J. Microbiol. Biotechnol. (2008), **18**(5), 952–957

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation and Characterization of Comprehensive Polychlorinated Biphenyl-Degrading Bacterium, *Enterobacter* sp. LY402

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Received: August 20, 2007 / Accepted: October 22, 2007

A Gram-negative bacterium, named LY402, was isolated from contaminated soil. 16S rDNA sequencing and measurement of the physiological and biochemical characteristics identified it as belonging to the genus *Enterobacter*. Degradation experiments showed that LY402 had the ability to aerobically transform 79 of the 91 major congeners of Aroclor 1242, 1254, and 1260. However, more interestingly, the strain readily degraded certain highly chlorinated and recalcitrant polychlorinated biphenyls (PCBs). Almost all the tri- and tetra-chlorobiphenyls (CBs), except for 3,4,3',4'-CB, were degraded in 3 days, whereas 73% of 3,4,3',4'-, 92% of the penta-, 76% of the hexa-, and 37% of the hepta-CBs were transformed after 6 days. In addition, among 12 octa-CBs, 2,2',3,3',5,5',6,6-CB was obviously degraded, and 2,2',3,3',4,5,6,6'- and 2,2',3,3',4,5,5',6'-CB were slightly transformed. In a metabolite analysis, mono- and di-chlorobenzoic acids (CBAs) were identified, and parts of them were also transformed by strain LY402. Analysis of PCB degradation indicated that strain LY402 could effectively degrade PCB congeners with chlorine substitutions in both *ortho*- and *para*-positions. Consequently, this is the first report of an Enterobacteria that can efficiently degrade both low and highly chlorinated PCBs under aerobic conditions.

Keywords: Biodegradation, polychlorinated biphenyl (PCB), aerobic

Polychlorinated biphenyls (PCBs) are one of the most prevalent and persistent groups of contaminants in the environment, accumulating in the biota, biomagnifying in the food chain, and causing multiple adverse health effects [12]. PCB molecules consist of a biphenyl nucleus carrying 1 to 10 chlorines, which can create more than 200 possible congeners that differ in the number and position of the chlorines. The less chlorinated congeners are usually less toxic, whereas the more chlorinated congeners show a higher toxicity. Thus, the remediation of PCB contamination in the environment has become extremely important, especially for highly chlorinated and recalcitrant coplanar PCBs.

The microbial degradation of PCBs is regarded as one of the most cost-effective and energy-efficient methods to remove PCBs from the environment. Therefore, much effort has been focused on the selection of highly efficient PCB-degrading bacteria. Many isolates have already been reported, including Gram-negative strains, such as *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Janibacter*, *Burkholderia*, *Acinetobacter*, *Comamonas*, *Sphingomonas*, *Paenibacillus*, and *Ralstonia*, and Gram-positive strains, such as *Arthrobacter*, *Corynebacterium*, *Rhodococcus*, and *Bacillus* [1–6, 9, 10, 13, 17–20]. However, most of these strains are only capable of degrading PCBs congeners that have five or fewer chlorines. Moreover. only a small number of strains have the ability to transform congeners with highly chlorinated (5 or more chlorines) and recalcitrant coplanar PCBs, yet their abilities are still limited. Accordingly, efforts are needed to isolate new microorganism strains that can bioremediate highly chlorinated PCBs contaminants in the environment.

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Materials and Methods

Chemicals and Media

Aroclors 1242, 1254, 1260, hexachlorobenzene (HCB), chlorobenzoic acids (CBAs), 2,2'-dichlorobiphenyl, and 2,3-dichlorobiphenyl were all purchased from AccuStandard Ltd. (New Haven, U.S.A.), whereas biphenyl (BP) and a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)+trimethylchlorosilane (TMCS) at the ratio of 99:1 were

pruchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). Pesticide-grade *n*-hexane was obtained from J. T. Baker (U.S.A.) and yeast extract from Oxoid Ltd. (Basingstoke, Hampshire, England). The other chemicals for the culture media were at least of analytical grade and purchased from Shanghai Chemical Agent Company (Shanghai, China).

