REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5/1/96 - 9/31/97	FINAL REPORT

4. TITLE AND SUBTITLE

Tuning Biphenyl Dioxygenase for extended Substrate Specificity and Enhanced Activity

5. FUNDING NUMBERS

N00014-96-10599

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5660

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

At the time when public concern about PCBs contamination in the environment is increasing, there is also an increasing interest in the development of novel biodegradation processes that can rapidly and effectively remove PCBs. However, some of the PCB congeners are known to be very resistant to aerobic biodegradation, particularly in the initial attack by the biphenyl dioxygenases. We seek to broaden the substrate specificity of biphenyl dioxygenase from Pseudomonas sp. LB 400, capable of degrading a broad spectrum of PCBs, was used as the starting template for these experiements. In the initial round of DNA shuffling between the bphA genes of Peudomonas LB400 and KF707, we were able to generate various chimeric biphenyl dioxygenases with extended specificity towards both 2,2',5 and 4,4' congeners. One clone in particular, has equal activity towards both congeners. Resulting analysis has revealed that random shuffling of sequences between the two genes as well as point mutation have occurred. These novel enzymes contained point mutations in regions common to LB400 and KF707 and cannot be predicted a priori. Additional results from subsequent rounds of DNA shuffling will be discussed.

14. SUBJECT TERMS	15. NUMBER OF PAGES
DNA Shuffling, PCB, aerobic degradation	4
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
U	U	U	UL

FINAL REPORT

GRANT#: N00014-96-10599

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GRANT TITLE: Tuning Biphenyl Dioxygenase for Extended Substrate Specificity and Enhanced Activity.

AWARD PERIOD: 1 May 1996 - 31 September 1997

OBJECTIVE: To generate a broad set of biphenyl dioxygenases with broadened substrate specificity and enhanced catalytic rate. We seek to apply sequential cycles of random mutagenesis with screening to identify possible structurally altered biphenyl dioxygenases with extended substrate specificity. In parallel, several chimeric dioxygenases will be created in order to explore and select for novel catalytic activity.

APPROACH: Highly chlorinated PCB congeners are known to be resistant to aerobic biodegradation, particularly the initial attack by the biphenyl dioxygenase. In addition, even minor differences in the DNA sequence affect the substrate range of the enzyme. In order to explore the sequence flexibility and to screen for novel dioxygenase with extended substrate specificity, random mutagenesis of the *bphA* gene coding for the large subunit of biphenyl dioxygenase from *Pseudomonas* LB400 will be carried out by error prone PCR. The mutant *bphA* fragments will be cloned into a low copy number vector and transformation into *E. coli* already carrying the *bphEFGBC* genes. Selection of clones with extended specificity towards different PCB congeners will be carried by spraying the plates with an etheral solution containing 5% of a particular PCB congener to be screened, and incubated until the formation of the yellow meta cleavage products conferred by the BphAEFGBC enzymes. This approach enables us to screen a large number of clones for extended specificity towards a large class of congeners. Promising clones from the first round of mutagenesis will be combined and further mutagenized by DNA shuffling. This approach enables us to carrying out recombination of positive clones and is much more flexible in searching the sequence space. In parallel, chimeric biphenyl dioxygenases will be generated by combining the *bhpA* gene from *Pseudomonas* LB400 with the that from *Pseudomonas* KF707 and KF715.

ACCOMPLISHMENTS: We developed a plate assay for selecting biphenyl dioxygenases with different congener specificities.

This assay was based on monitoring the formation of yellow meta cleavage products from biphenyl or PCBs. Initially, *E. coli* transformed with pCEC11 (plasmid pUC18 with *bphEFGBC* on an *EcoR*1-*Sac*1 fragment) and plasmid pCA31(plasmid pK194 harboring the chromosomal *Nsi*1-*EcoR*1 fragment of *bphA* gene) were sprayed with biphenyl and was shown to form yellow color on plate. Subsequently, yellow color formation was also detected towards 2,3-dichloro-biphenyl and to a lesser extend towards 2,2',5-trichloro-biphenyl, thus verifying the suitability of the screening assay.

Extensive work was invested to improve the cloning efficiency of the *bphA* gene derived from PCR. Primers have been designed flanking the multicloning site of the plasmid pCA31. Error prone PCR was carried using pCA31 as the template DNA. Mutation rates ranging from 0.4% to 2% were selected depending on the ratio between dATP/dGTP and the presence of manganese. The PCR fragments were purified, restricted with *Hind*III and *EcoR*1 and ligated into pK194 previously cut with the same enzymes, and transformed into *E. coli* previously transformed with pCEC11 to establish a mutant *bphA* gene library. Transformants were plated on LB-agar containing ampicillin and kanamycin as selection markers and incubated overnight at 37°C. About 75 % of these transformants turned yellow after treatment with biphenyl due to the formation of the yellow meta cleavage product. Approximately 1,000 transformants with the *bphA* insert was obtained per week.

We have also shown that the *bphA* fragment from Pseudomonas KF707 can be used to complement the *bphEFGBC* genes from LB400. The resulting *E. coli* carrying these genes turned yellow when treated with biphenyl. Moreover, yellow meta products were also detected with 4,4'-dichloro-biphenyl, a congener not degraded by LB400. The initial screening of mutant *bphA* fragment from LB400 will be carried out with 4,4'-dichloro-biphenyl.

