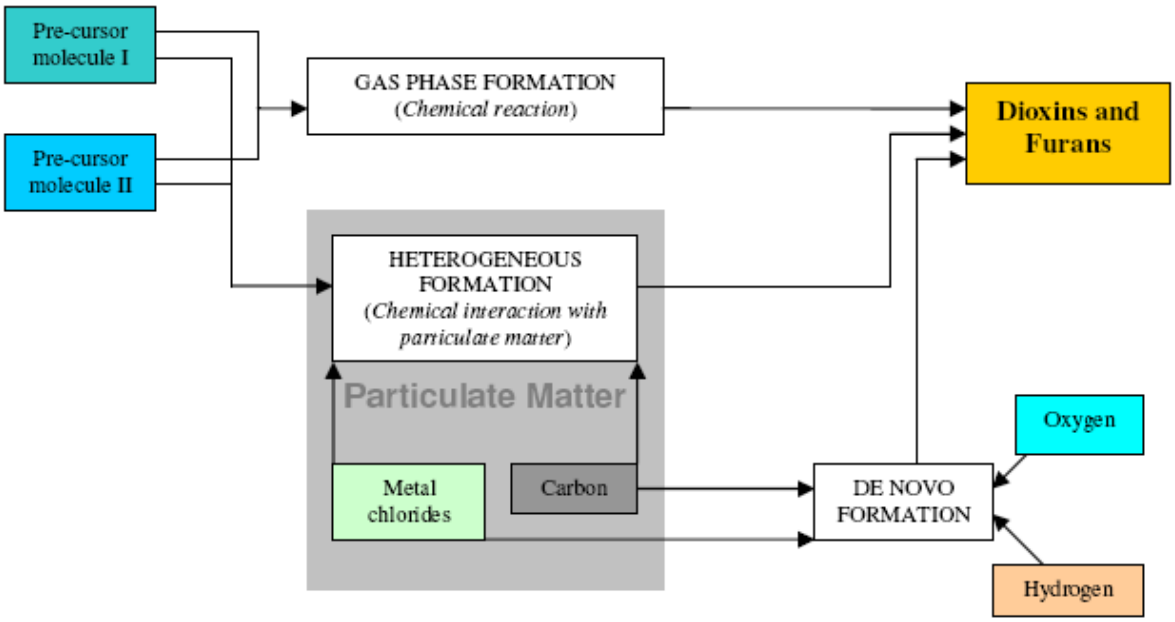

Figure 2.6: The three main PCDD/F formation routes (Environment Australia, 2003)

2.7.4 Control of dioxins and furans emission

Prolonged exposure of particulate matter retained by particulate control devices (PCD) to high temperature between 200ºC to 800ºC might form dioxins and furans with maximum formation rate was observed between 300ºC to 400ºC (Tupperain *et al*. 1998; McKay 2002). However, with new EU legislation for incinerators, power plants and kiln, the temperature to be used must in excess of 850ºC, which make the formation of dioxins and furans within furnace not possible due to the destruction of the compounds. As an addition, under EU Waste incineration Directive, in order to eliminate PCDD/Fs emission to air, PCDD/Fs have been increasingly concentrated in fly ash (Landesumweltamt Nordhein-Westfalen, 1997). Air pollution devices used to control PCDD/Fs emission can include electrostatic precipitators, bag filter, scrubbers, activated carbon injector and cyclone filter (Table 2.2)

Table 2.2: Air pollution control devices for PCDD/F emission control (Adapted from Sutherland, 2007)

APCD	Description	Efficiency
Activated carbon (Buekens and Huang, 1998; Environment Agency, 2002; McKay, 2002)	Injection of carbon powder into the flue gas can significantly adsorb and remove dioxin, furan, mercury and other heavy metal emissions. Used in UK MSW incinerators.	Removal efficiencies exceed 95% of PCDD/Fs from flue gas; satisfies EU regulation
Electrostatic precipitator (Environment Agency, 2002; McKay, 2002)	An ESP employs an electrical field, which charges and retains particulate matter.	ESPs in incinerators widely replaced by activated carbon and scrubber facilities during 1990s, due to weak PCDD/F control.
Fabric Filter (Fell, 1998; McKay, 2002)	6-8 inch diameter fabric barriers composed of woven fibre-glass material, usually arranged in series retrain particulate matter	Traps particle-bound dioxins and furans only
Scrubber (McKay, 2002; Stanmore, 2004)	Scrubbers are primarily designed to remove acidic (acid rain) gases, namely nitrous and sulphurous dioxides. These incorporate lime, which can be sprayed as a powder or as a slurry.	Lime (CaO) injection suppresses PCDD/F emissions, although $CaCO_3$ is less successful.
Selective Catalytic Reduction (SCR) (Buekens and Huang, 1998; McKay, 2002)	Used to treat nitrous gases. The catalysts used in SCR devices (e.g. titanium, vanadium, tungsten) also decompose organohalogens (e.g. PCDD/Fs).	Temperatures exceeding 200°C must be contained, thus PCDD/F emissions may increase

2.7.5 Health effects towards dioxins and furans exposure.

Dioxin was regarded to be the most toxic chemicals in existence by media and regulators even though it has been both inaccurate and difficult to prove. However, during 1970s and 1980s poisoning incidents during the Vietnam War and Seveso factory accident where individuals who were heavily exposed to TCDD exhibited severe deformities, skin lesions and several other symptoms incited many researchers to investigate the effects of PCDD/Fs (Bates, 1999).