The defined mineral salt medium (MSM) was prepared based on the medium described by Bedard *et al.* [3]. The MSM supplied with biphenyl crystals (MSM-B) was used for the selection and enrichment of the bacterial isolates. The biphenyl on MSM-B agar plates was applied to the lids of inverted Petri dishes.

Isolation and Enrichment of Bacteria

The soil used for the isolation of PCB-degrading bacteria was obtained from the subsurface of oily sludge at a transformer-dismantling site with a more than 20-year history in the southeastern region of China (Wenling, Zhejiang). The contaminated soil (2 g) was placed in 50 ml of MSM-B and incubated at 30°C for 6 days. Ten ml of the culture was then transferred to 50 ml of fresh MSM-B and incubated under the same conditions. After repeating this procedure 7 times, the culture was diluted to 10^{-6}, and 50 µl of the solution was inoculated onto a MSM-B agar plate and left for 5 days. Each colony that appeared different in morphology was further incubated in MSM-B and screened based on its rate of degrading 2,3',4,4'-chlorinated biphenyls (CBs). Strain LY402 with the highest 2,3',4,4'-CB degradation rate was then finally selected for the following PCB degradation experiments.

Genotypic and Phenotypic Analyses

The 16S rDNA of strain LY402 was amplified from genomic DNA using a TaKaRa 16S rDNA Bacterial Identification PCR Kit (Takara Bio, Japan) and sequenced using an ABI PRISM 3730XL DNA analyzer (Applied Biosystems, CA, U.S.A.). The sequence was then aligned using CLUSTALW with all the parameters set at their default values [16]. Phylogenetic analyses were performed using the neighbor-joining method [21], and the graphics for the phylogenetic trees were produced using the Treeview program [14]. The 16S rDNA sequence was deposited in GenBank under Accession No. DQ659161.

The morphological properties of the cells were examined by scanning electron microscopy (SEM). The physiological and biochemical characteristics were measured as described by *Bergey's Manual of Systematic Bacteriology* [15].

Degradation of Commercial PCB Mixtures (Aroclors)

The Aroclors 1,242, 1,254, and 1,260 were mixed at a ratio of 1:1:1 for the degradation experiments. Acetone (0.2 ml) containing 6 µg of PCBs was added to each 12-ml glass vial. The acetone was allowed to evaporate under a fume hood. The LY402 cells grown on MSM-B were transferred to fresh MSM at an OD_{600} of 1.0 and added to the vials, which were then incubated at 30°C on a shaker at 150 rpm. The control cells were heat-killed. Each biodegradation assay was carried out two times.

Analytical Methods

To stop the degradation reactions immediately, one drop of 70% perchloric acid was added to the vials, and then 0.1 ml of hexane containing hexachlorobenzene at 1.0 mg/ml was added as the internal standard, two volumes of *n*-hexane were added to extract the residual PCBs in the medium, and 0.5 g of sodium sulfate was added to prevent the formation of a stable emulsion. Foil-lined caps were used for this procedure. The samples were all shaken horizontally on an orbital shaker at 300 rpm for 30 min, and then a 1-ml aliquot of the hexane layer was transferred from the vials for a GC/ECD analysis.

The extracts were analyzed for their PCB concentrations using an HP 6890 (Agilent, CA, U.S.A.) GC equipped with an autoinjector, ^{63}Ni electron capture detector, and DB-1701 capillary column (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 µm; J&W Scientific, Folsom, CA, U.S.A.). The samples (1 µl) were injected using an autoinjector in the splitless mode, while the temperature of the splitless injector was maintained at 290°C and the detector temperature was set at 310°C. N_2 was used as the carrier gas. The column temperature program was started at 150°C, then increased 1.1°C/min to 280°C, and finally run at 280°C for 20 min. The identification of the congeners was obtained according to the previous report by Zhang *et al.* [22]. To calculate the degradation rate for each PCB congener, every individual PCB peak was calibrated by its relative response factors (RRF) [11]. All the calibration peaks were then normalized to a HCB peak and compared with the control. The equation used was

$$\% \text{ Degradation} = 100 - [(A_i)_{sample}/(A_i)_{control}]/[(A_{HCB})_{sample}/(A_{HCB})_{control}] \times 100\% \text{ and}$$

$$\% \text{ Total Degradation} = 100 - \frac{\sum_{i=1}^{n}\left(\frac{Ai}{RRFi \times A_{HCB}}\right)_{sample}}{\sum_{i=1}^{n}\left(\frac{Ai}{RRFi \times A_{HCB}}\right)_{control}} \times 100\%$$

where A_i is the area of a PCB congener peak in the sample.

