DOE/PC/89903--T7

MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL DESULFURIZATION
Contract # DE-AC22-89PC89903

Cloning and Expression of the Sulfoxide/Sulfone/Sulfonate/Sulfate
Genes in *Pseudomonads* and *Thiobacillae*

Tenth Quarterly Report
Due: January 20, 1992
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The original conception of the work was that genetic determinants of the sulfoxide/sulfone/sulfonate/sulfate ("4S") pathway in *Pseudomonas* spp. would be cloned *in vivo* and then transferred to *Thiobacillus* spp. This ambition remains an appealing prospect; however, fulfilling that ambition has been confounded by an instability observed in the Dbts⁺ phenotype in *Pseudomonas* spp. But the persisting interest in the phenotype has lead to isolation of fresh strains which have a Dbts⁺ phenotype. One strain in particular, N1-36, has been the focus of extensive characterizations in long-term cultures. During the present quarter, seven cultures maintained in a "fermentor" for a week or longer have been run to determine rate and extent of growth, extent of conversion of dibenzothiophene (DBT) or dibenzosulfone (DBTO₂) to monohydroxybiphenyl (OH-BP), effect of pH maintained at 6.0, and the effect of adding glucose to cultures in which the amount of glucose had been diminished by bacterial consumption.

In addition, a study of the effectiveness of using R68.45 as a vehicle for *in vivo* cloning of genes was completed this semester. The data demonstrate that the physical organization of an R prime (derived from R68.45) is altered as it is passed intergenerically.

Introduction of Dbts⁺ determinants into *Thiobacillus* spp. continues to be an important goal. Accordingly, a characterization of the genomic organization of two species, *T. novellus* and *T. acidophilus*, has been initiated. Conditions for digestion with rare cutting restriction endonucleases and pulsed-field gel electrophoresis of the products have been determined.

Finally, during this period--and in anticipation of the conclusion of the project--goals for the remaining period of the contract were evaluated. A new series of milestones has been established.

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Production of monohydroxybiphenyl (OH-BP) by N1-36

N1-36 is a gram positive short rod which has been presumptively identified as a *Rhodococcus* spp. The ability of the organism to generate OH-BP from DBT has been confirmed by a colorimetric assay, by HPLC, and by GC-mass spectroscopy.

During the past quarter, N1-36 has been grown repeatedly in New Brunswick Scientific Bio-Flo III or C-30 "fermentors". These "fed batch" cultures were maintained in the fermentors from 180 to 210 hours. 100 μM DBTO₂ or 100 μM DBT served as the sole known source of sulfur. A variety of parameters were monitored including: optical density at 660 nm, number of colony forming units per ml, pH, concentrations of dissolved oxygen, glucose, DBT or DBTO₂, and OH-BP, and intensity of fluorescence.

The following observations are relevant:

- Using a 5 % inoculum, stationary phase was typically achieved in approximately forty hours.
- When DBTO₂ was the substrate, the yield of OH-BP between 40 and 100 hours hovered around 60-65 μM ; the yield then declined to approximately 50 μM . When DBT was the substrate, the yield of OH-BP peaked at 40 to 50 hours at a concentration of 40 μM and then gradually declined over the next 140 hours to approximately 30 μM .

Previous batch cultures had indicated that pH 6.0 was effective for the production of OH-BP. Hence, in later fed batch cultures, the pH was maintained at 6.0. A further change was the addition of 0.3% glucose at 26 to 27 hours, a time corresponding to late exponential phase. (The glucose was added to determine if the presence of substrate would offset the decline in OH-BP which was observed in the latter half of incubation. At the time of the experiments [December 1991], some observations suggested that OH-BP may function as a substrate after exhaustion of glucose. Data obtained in the present quarter do not support this interpretation.)

Representative data appear on page 3. The upper panel presents data from fed batch culture 5; the data show the rapid consumption of DBTO₂, the appearance of OH-BP, and the subsequent decline in OH-BP concentration which occurs during stationary phase. The lower panel (fed batch culture 6) shows the consumption during stationary phase of the 0.3% glucose which was added at 26 hr. (Concentrations of OH-BP were not measured until January 1992; hence, they will be presented in the next quarterly report. The data will show that the concentration of OH-BP declines in the presence of glucose.) The culture depicted in the lower figure had 100 μM DBTO₂ as the sole known sulfur source; the approximate number of CFU ml⁻¹ was 1×10^{10} . When 100 μM DBT served as the sole known sulfur source (fed batch culture 7), the approximate number of CFU ml⁻¹ was 7×10^9 .