Among the health effects that were reported due to dioxins and furans exposure the most extensive research have been done is focussed on cancer. Dioxin is also known for its ability to damage the immune system and interfere with hormonal systems Dioxins exposure has been linked to birth defects, inability to maintain pregnancy, decreased fertility, and reduced sperm counts endometriosis, diabetes, learning disabilities, immune system suppression, lung problems, skin disorders, lowered testosterone levels and much more (People's report on dioxin, 1999)

There is a lot of evidence showing that dioxin exposure can cause cancer in human. For some human populations, the danger of cancer began to rise noticeably when the dioxin body burden reaches 109ng/kg. In 1985, EPA established a stringent allowable intake level of dioxin for human where an average person is allowed to consume not more than 0.42 pg/day. However, due to widespread of dioxin contamination in various levels of the food chain, it was reported that average American consumed dioxin in daily quantities ranging from 3 to 50 pg which exceeded the EPA's safe dose by 7-fold to 120 fold. In 1997, IARC, WHO published their research into dioxins and furans, subsequently announced on February 14, 1997 that the most potent dioxin 2,3,7,8-TCDD is considered as Class I carcinogen (Known human carcinogen). US National Toxicology Program (January 2001) reclassified 2,3,7,8- TCDD from "Reasonably Anticipated to be a Human Carcinogen" to "Known to be a Human Carcinogen" which was upgraded to its potential carcinogenicity. Reanalysis of the cancer risk from dioxin reaffirmed that there is no known "safe dose" or threshold below which dioxin will not cause cancer. There are also study by Warner and coworkers (2002) shows that dioxin is related to increased incidence of breast cancer.

Other disease related to exposure of dioxins in human in chloracne. It was first appeared in 1930s among workers dealing with pesticides and PCBs manufacturing but only being identified caused by dioxins only after 1960. Chloracne produces skin eruptions, cysts and pustules. In human, chloracne has occurred at body burden as low as 96ng/kg and as high as 3000ng/kg. Researchers at National Institute for Occupational Safety and Health (NIOSH), US found that the levels of testosterone circulating in the blood of male workers who exposed to dioxins were reduced. Even though the reduction was statistically significant, however the reduction was small and still remained within the range that is considered normal.

2.7.6 Human exposure to dioxins

Dioxins exposure to human is tremendous, almost everything that people comes into contact may contain at least one type of dioxins but whether the amount exposed to human considered safe is still unclear. Dioxins is fat-soluble and easily accumulates in food chain which mainly (97.5%) found in meat and dairy products. In aquatic environment, these toxins bioaccumulate in the living organisms and the amount can be enormous compare to surroundings due to its hydrophobicity. For an instance the dioxins levels in fish are 100, 000 times that of the surrounding environment. A survey by the Danish National Food Agency in 1987 shows higher I-TEQ/kg fat in fish products with 58.3 ng I-TEQ/kg fat in herring and 40.9 ng I-TEQ/kg fat in cod liver while the average for dairy product and meat is between 0.5 to 3.8 ng I-TEQ/kg fat (Büchert, 1988). In 1991, the WHO has estimated that the average European adult weighing 70kg has dioxin intake through food of 132 pg I-TEQ/day where 48 pg I-TEQ/day itself was from milk packaging.

The scary threat of dioxins exposure in the workplace and environment faced by industrialised countries in Europe, the US and Japan has initiated more and more research on reduction of the dioxin release to environment and remediation technology of dioxin compounds. Workers in some industrial plants may be exposed to much more dioxins that the average population. Antonson *et al.* (1989) reported that in some steel industries at Sweden, the air levels were 1-20 pg N-TEQ/m3 while Schick and Conrad (1994) reported that dioxins concentration around the melting furnaces at cooper/precious metals recycling and aluminium recycling industries in Germany were 1.5-10.8 pg I-TEQ/m3 and 8.2-22.6 ph I-TEQ/m3 respectively.

Textile industries were considered as the most likely source of dioxins exposure towards workers. The bleaching of textiles with chlorine compounds might be possible source of dioxins. Contamination of certain dyestuffs based on chloroanilins and anthraquinones (eg: HeliogenBluea, Chloranil and Carbazole Violet) were also possible sources of dioxins from textile industries. The Danish EPA (1996) has calculated that exposure to a new t-shirt every week will expose the individual to an average of 70 pg N-TEQ/ week (range 4-520 pg/week).

In Canada, the daily intake of dioxins of 0.05 pg N-TEQ was estimated from pharmaceuticals, cosmetics and passive smoking among other sources. In addition use of chlorine bleached toilet paper contributed with daily intake of 0.25 pg N-TEQ by skin absorption (Birmingham *et al.* 1989). Dioxins intake through inhalation has also been studied. Papke *et al.* (1989) reported that 1-3 pg I-TEQ/m3 of dioxins was determined in the rooms with woods treated with pentachlorophenol many years before. In house dust dioxin levels were around 500 ng I-TEQ/kg,

which are 10 times more than in the forest soils (Rotard *et al*. 1991). According to the WHO, the adults daily inhales will be around 10% of the tolerable daily intake (TDI) of 10pg I-TEQ/kg b.w./day. Cigarette smoke may contain range from 0.1 pg I-TEQ/cigarette to 1.8 ng I-TEQ/m3 (Muto and Takizawa, 1992; WHO, 1991: Löfroth and Zebühr, 1992).

2.7.7 Level of dioxins and furans in the environment

The concentration of PCDD/Fs in the environment occurs across a wide spectrum from a few trillionths of grams per kg to nearly 0.1grams per kg. Typical soils and sediments are usually characterised by I-TEQ concentrations of a few (tens of) ng PCDD/Fs per kg (Rose *et al*. 1997; Bates 1999; European Commission, 2003). Heavily polluted soils and sediments especially near currently operating or former incinerators and organo-chemical plants may be enriched with PCDD/Fs, with concentrations which are several orders of magnitude more (Lorber *et al*. 1998; Watson, 1998; Krauss *et al*. 1999; Environment Agency, 2002). The most extreme concentrations of PCDD/Fs tend to occur in soils outside major organo-chemical plants, or where waste oils and/or sludge were dumped (ATSDR, 1994; 1998).