Identification of PCB Metabolite

As with the Aroclor degradation procedure, 12.5 mg/l of 2,2'-chlorobiphenyl (CB) and 2,3-CB were treated with the LY402 for 6 h, respectively. Thereafter, the cell suspensions were acidified with HCl to obtain a pH of 2, and extracted twice with 3 ml of ethyl acetate. Sodium sulfate (0.4 g) was then added to remove any water, and the extracts were dried under a gentle stream of nitrogen. Next, the residual was dissolved in 100 µl hexane, derived by adding 100 µl of BSTFA+TMCS (99:1), and incubated at 60°C for 15 min. Finally, hexane was added to 1 ml of the samples for metabolite analyses using GC/MS (HP 6890N GC with HP 5975 Inert MSD), equipped with a HP-5 column (30 m×0.25 mm×0.25 µm). The mass spectrometer was used in the 70-eV electron impact mode, and a quadrupole mass filter (150°C) was used to scan from *m/z* 50 to *m/z* 550 per second. The injector and ion source temperatures were 290°C and 230°C, respectively. The carrier gas was helium at a flow rate of 1 ml/min. The temperature program started at 80°C for 1 min, and then increased 25°C/min to 140°C, 8°C/min to 220°C, and finally 15°C/min to 280°C.

RESULTS

Isolation and Characterization of Strain *Enterobacter* sp. LY402

The 16S rDNA gene sequences of LY402 were aligned with the sequences deposited in the GenBank database