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Bacth-5 (Fed Batch)

N1-36

N1-36

Batch-6

N1-36 was streaked on agar surfaces of plates containing (i) no carbon source, (ii) OH-BP or glucose or ethanol as sole carbon source, (iii) ethanol with each of the other carbon sources, and (iv) all three compounds together. No growth was seen with no carbon source or OH-BP as carbon source. Glucose supported growth after four days. Ethanol alone or with either or both of the other carbon sources supported visible growth after two days.

The data regarding N1-36 are observations obtained by Ms. Ping Wang, a Ph. D. student in the Department of Molecular Biology at Lehigh University.

R68.45 as a vehicle for *in vivo* cloning

R68.45 is a broad-host-range plasmid which has the capacity to form primes, that is plasmids which have acquired additional genetic information from the chromosome of a species in which it resides or from other plasmids. This capacity is the basis of *in vivo* cloning. *In vivo* cloning remains a very important prospect because the process is "natural" and, hence, not subject to some of the (social and political) constraints imposed on genetic organizations achieved by *in vitro* cloning.

An aspect of the contracted research is movement of stable genetic determinants for the Dbts⁺ phenotype in *Pseudomonas* spp. to a *Thiobacillus* spp. Because the Dbts⁺ phenotype is demonstrably unstable, a "model system" has been substituted for analysis of the efficacy of using R primes to transfer genes interspecifically and intergenerically. The "model" uses R primes which carry genes which can compensate for methionine auxotrophy. More specifically, *P. putida* PRS 2003 was used as a source of R primes which upon interspecific conjugal transfer converted Met⁻ mutants of *P. aeruginosa* 27853 to Met⁺. Subsequently, intergeneric crosses were used to transfer these R primes from *P. aeruginosa* to *E. coli* B and then back to *P. aeruginosa*.

Three independent matings between *P. putida* PRS 2003 and Met⁻ strains of *P. aeruginosa* were used to generate R-*met* primes. The primes were genetically stable under non-selective conditions; physical characterization by gel electrophoresis showed the three primes selected for characterization, pPM4, pPM15, and pPM21, to be slightly larger than R68.45. Genetic data from such crosses indicate that R-*met* primes form with a frequency of 10⁻⁴ to 10⁻⁷ primes per plasmid transfer.

pPM4, pPM15, and pPM21 were transferred from *P. aeruginosa* 27853 to *E. coli* B and then back to *P. aeruginosa* 27853. In the latter cross, the frequencies with which a marker characteristic of R68.45, resistance to carbenicillin [Cb^r], and a chromosomal marker acquired by the R-prime [met⁺] were monitored. The data are summarized in the table on the following page:

<u>R-prime</u>	<u>Frequency of</u> <u>Cb^r transfer</u>	<u>Frequency of</u> <u>met⁺ transfer</u>	<u>plasmid marker and</u> <u>chromosomal marker</u>
pPM4	1.6×10^{-3}	1.1×10^{-5}	1.5×10^2
pPM15	2.3×10^{-4}	6.6×10^{-7}	3.5×10^2
pPM21	7.0×10^{-5}	2.3×10^{-7}	3.0×10^2

pPM4, pPM15, and pPM21 were also characterized physically by agarose gel electrophoresis. Initially, the plasmids isolated from *E. coli* B appeared to have the same size as the R-met primes isolated from *P. aeruginosa* 27853 (see, respectively, left-hand and right-hand panels in figure on following page). But after subculturing the *E. coli* strains, the plasmid appeared to become smaller (see figure on page 7). Digestion of R68.45 with restriction endonucleases *Hind*III and *Sal*I produces three bands of approximately 30 kb, 19 kb and 6.5 kb. Digestion of the smaller plasmids from *E. coli* B with these enzymes yields the two larger fragments and a third fragment which is smaller than 5 kb (data not shown). Notably, the 6.5 kb fragment contains a tandem duplication of an insertion sequence (IS21); this duplication has been proposed to be a functional site for formation of R-primes.

The data regarding R68.45 and R-met primes are observations obtained by Ms. Patricia Ann McQueney.