In secondary and waste materials, incinerator bottom ash (IBA) and pulverised fuel ash (PFA) may contain PCDD/Fs with concentration exceeding 50ng TEQ/kg which can approach several thousand ng I-TEQ/kg or above (Danish EPA, 1997; Abad *et al*. 2000; McKay, 2002; Gan *et al*. 2003; Dawy, 2004). Sewage sludge can also contaminated with dioxins and furans with a wide spectrum of concentrations, from below 50ng I-TEQ/kg to over 1000ng I-TEQ/kg, depending on the source of the effluent. Sewage sludge from treatment plants processing urban and/or

industrial effluent can be more enriched in dioxins and furans (Sewert *et al*. 1995; Danish EPA, 1997; Dyke *et al*. 1997; LUA NRA, 1997; European Commission, 2003). One contributing factor to the varying dioxin (PCDDs) content in sewage sludge is wash-off from urban roads, as particulate matter originating from diesel and petrol tanks containing PCDDs (ATSDR, 1998). Particulate matter from vehicles or deposited from the atmosphere is a prime source of PCDD/Fs in effluent (Sewert *et al*. 1995).

Incinerator fly ashes of all types may contain concentrations which are consistently above 50ng I-TEQ/kg. Some types of ash may be suitable; for example sewage sludge ash samples (SSA) analysed by Halliday (2002) contained negligible concentrations. However data for other SSA samples was not found, so no further conclusions can be drawn. In contrast, likewise, foundry/smelter dust and wood incineration ash may contain highly variable concentrations, although the data supporting this is limited (Danish EPA 1997; Dyke *et al*. 1997; Wunderli *et al*. 2002; Mc Kay 2002).

2.8 Microbial degradation of dioxins

Microorganisms play an important role in the degradation and mineralization of xenobiotic and aromatic compounds in natural environments. Microorganisms naturally transform organic compounds and re-introduce them into the global carbon cycle. Biological method using particular microorganisms or microbial consortia capable of dioxin transformation and degradation have greater appeal than physico-chemical ones in their potential application for

environmental remediation and considered a feasible method as an alternative to other expensive physico-chemical approaches.

Biodegradation of dioxins has been extensively studied in several microorganisms. There are several microbial mechanisms responsible for biodegradation of dioxins including oxidative degradation by dioxygenase-containing aerobic bacteria, bacterial and fungal cytochrome P-450 and fungal lignolytic enzymes, reductive dechlorination by anaerobic bacteria and direct ether ring cleavage by fungi containing etherase-like enzymes. Dioxin degradation has been characterised in aerobic bacteria that contain aromatic ring hydroxylating dioxygenase enzymes.

Investigation into the microbial degradation of TCDD was initiated by Kearny *et al*. (1972), Matsumura and Benezet (1973) and Ward and Matsumura (1978) and subsequent studies have defined the various aspects of dioxin degradation by numerous microorganisms.

2.8.1 Biodiversity of dioxin-degrading bacteria

Large numbers of bacterial strains that are capable of degrading dioxins, furans and their analogues via dioxygenation have been isolated and characterised. Almost all of dioxins and furans degrading bacteria are from phyla Proteobacteria and Actinobacteria. Both lateral and angular dioxygenation systems are found in species of different phylogenetic groups which shows that dioxygenase-involved degradation systems for aromatics are transferable across the genetic boundary of bacterial species by lateral gene transfer.

Among members of the phylum of Proteobacteria, one of the best characterised dioxins and furans degrading bacteria is alphaproteobacterium, strain RW1 which was initially identified as *Sphingomonas sp* but later known as *Sphingomonas wittichii*. Another Sphingomonad that able to degrade dioxins and furans is *Sphingomonas yanoikuyae* which belong to genus *Sphingobium* even though all other members of Sphingobium did not show any evidence on degrading dioxins and furans. Family Sphingomonadaceae includes large number of species that have been reported to be capable of degrading and assimilating dioxins-like compounds. The reason why many species of Sphingomonads have the ability to degrade dioxins and related aromatics is not known but it is considerable interest whether the presence of glycosphingolipids of the cell surfaces of these bacteria are related to the affinity to aromatic compounds.

Other Gram negative bacteria reported as dioxins and furans degraders are from class Betaproteobacteria such as *Burkholderia* and *Ralstonia* and also from class Gammaproteobacteria such as *Pseudomonas.*

Another rich source of dioxins and furans degrading bacteria is from phylum Actinobacteria which is Gram positive and high G+C content. Among the bacteria that able to degrade dioxins and furans from this phylum are from genera of *Rhodococcus* and *Terrabacter*. Different species of these genera have been isolated and characterised. Another Gram positive bacteria from phylum Firmicutes which have low G+C content is not really known on their potential to degrade dioxins and furans. However, there are reports on degradation of 2,3,7,8-TCDD by *Bacillus megaterium* (Matsumura and Benezet, 1973; Quensen and Matsumura, 1983) and biosorption of 1,2,3,4-TCDD and various PCDF congeners by *Bacillus pumilis* (Hong *et al*.

2000). The bacterial strains that are capable of degrading dioxins, furans and their analogues via dioxygenation were listed in Table 2.3.