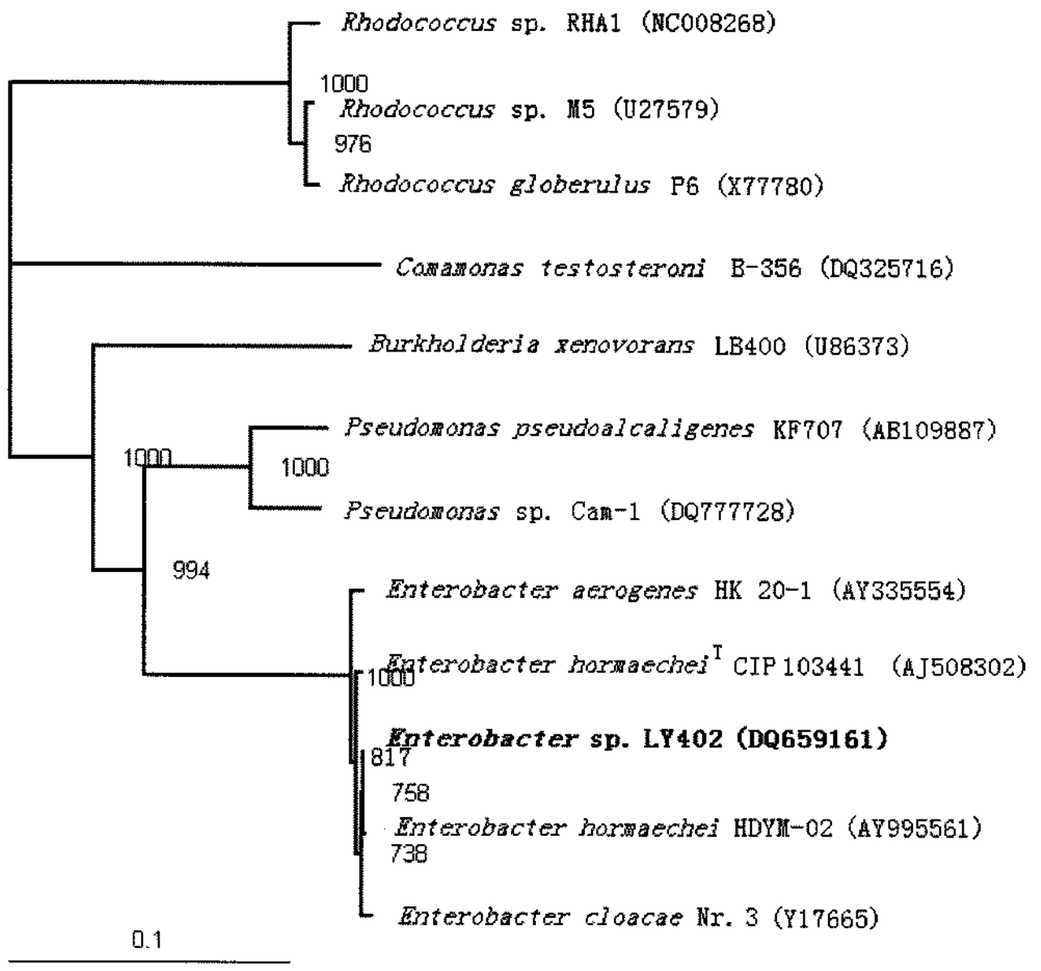

Fig. 1. Phylogenetic neighbor-joining tree for *Enterobacteria* sp. LY402 obtained from 16S rDNA sequence analysis.
The GenBank accession number for LY402 is DQ659161. The scale bar denotes 10 nucleotide substitutions per 1,000 nucleotides. Bootstrap values from 1,000 analyses are shown at the nodes.