In the intitial round of error-prone PCR, approximately 10,000 clones were screened and only one clone showed improved activity towards 4,4' congener. In contrast, with DNA shuffling between the *bphA* genes of *Peudomonas* LB400, KF707 and KF715, we were able to generate various chimeric biphenyl dixoygenases with extended specificity towards both 2,2',5 and 4,4' congeners (Fig. 1). Most remarkedly is clone DS13.9K which metabolized the two PCBs equally well based on the photometric measurement. To understand the molecular basis for the extended substrate range, the *bphA* DNA for clones # DS2.5K, DS8.5K, DS9.5K, DS10.2K, and DS13.9K was sequenced. For the first four clones only a region critical for substrate specificity was sequenced, for clone DS13.9K the entire coding region of *bphA* was sequenced. Though elevated activity was observed for DS2.5K, DS9.5K, and DS10.2K toward 2,2',5-trichlorobiphenyl, DNA sequence comparison showed that region III and region IV were similar

with the sequence of *bphA* from *Pseudomonas pseudoalcaligenes* KF707. Random shuffling of sequences as well as point mutations have occurred. Some of the point mutations occurred in regions common to LB400, KF707, and KF715, and cannot be predicted *a priori* from sequence information alone. These data suggest that amino acid residues outside of region III and IV are also involved in substrate recognition. Due to high homology between the DNA sequences of the two parental genes the extend of DNA shuffling for the entire *bphA* gene from clone DS13.9K is difficult to estimate. However, seven point mutations were observed of which three led to amino acid substitutions. Most remarkably is the shuffling of region III which is homologous to the KF707 DNA, whereas region IV is homologous to LB400 DNA. Thus this mutant resembles the mutant BDE335-5 which was produced by site directed mutagenesis and also showed activity towards both 4,4'-dichlorobiphenyl and 2,2',5 trichlorobiphenyl. We are currently testing the mutants we have obtained so far for their ability to degrade higher chlorinated PCBs using gaschromatography. Furthermore, the mutated *bphA* genes can be used as parental sequences in another round of DNA shuffling to further relax the substrate spectrum of the various mutant biphenyl dioxygenases.

CONCLUSIONS: During the grant period, we have successfully demonstrated the usefulness of directed evolution towards improving aerobic PCB degradation. With the ease of the screening procedure developed, we anticipated that novel and flexible biphenyl dioxygenases with relaxed congener specificity can be created in subsequent cycles of mutagenesis.

SIGNIFICANCE: At the time when the public concern about PCBs contamination in the environment is increasing, there is also an increasing interest in developing novel biodegradadtion processes that can rapidly and effectively remove PCBs. Although many aerobic microorganisms are capable of degrading PCBs, many PCBs are resistant to the initial attack by the biphenyl dioxygenase. We have developed a directed evolution approach to fine tune the biphenyl dioxygenase not only to extend substrate specific but also to understand the structure-activity relationship of this enzyme. This information will in the future enable more rational approaches to design the biphenyl dioxygenase.

AWARD INFORMATION: Dr. Wilfred Chen received the 1997 NSF Career Award.

PUBLICATIONS AND ABSTRACTS:

1. Fredi Bruhlmann and Wilfred Chen. 1997. Tuning Biphenyl Dioxygenase For Enhanced PCB Degradation. Abstr. Amer. Instit. of Chem. Engineers 1997 National Meeting.

2. Fredi Bruhlmann and Wilfred Chen, Tuning Biphenyl Dioxygenase for Enhanced PCB Degradation. Manuscript in preparation.

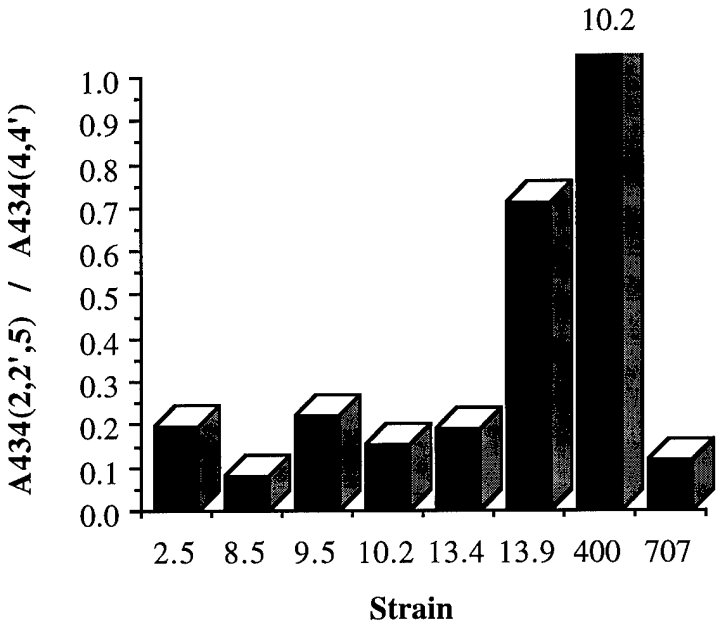

Fig. 1 Substrate specificity between the various mutant biphenyl dioxygenases and wild type biphenyl dioxygenases changed as seen by different activity ratios toward 2,2',5-trichlorobiphenyl and 4,4'-dichlorobiphenyl. Formation of the yellow meta cleavage product was measured spectrophotometrically at 434 using the cell-free culture supernatant of an overnight culture incubated with 0.5 mM of each PCB congener.