Table 2.3: Bacteria strains that able to degrade dioxins, furans and their analogues (Adapted from Hiraishi, 2003)

Phylogenetic group/species/strain	Subtrate	Mode of dioxygenation
Class Aiphabacteria		
Novosphingobium aromativorans IFO 15084T	DF	lateral
Novosphingobium stygium IFO 16085T	DF	lateral
Novosphingobium subterraneum IFO 16086T	DF	lateral
Porphyrobacter sanguineus IAM 12620T	DF	lateral
Sphingobium yanoikuyae B1	DD,DF,MCDD/Fs	lateral
Sphingomonas sp HH19k	DF	angular
Sphingomonas sp HH69	DD,DF,MCDD/Fs, DCDD/Fs, TrCDD/Fs, TCDD/Fs	angular
Sphingomonas sp HL7	DF	NA
Sphingomonas sp KA1	MCDD, DCDD, CAR	NA
Sphingomonas sp RW16	DF	angular
Sphingomonas sp SS3	DE	angular
Sphingomonas sp SS31	MethylDE	angular
Sphingomonas sp SS33	DCDE	angular
Sphingomonas sp CDH-7	CAR	angular
Sphingomonas wittichii RW1	DD,DF, 9-FN, MCDD/Fs, DCDD/Fs, TrCDD/Fs, TCDD	angular
Class Betaproteobacteria		
Burkholderia cepacia F297	DF, DBT, FN	lateral
Burkholderia cepacia ET4	DE	lateral
Burkholderia sp . JB1	MCDD/F	angular
Burkholderia sp LB400	DD, DF	angular
Ralstonia eutropha DSM 5536	MCDD/F, DCDD/F, TrCDD/F	NA
Ralstonia sp DSM 6708	MCDD/Fs, DCDD/Fs, TrCDD/Fs, TCDD/Fs	NA
Ralstonia sp RJGII.123	CAR	angular
Ralstonia sp SBUG290	DF, BP	lateral
Class Gammaproteobacteria		
Erwinia sp CU3614	DE, MCDD, DCDD	NA
Klebsiella sp HL1	DF	NA
Pseudomonas criciviae S93B1	DE	NA
Pseudomonas fluorescens BS243	DF	lateral
Pseudomonas fluorescens TTC1	DF, DBT	lateral
Pseudomonas mendocina MC2	9-Flourenone	NA
Pseudomonas pseudoalcaligenes POB310	Carboxy DE	NA
Pseudomonas putida BS291	DF	lateral
Pseudomonas putida G7	DF, FN, DBT	lateral
Pseudomonas putida PH-01	DF	angular
Pseudomonas resinovorans CA10	CAR, DD, DF	angular

Pseudomonas sp CA06	CAR	angular
Pseudomonas sp F274	DF, FN	angular
Pseudomonas sp HL7b	FN	lateral
Pseudomonas sp LD2	CAR	angular
Pseudomonas sp NSS2	Carboxy DE	angular
Pseudomonas stutzeri ATCC 31258	CAR	angular
Pseudomonas stutzeri OM1	CAR	angular
Phylum (class) Actinobacteria		
Arthrobacter sp DBTS2	DBT, 9-Flourenone	angular
Arthrobacter sp F101	FN	lateral
Brevibacterium sp DO	DBT, FN	angular
Brevibacterium sp DPO220	DF, FN	angular
Microbacterium sp YK18	DF, FN	NA
Nocardioides sp NSA1-2	DF, FN	lateral
Rhodococcus erythropolis SBUG 271	DF, BP	lateral
Rhodococcus opacus SAO101	DF, DD, MCDD, DCDD	NA
Rhodococcus sp TUT581	DF	lateral
Rhodococcus sp YK2	DF	NA
Terrabacter (Janibacter) sp DPO1361	DF	angular
Terrabacter (Janibacter) sp DPO360	DF	angular
Terrabacter (Janibacter) sp DBF63	DF, DD, FN	angular
Terrabacter (Janibacter) sp YK3	DF	angular
Phylum Firmicutes		
Baccillus megaterium ATCC13368	2,3,7,8-TCDD	NA