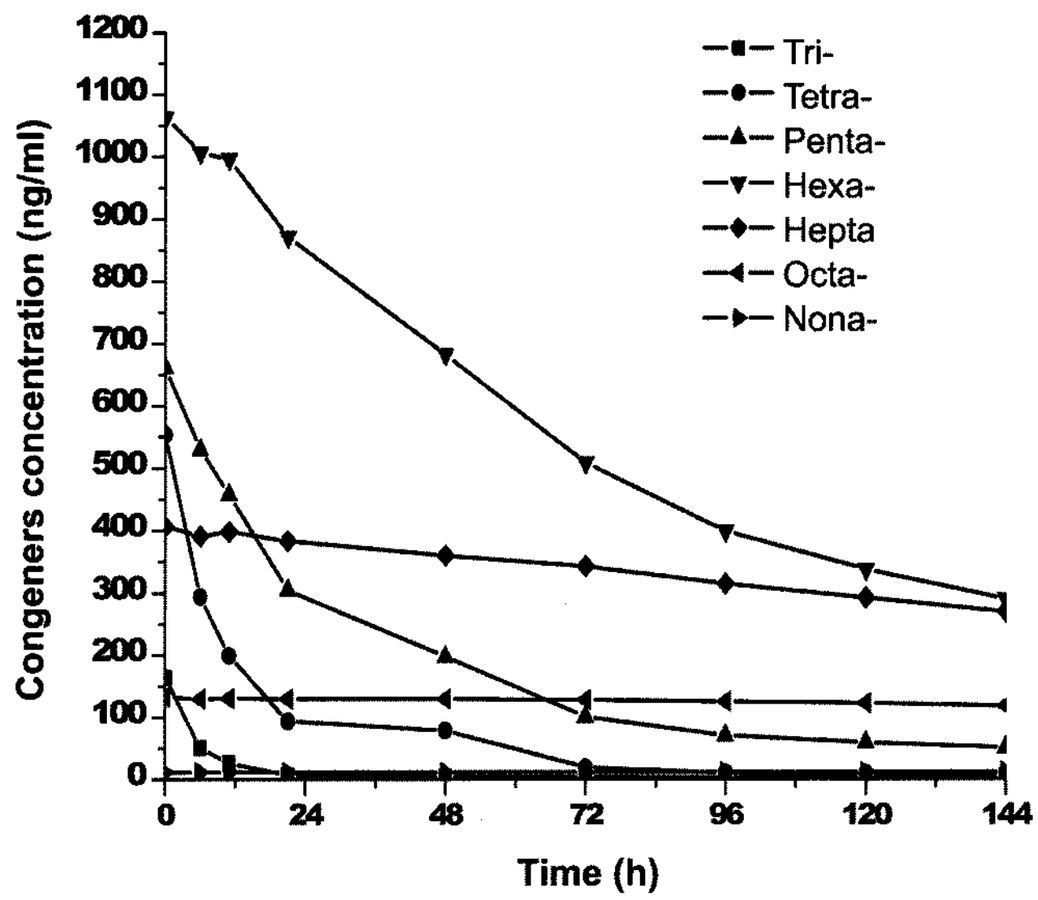

Fig. 2. Time course for degradation of PCBs by *Enterobacteria* sp. LY402.
Three μg/ml Aroclors (1242:1254:1260=1:1:1) was incubated with heat-inactivated or active LY402 at 30°C. Data are averages of two experiments.

using a BLAST search and revealed to be 99% identical to *Enterobacter hormaechei* HDYM-02 (GeneBank Accession No. AY995561). The results of the phylogenetic analysis also showed that strain LY402 belonged to the genus *Enterobacter* (Fig. 1). The bacterium was a Gram-negative rod, facultative anaerobe, catalase positive, and oxidase negative. The strain was further determined by growth on 95 carbon sources using a Biolog MicroStation. The profiles of the enzymes produced from the strain and carbohydrate utilization also proved it was a species of *Enterobacter*.

Table 1. Comparison of degradative competence at different chlorine positions including published strains considered to have strong PCB-degrading ability.

PCB congener	Gram-negative bacteria			Gram-positive bacteria	
	LY402[a]	H850[b]	LB400T[c]	KBC101[d]	RHA[e]
Ortho-chlorinated PCBs					
2,5,2'-CB	99	100	100	100	98
2,5,2',5'-CB	99	100	100	72	76
2,4,5,2',5'-CB	98	85	100	58	29
2,3,5,6,2',5'-CB	97	40	–	–	27
2,3,4,5,2',3',5'-CB	46	0	–	–	0
Para-chlorinated PCBs					
4,4'-CB	89	100	25	–	95
2,4,2',4'-CB	99	65	81	33	83
2,4,3',4'-CB	98	40	43	58	99
3,4,3',4'-CB	73	<40	6	56	0
2,4,5,2',4',5'-CB	89	20	41	11	0
2,3,4,6,2',3',4'-CB	21	0	–	–	0

The values represent the percents of degradation. The hyphens indicate that the results were not reported.
[a]Data are averages of two experiments.
[b]Results for *Alcaligenes eutrophus* H850 reported by Bedard *et al.* [4].
[c]Results for *Burkholderia xenovorans* LB400T reported by Gibson *et al.* [10].
[d]Result for *Paenibacillus* sp. KBC101 reported by Sakai *et al.* [7].
[e]Result for *Rhodococcus* sp. RHA1 reported by Seto *et al.* [18].

Degradation of Commercial PCB Mixtures (Aroclors)

During the 6-day degradation experiments, the PCB concentrations in the vials were monitored on days 2, 3, 4, 5, and 6. For the control sample, the chromatogram showed 91 clearly separated congener peaks in the control sample, whereas 79 of the 91 congeners were shown as degraded to various degrees by LY402. Moreover, based on calculating the molecular mass of all the congeners, 75.5% of the mixture was found to be transformed at the end of the 6 days. Almost all the tri- and tetra-CB congeners, except for 3,4,3',4'-CB, were completely degraded after 3 days (Fig. 2, Table 1). With the development of time, 73% of 3,4,3',4'-CB, 92% of the penta-, 76% of the hexa-, and 37% of the hepta CBs were transformed after 6 days (Fig. 2, Table 2). In contrast, among the 11 octa-CB congeners, only 2,2',3,3',5,5',6,6-CB was obviously degraded by 58%, whereas 2,2',3,3',4,5,6,6'- and 2,2',3,3',4,5,5',6'-CBs were just slightly transformed (Table 2). For the nonaclorinated PCBs, only 2,2',3,3',4,4',5,5',6-CB was

Table 2. Degradation of highly chlorinated PCBs by *Enterobacter* sp. LY402 after 6 days.

Congener No.	Congener	% Degradation (SD)
136	2,2',3,3',6,6'	96(2)
151	2,2',3,5,5',6	97(1)
135	2,2',3,3',5,6'	90(0)
147,144	2,2',3,4',5,6/2,2',3,4,5',6	95(1)
149	2,2',3,4',5',6	80(1)
131	2,2',3,3',4,6	88(13)
134	2,2',3,3',5,6	95(0)
114,146	2,3,4,4',5/2,2',3,4',5,5'	90(0)
153	2,2',4,4',5,5'	89(0)
179,132	2,2',3,3',5,6,6'/2,2',3,3',4,6'	83(0)
141,176	2,2',3,4,5,5'/2,2',3,3',4,6,6'	72(1)
137	2,2',3,4,4',5	64(3)
130	2,2',3,3',4,5'	85(0)
178,138,163,164	2,2',3,3',5,5',6/2,2',3,4,4',5'/ 2,3,3',4',5,6/2,3,3',4',5',6	53(3)
175	2,2',3,3',4,5',6	65(4)
187	2,2',3,4'5,5',6	70(2)
183	2,2',3,4,4',5',6	83(2)
129	2,2',3,3',4,5	31(4)
185	2,2',3,4,5,5',6	73(1)
167	2,3',4,4',5,5'	26(1)
202	2,2',3,3',5,5',6,6'	58(1)
174	2,2',3,3',4,5,6'	26(4)
128	2,2',3,3',4,4'	45(3)
177	2,2',3,3',4',5,6	42(3)
171	2,2',3,3',4,4',6	21(4)
173	2,2',3,3',4,5,6	28(4)
156	2,3,3',4,4',5	16(4)
172	2,2',3,3',4,5,5'	46(2)
157	2,3,3',4,4',5'	0
180,197	2,2',3,4,4',5,5'/2,2',3,3',4,4',6,6'	24(3)
193	2,3,3',4',5,5',6	25(5)
200	2,2',3,3',4,5,6,6'	15(3)
191	2,3,3',4,4',5',6	0
198	2,2',3,3',4,5,5',6	0
199	2,2',3,3',4,5,5',6'	26(2)
170,190	2,2',3,3',4,4',5/2,3,3',4,4',5,6	0
203,196	2,2',3,4,4',5,5',6/ 2,2',3,3',4,4',5,6'	0
208	2,2',3,3',4,5,5',6,6'	0
207	2,2',3,3',4,4',5,6,6'	0
189	2,3,3',4,4',5,5'	0
195	2,2',3,3',4,4',5,6	0
194	2,2',3,3',4,4',5,5'	0
205	2,3,3',4,4',5,5',6	0
206	2,2',3,3',4,4',5,5',6	0

Three μg/ml Aroclors (1242:1254:1260=1:1:1). Data are the averages of two experiments. Less than 15% degradation was not considered significant and was not reported.