2.8.2 Biodegradation via lateral dioxygenation

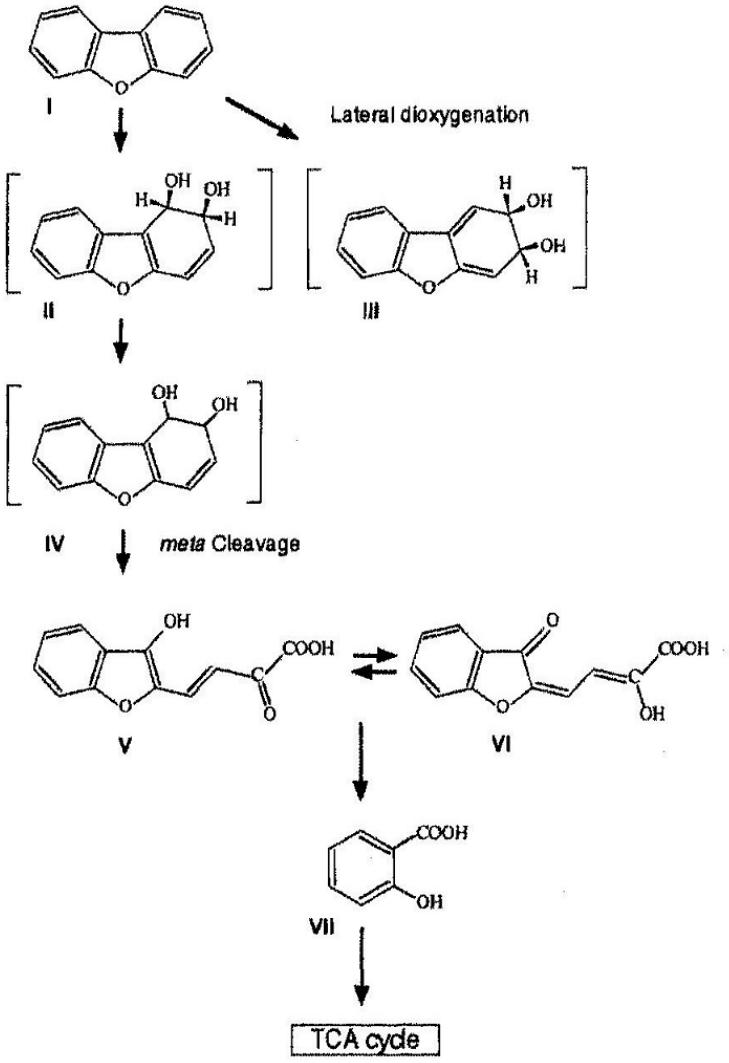

Figure 2.7: Metabolic pathway of dibenzofuran degradation via lateral dioxygenation (route via 1,2-dioxygenation). Metabolites from dibenzofuran (I) are as follows: II) 1,2-dihydroxydibenzofuran; III) 2,3-dihydro-2,3-dihydroxydibenzofuran; IV) 1,2-dihydroxydibenzofuran; V) 2-oxo-4-(3'-hydroxybenzofuran-2'-yl)-but-3-enoic acid; VI) 2-hydroxy-4-(3'-oxo-3' H-benzofuran-2'-yliden)-but-2-enoic acid; VII) salicylic acid (Adapted from Hiraishi, 2003).

The initial step of degradation by naphthalene and biphenyl- degrading strains of *Pseudomonas* and *Beijerinckia* sp which were reclassified as *Pseudomonas putida* and *Sphingobium yanoikuyae*, respectively transformed dibenzo-p-dioxin, dibenzofuran and monochlorinated congeners to cis-dihydroxylated compounds. The dioxygenation attack occurred at the lateral 1,2 and 2,3 position at one of the aromatic rings with high regiospecificity. Figure 2.7 shows lateral dioxygenation of dioxin, furan and their analogues.

Naphthalene dioxygenase from *Pseudomonas putida* which was expressed in recombinant strain showed that salicylate induced cells oxidized dibenzofuran to cis-1,2-dihydrodiol as major product and small amount of cis 2,3-dihydrodiol as minor products (Resnick and Gibson, 1996). The products of dibenzofuran oxidation, dihydrodiols are then converted to dihydroxy compounds where it has been meta-cleaved between carbon atom 1 and 9b. The cleavage of the aromatic ring is known to be associated with the production of a polar yellow metabolite that has an absorption maximum at around 460nm at pH 7.0 which was identified as the enol of 2-oxo-4-[2'-(3'-hydroxy) benzofuranyl]-but-3-enoic acid by Selifonov *et al.* (1992) when using naphthalene grown strain of *Pseudomonas flourescens* and *Pseudomonas putida*. *Ralstonia* SBUG 290 also shows the degradation of dibenzofuran via lateral dioxygenation when grown in the presence of biphenyl with production of yellow metabolite identified as 2-hydroxy-4(3'-oxo-3' H-benzofuran-2'-yliden)-but-2-enoic acid (Becher *et al*. 2000) which showed a pH-dependent shift (pH 3 to pH 7) and indicates a keto-enol tautomerism of a meta-cleavage products. *Rhodococcus erythropolis* SBUG 271, able to co-oxidize dibenzofuran with concomitant production of yellow ring-cleavage metabolite when grown with biphenyl (Stope *et al*. 2002). Biodegradation of structural analogues of dibenzofuran via lateral dioxygenation has also been

reported in several bacterial strains. Diphenyl ether was metabolised by *Burkholderia cepacia* ET4 with similar early steps for catabolic pathway of biphenyl (Pfeifer *et al*. 1989; 1993). Cell suspensions of *Pseudomonas sp* strain HL7b transformed flourene to a yellow ring-cleavage metabolite showing an absorption max at around 460nm (Foght and Westlake, 1988). A similar result was obtained with *B. cepacia* strain F297 supplemented with fluorene and dibenzothiophene a sole carbon source and energy. Two productive routes and one non-productive route were found in *Arthrobacter* sp. strain F101 for fluorene metabolisms. The initial dioxygenase attack at the 1,2 and 3,4 positions of the 2 productive routes yield the corresponding dihydrodiols with the formation of yellow polar metabolite which indicate the keto tautomerism characteristics of meta-cleavage while the other route which is non-productive was initiated by monooxygenation at C-9 to give 9-fluorenol and then 9-fluorenone. Most reports show that degradation of dibenzofuran and its relative via lateral dioxygenation is by cometabolisms and usually unable to degrade dibenzofuran completely because it leads to dead-end products. However, recent research shows that some bacteriochlorophyll-containing bacteria are able to grow in dibenzofuran as sole carbon source such as *Porphyrobacter sanguineus* (Hiraishi *et al*. 2002) and *Sphingobium yanoikuyae*.

2.8.3 Biodegradation via angular dioxygenation

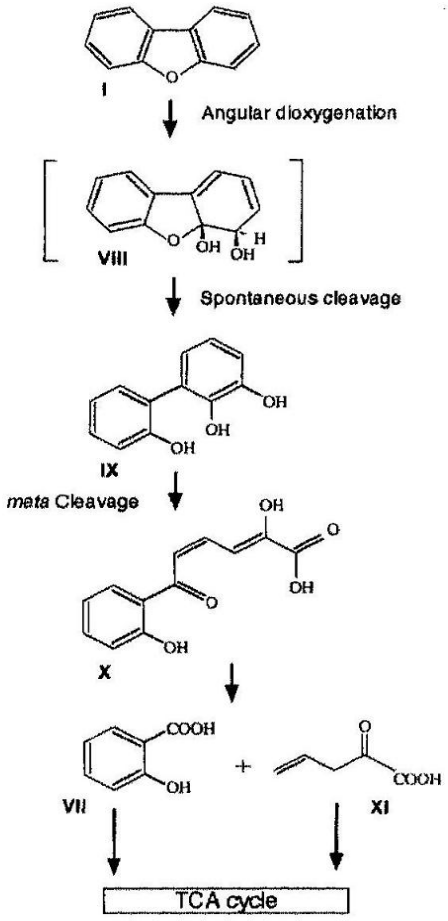

Figure 2.8: Metabolic pathway of dibenzofuran degradation via angular dioxygenation. Metabolites from dibenzofuran (I) are as follows: VIII) 4,4a-dihydro-dihydroxydibenzofuran; IX) 2,2",3-trihydroxybiphenyl; X) 2-hydroxy-6(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; XI) 2-oxo-4-pentenoate; VII) Salicylic acid (Adapted from Hiraishi, 2003)