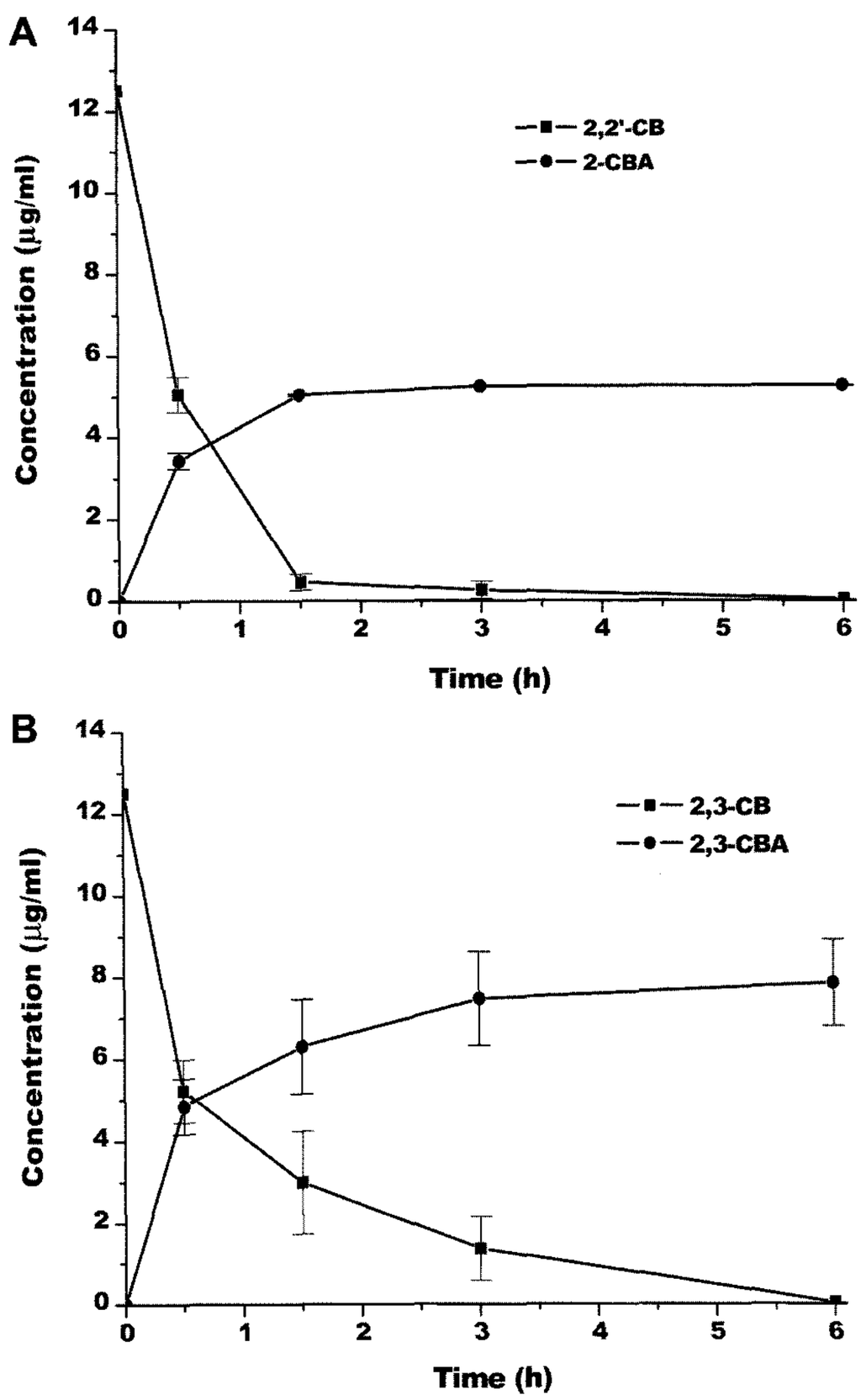

Fig. 3. Time course for degradation of 2,2'-CB and 2,3-CB and production of their metabolites by resting cells of *Enterobacter* sp. LY402.

A. 2,2'-CB (12.5 μg/ml) and its metabolite 2-chlorobenzoic acid (2-CBA). **B.** 2,3-CB (12.5 μg/ml) and its metabolite 2,3-chlorobenzoic acid (2-CBA). Data are the averages of three experiments. The bars denote the standard errors.

detected in the Aroclor mixtures and it was not degraded by LY402 (Table 2).

Intermediate Metabolites of PCBs

To understand the metabolism pathways, 2,2'-CB and 2,3-CB were used for a degradation metabolite analysis based on GC-MS. Peaks at 8.380 min and 10.6 min corresponding to 2-CBA and 2,3-CBA, respectively, were observed after 2,2'-CB and 2,3-CB were treated with LY402 for 6 h. A mass analysis further confirmed that the accumulations were 2-CBA and 2,3-CBA. The results demonstrated that the LY402 rapidly metabolized both 2,2'-CB and 2,3-CB and produced the corresponding chlorobenzoic acids. The concentrations of 2-CBA and 2,3-CBA also increased accordingly with the rapid depletion of 2,2'-CB and 2,3-CB (Fig. 3). After 6 h, strain LY402 almost completely transformed the 2,2'-CB and 2,3-CB, yet only 60% of 2-CBA and 73% of 2,3-CBA were detected (Fig. 3), indicating that strain LY 402 was capable of further CBA degradation.

Discussion

Strain LY402 described in this paper showed a strong ability to degrade a wide range of congeners in commercial PCBs mixtures. All previously reported bacterial strains with a PCB-degrading capacity are generally only able to degrade congeners with five or fewer chlorine substitutions, and show very little or no ability to degrade highly chlorinated congeners (5 or more chlorines) and coplanar PCBs. In contrast to such previously reported strains, strain LY402 was shown to be capable of transforming 92% of the penta-, 76% of the hexa-, and 37% of the hepta-CBs, while also slightly degrading some of the octa-CBs (Fig. 2, Table 2). The toxicological and persistent properties of PCB congeners are dependent on the number of chlorines and their substitution positions. Thus, the transformation profile for five ortho- and six *para*-substituted PCB congeners, when using LY402, was compared with those reported for strong PCB degraders (Table 1). Whereas both LB400T and KBC101 showed a low transformation activity with *para*-substituted congeners, KBC101 and H850 showed a moderate activity with *para*-chlorinated congeners, yet, their abilities to degrade coplanar 3,4,3',4'- or high chlorinated CBs is limited (Table 1). In contrast, strain LY402 not only readily transformed 3,4,3',4'-CB, but also showed an ability to degrade both *ortho*- and *para*-chlorinated congeners in a much wider range of PCB congeners (Table 1). LY402 was able to degrade 97% of 2,3,5,6,2',5'-CB (*ortho*-) and 46% of 2,3,4,5,2',3',5'-CB (*ortho*-), plus 89% of 2,4,5,2',4',5'-CB (*para*-) and 21% of 2,3,4,6,2',3',4'-CB (*para*-). In addition, 73% of 3,4,3',4'-CB was also degraded (Tables 1 and 2). All the results indicated that LY402 had a stronger PCB transformation activity. Therefore, the degradation rates for strain LY402 were faster than those for all the other previously reported strains [1–6, 9, 10, 13, 17–20].

Identifying the CBAs indicates which positions and biphenyl rings were first subjected to the degradation enzymes. From the metabolite analysis of the degradation of 2,2'-CB and 2,3-CB, 2-CBA and 2,3-CBA were generated. This finding indicates that this particular strain was not different from many other well-known strains on degradation of PCBs [8]. In addition, since the levels of 2-CBA and 2,3-CBA were lower than the amount of the corresponding CBs (Fig. 3), it was postulated that strain LY402 was capable of further CBAs degradation. Thus, it would seem that strain LY402 is capable of degrading certain metabolites and continuing to use such metabolites as substrates for biological activity. According to the published data, the aerobic oxidative destruction of PCBs involves two clusters of genes that are responsible for the transformation of PCB congeners into CBAs and the degradation of CBAs, respectively. Thus, since strain LY402 was shown to be capable of transforming many PCB congeners and 2-CBA and 2,3-CBA (Figs. 2 and 3), this would seem to indicate the involvement of these two gene clusters in strain LY402 thereby allowing more high-efficiency and wider range of PCB congeners degradation. Previous studies have also shown more efficient degradation of monochlorinated biphenyls by bacterial consortia and the presence of *Pseudomonas cepacia* is capable of degrading CBA produced by PCB-degrading strains [7]. Thus, further research about genes of LB400 to degrade both PCB and CBA is needed.

In conclusion, this is the first report of the degradation of highly chlorinated- and coplanar-PCBs by a genus of *Enterobacter* under aerobic conditions. Strain LY402 was also found to be effective in degrading complicated PCB mixtures including a wide range of congeners from Aroclors 1,242, 1,254, and 1,260, plus the most toxic coplanar PCBs. Therefore, strain LY402 offers great potential as regards removing PCB contaminants from the environment.

Acknowledgment

This work was supported by the National High Technology Research and Development Program (863 program, 20375006) from the Ministry of Science and Technology of the P. R. China.

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