In angular dioxygenation (Figure 2.8), initial dioxygenase attack at the angular position on two carbon atoms adjacent to the ether bridge with high regioselectivity and specificity (Engesser *et al*. 1989; Strubel *et al*. 1989). Transformation of a dibenzofuran analogue by Gram-positive bacterium, genus *Janibacter*, provides an evidence of dioxygenase that attack at unusual angular

position. Angular dioxygenation was found in biodegradation pathways for different aromatics in several species of the phyla Actinobacteria and Proteobacteria. The most studied organism in angular type of dioxygenation is *Sphingomonas wittichii* RW1 where the metabolic routes for dibenzo-p-dioxin and dibenzofuran have been proposed (Wittich *et al*. 1992).

Initial step of degradation of dibenzofuran/dibenzo-p-dioxin is dioxygenation attack at 4 and 4a positions of one of the aromatic rings which produce corresponding cis-dihydrodiols. The hemiacetal products of dibenzofuran and dibenzo-p-dioxin are spontaneously transferred to 2,2',3-trihydroxybiphenylether and 2,2',3-trihydroxybiphenyl. The dihydroxylated rings of the products were meta-cleaved by an extradiol dioxygenase which subsequently hydrolyzed to yield catechol and salicylic acid respectively.

Angular dioxygenase attack has also been reported in the biodegradation of several structural analogus of dibenzofuran such as hydroxylated, methoxylated and acetoxylated dibenzofuran which were transformed to the corresponding salicylate by *Sphingomnas sp* HH69 (Harms *et al*. 1995). The biodegradation of dioxin-related compound via angular dioxygenation and its biotechnological implication have recently been reviewed (Nojiri *et al*. 2001, Nojiri and Omori, 2002).

2.8.4 Biodegradation of chlorinated congeners

Even though a number of studies have demonstrated the ability of *Sphingomonas wittichii* RW1 to degrade chlorinated dibenzo-p-dioxin and dibenzofuran congeners but it is limited to several mono and dichlorinated dibenzo-p-dioxins/dibenzofurans (Wilkes *et al*. 1996) but not highly chlorinated congeners. Despite its ability to degrade dichlorinated, it is unable to grow with these congeners as carbon and energy source due to non-permissive steps in the degradation pathway for the metabolites produced from these compounds. Complete mineralization of 4-MCDF was found in a co-culture of RW1 and a 3,5-dichlorosalicylate degrading bacteria, *Burkholderia sp* JWS where dioxygenolytic attack was took place at the non-halogenated aromatic ring and subsequently resulted in stoichiometric production of the dead-end metabolite 3-chlorosalicylate and was then utilized by JWS as carbon and energy source (Arfmann *et al*. 1997). Catabolism of 2,7-DCDF was transformed by RW1 into 2 metabolites; 6-chloro and 7-chloro 2 methyl-4H-chromen-4-one while 2,4,8-TrCDF was transformed to 6,8-dichloro-2-methyl- chromen-4-one. Bacterial strain other than *Sphingomonas wittchii* RW1, have also been reported to be capable of degrading chlorinated dibenzofurans, dibenzo-p-dioxins and DEs. Among them are *Sphingomonas sp* strain SS33 (2,4 and 4,4-dichlorinated DE) and *Burkholderia sp* JB1 (2-MCDD). The influence of the substitution pattern on the biodegradation of all 210 congeners of PCDD/DFs was studied by Schreiner *et al*. (1997) and concluded that bacterial degradation rates for dioxins depends upon the configuration of the isomers and decrease with increasing degree of chlorination of the aromatic rings. A chlorine substitution at positions 1, 4, 6 or 9 interferes with the dioxygenolytic attack on the diaryl ether linkage and retarding the degradation of target chlorinated compounds. Thus it is likely that the potential for complete dioxin degradation of a

single wild strain of dioxygenase producing aerobic bacteria is restricted to TrCDD/Fs and lower chlorinated congeners in general.

2.8.5 Dioxin degradation by *Burkholderia xenovorans* LB400

L'Abbe *et al* (2005) investigated the ability of *Burkholderia xenovorans* LB400 in degradation of Dibenzo-p-dioxin (DD) and Dibenzofuran (DF). Biphenyl dioxygenase of *Burkholderia xenovorans* LB400 was isolated and expressed in *E. coli* produces dihydro-dihydroxy-dibenzofuran as a major metabolite, which resulted from the lateral oxygenation of dibenzofuran and 2,2',3- trihydroxybiphenyl was the minor metabolite. However, 2, 2',3-trihydroxybiphenyl ether is a sole metabolite of dibenzo-p-dioxin degradation using biphenyl dioxygenase of LB400. Dibenzofuran and dibenzo-p-dioxin which were regarded as analogues of doubly ortho-substituted biphenyls or diphenylethers were attacked at the "quasi ortho" carbon (the angular position) and its neighbour. Angular attack by the biphenyl dioxygenase was the main route of dibenzo-p-dioxin oxidation but lateral dioxygenation was the major reaction with dibenzofuran. Dioxygenation of chlorinated biphenyls (Cl-Bs) has been characterised by Seeger and co worker which was catalysed by *bph*-encoded biphenyl dioxygenase of *Burkholderia xenovorans* LB400 and *Rhodococcus globerulus* P6. The attack of biphenyl dioxygenase of LB400 on symmetrical ortho-substituted biphenyls or quasi ortho substituted biphenyl analogues such as dibenz-p-dioxin and dibenzofuran has been investigated by Seeger and co-workers (2001).

The study demonstrated that angular attack by BPDO was the main route of dibenzodioxin oxidation while lateral dioxygenation leading to dihydrodiols was the major reaction with

dibenzofuran. Ring-hydroxylating dioxygenase are quite versatile with respect to the substrates accepted as well as to the type of reaction catalysed.

Bph-encoded biphenyl dioxygenase (BphAs) of LB400 was characterised to catalyse the dioxygenation of chlorinated-biphenyls (Cl-Bs). BPDO able to attack chlorinated ortho carbons of some biphenyls (Seeger *et al*. 2001; Haddock *et al*. 1995; Seeger *et al*. 1995; Seeger *et al*. 1999). This type of attack, leading to elimination of the ortho chlorine is theoretically can reduce the toxicity, increase solubility and enhance the enzymatic turnover of the metabolites. Dibenzofuran may be regarded as a biphenyl derivative in which carbons 2 and 2' are oxygenated in a specific way, namely by the formation of an ether bridge. The results show that the dibenzofuran molecule is not preferentially deoxygenated at position 4 and 4a but the main product originated from attack at carbons 1 and 2. Unlike dibenzofuran, dibenzo-p-dioxin was preferentially deoxygenated at the angular position. However, product quantities, as deduced from total ion chromatogram peak areas, suggest that the absolute yields of angular dioxygenation were similar with both compounds and that the lateral attack was strongly disfavoured with dibenzo-p-dioxin.

2.9 Biphenyl dioxygenase

Biphenyl dioxygenase encoded by *bphA* gene is a Rieske-type, 3 component enzyme, composed of a terminal dioxygenase and an electron transfer chain. Terminal dioxygenase consist of a large and a small subunit associating as a $\alpha 3\beta 3$ heterohexamer while electron transfer chain consists of ferredoxin and ferredoxin reductase which involve in electron transfer from NADH to reduce

the terminal dioxygenase. BphA encoded by *bphA1* and *bphA2* for a large and a small subunit of the terminal dioxygenase while *bphA3* and *bphA4* encode for ferredoxin and ferredoxin reductase respectively. The large subunit of terminal dioxygenase is crucially involved in the substrate specificity of biphenyl dioxygenase (Kimura *et al.* 1998; Kumamaru *et al.* 1998). Therefore, evolutionary molecular engineering has been applied to large-subunit genes of different origins. Evolved biphenyl dioxygenase show not only enhanced and expanded degradation for PCB but also other related compounds.

2.9.1 Structural versatilities

The *bph* genes are present on bacterial chromosomes, plasmids and transposons. The typical *bph* gene cluster as found in *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia xenovorans* LB400 are shown in Figure 2.9. The *bph* gene cluster can be very similar and also very different in terms of gene organisation and structure of each gene (Figure 2.9). This shows that *bph* gene cluster can move among bacterial species and evolved from a common ancestor.

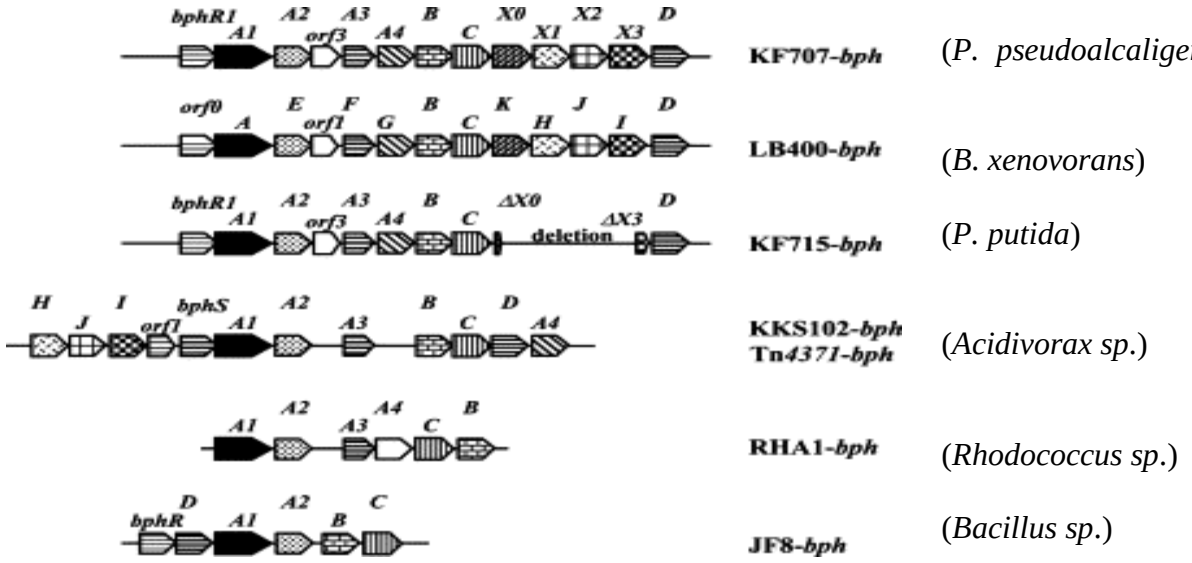

Figure 2.9: Organisation of *bph* gene clusters encoding upper pathway enzymes in various bacterial strains; *P. pseudoalcaligenes* KF707, *B. xenovorans* LB400, *P putida* KF715, *Acidivorax* sp. KKS102, *Rhodococcus* sp. RHA1 and *Bacillus* sp. JF8. (Furukawa and Fujihara, 2008).

2.9.2 Functional versatilities

Aromatic ring-hydroxylating dioxygenase involved in initial oxygenation are of particular importance because this reaction destabilizes the aromatic ring and initiates the degradation of aromatic compounds. The terminal dioxygenase of BphA activates molecular oxygen and introduces it to the substrate while the reductase chain transfer electron from NADH to the terminal dioxygenase. Terminal dioxygenase can be homomultimer or heteromultimer which comprise of a large and small subunit. The biphenyl dioxygenase of *Burkholderia xenovorans* LB400 which is similar to KF707 is a class IIB-type, 3 components enzyme consisting of 4 subunits; a large subunit (BphA1) and a small subunit (BphA2) of terminal dioxygenase, a

ferredoxin (BphA3) and a ferredoxin reductase (BphA4). Biphenyl dioxygenase of LB400 and KF707 has been extensively studied with the respect to the degradation of PCBs. However the range of PCB congeners oxidized by the *Burkholderia xenovorans* LB400 enzyme is much wider than that oxidized by *Pseudomonas pseudoalcaligenes* KF707 enzyme.

Pseudomonas pseudoalcaligenes KF707 BPDO has a higher activity for several di-para-substituted-PCBs while *Burkholderia xenovorans* LB400 BPDO has remarkable ability to oxidize PCB congeners that contain up to 4 chlorines by introducing 2 hydroxyl groups at either 2, 3 or 3, 4 positions. The specificity of the LB400 BPDO for PCBs was correlated with the relative positions of the chlorine substituents on the aromatic rings rather than with the number of chlorine subsituents on the rings (Arnett *et al.* 2003). The attack by BPDO of LB400 on several symmetrical ortho-substituted biphenyl or quasi-ortho substituted biphenyl analogues such as dibenzo-p-dioxin and dibenzofuran was also investigated.

2.9.3 Regulation of *bph* genes

Even though there is a lot of information regarding the biochemical and genetic analogues of the *bph* genes, the knowledge concerning regulation is remained limited. The regulation of *bph* gene cluster in KF707 was reported by Watanabe and coworkers (2000; 2003). In this study, two regulatory systems were identified. There are *bphR1*-dependent transcription for *bphR1* itself and *bphR2*-dependent transcription of *bphA1A2(orf3)A3A4BC*. The transcription of the *bph* locus of LB400, where *bph* genes are very similar to KF707 was investigated by Beltrametti and coworkers (2001). The ORF0 protein which corresponded to KF707 *bphR1* indicates the

activation of the *bphA1* promoter. Even though the *bph* genes of LB400 and KF707 are nearly identical, the regulatory mechanisms at the *bph* genes are totally different. The *bph* gene which was carried by Tn4371 transposon allows *Ralstonia metallidurans* CH34 to use biphenyl as sole carbon and energy. In *Ralstonia metallidurans* CH34, the genes involved in benzoate degradation are chromosomal. Sigma-54 consensus binding sites were found upstream of the Tn4371 *bph* gene and the closely related gene in *Pseudomonas sp* KKS102. Merlin *et al.* (1997) transferred plasmid RP4::Tn4371 into a mutant of *Alcaligenes eutrophus* H16 that lacks of sigma-54 and observed that the sigma-54 mutant exconjugant could not grow on biphenyl which is contrast with wild-type *Alcaligenes eutrophus* H16, indicating the dependent of Tn4371 *bph* gene expression on sigma-54. *Ralstonia eutropha* A5 (Shields *et al*. 1985) and *Pseudomonas sp* KKS102 (Kimbara *et al*. 1989) shows direct common descent of their *bph* gene cluster with Tn4371, even though they were isolated from very different geographical location. It is likely that the Tn4371/KKS102 *bph* gene organisation is widely represented among PCB-degrading bacteria.

2.9.4 *Bph* expression is under the control of the σ54 RNA Polymerase

In the *Pseudomonas sp* KKS102 *bph* cluster (Kikuchi *et al*. 1994), 2 putative σ^{54}-dependent promoters were recognised. Analysis of the nucleotide sequence in and around the Tn4371 *bph* gene cluster also revealed 2 consensus sequences from the binding of the sigma subunit of RNA polymerase σ^{54}, located 270bp upstream of *bphE* and orf4 upstream of *bphA1*. The organization of σ^{54} in LB400 is different from KKS102/KF707 where 2 putative σ^{54} were located in

chromosome 1 and chromosome 2 instead of clustered in transposon as Tn4371 or plasmid. Tn4371 *bph* gene expression was demonstrated under the direct or indirect control of σ-^{54} RNAP.

Despite all the information such as presence of σ^{54}-associated RNA, polymerase binding consensus sequence as well as indications for presence of regulatory element in the neighbourhood of the structural genes which show the existence of a specific control through biphenyl (or/and metabolites) via a specific regulator combined with σ^{54}-dependent global control.

Merlin *et al* (1997) observed that *rpoN* mutant of H16 cannot grow on minimal media supplemented with biphenyl as sole carbon source which is contrast with the wild type H16 suggesting the control of σ54 in the expression of the Tn4371 *bph* gene in H16. However, Mouz *et al* (2001) shows that *bphC* transcription (product of Tn4371/*bphC*) is not affected with the loss of *rpoN* function in *rpoN* mutant of CH34 directly or indirectly. Nevertheless, when provided with biphenyl as sole carbon and energy source in liquid cultures, the *rpoN* mutant grew very poorly compared to wild type.