Pulsed-field gel electrophoresis of chromosomes from *Thiobacillus* spp.

Preliminary data indicate that the chromosome of *T. novellus* 8093 can be cut with *Spe*I and *Dpn*I and possibly *Dra*I. The chromosome of *T. acidophilus* 27807 can be cut with *Spe*I and *Xba*I. The conditions presently favored are a 20 cm gel run at a 130 volt potential for 52 hr with a ramp of 5 sec $\rightarrow$ 120 sec. Under these conditions, lambda ladders through 460 kb can be resolved as well as all but the largest of the *Saccharomyces cerevisiae* chromosomes. Further, patterns similar to if not identical with published results are obtained when the *P. aeruginosa* PAO chromosome is digested with *Dpn*I and *Spe*I; however, there is a noticeable level of material in the lanes which binds ethidium bromide nonspecifically. Adenovirus is being used as an additional standard. This DNA has been successfully cut with *Sma*I, *Ssp*I, *Pst*I, *Sfi*I, *Nhe*I, and *Spe*I. Success was not achieved with the following enzymes: *Dpn*I (presumably because methylation of the DNA is required), *Not*I (an insufficient number of fragments was observed), *Pvu*I (the two largest bands were missing), *Dra*I (failed to cut for no known reason), *Xba*I (produced an insufficient number of bands), and *Sal*I (failed to give complete digestion). Methylation of Ad2 DNA has been achieved. The effect of the methylation is to block *Sal*I sites.

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Prime plasmids isolated from *P. aeruginosa* 27853-11s transconjugants. Electrophoretic separation of plasmid DNA isolated from R-net containing *P. aeruginosa* transconjugants. (lane 1 - λ HindIII, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-11s pPM4, lane 4 - *P. aeruginosa* 27853-11s pPM7, lane 5 *P. aeruginosa* 27853-11s).

E. coli B transconjugant containing the plasmid pPM21. Plasmid DNA isolated from an *E. coli* B transconjugant following mating with *P. aeruginosa* 27853-1s pPM21. (lane 1 - λ HindIII, lane 2 - *P. aeruginosa* 27853-1s pPM21, lane 3 - *E. coli* B transconjugant, lane 4 - *E. coli* B).

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Plasmids isolated from the *E. coli* B transconjugants after storage in the absence of selection as compared to the plasmid isolated from the *P. aeruginosa* met⁺ transconjugants. Isolated plasmids were electrophoresed through a 1% agarose gel. (lane 1 - λ HindIII, lane 2 - *P. aeruginosa* 11s pPM4, lane 3 - *E. coli* B pPM4, lane 4 *P. aeruginosa* 1s pPM15, lane 5 - *E. coli* B pPM15, lane 6 *P. aeruginosa* 1s pPM21, lane 7 - *E. coli* B pPM21).

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Plasmids from *T. acidophilus* 27807 have been isolated, purified on CsCl gradients, and electrophoresed through agarose. The plasmids were larger than R68.45 which is approximately 60 kb. The *T. acidophilus* plasmids were digested with *Spe*I and *Xba*I and electrophoresed. Bands in a range from ≈ 18 kb to ≈ 35 kb were observed. These data will be important when attempting to discern which components from a genomic digest come from chromosomes and which come from plasmids.

The data on pulsed-field gel electrophoresis were generated by Ms. Diane Dutt, a technician and graduate student in the Department of Molecular Biology at Lehigh. (Photographs of recently-prepared, aesthetically-pleasing gels will be included in the next quarterly report.)

Miscellaneous

An abstract entitled, Analyses of Monohydroxyl Biphenyl Production from Dibenzothiophene by New Desulfurizing Bacteria, was submitted to the "general meeting" of the American Society for Microbiology.

In consultation with Mr. William P. Barnett, new timelines were established. They appear (with comments) on the following page.

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MILESTONE SCHEDULE/STATUS REPORT

Selection and preliminary characterization of fresh *Pseudomonas*
isolates which stably express the DbtS⁺ phenotype:

28-----29-----30-----31-----32-----33-----34-----35-----36

Little progress; this task will be emphasized during the next five months.

Modeling R' formation and transfer in *Pseudomonas* spp.:

28-----29-----30-----31-----32-----33

Efforts with this task are nearly complete.

Kinetics and site of monohydroxybiphenyl production by N1-36:

28-----29-----30-----31-----32-----33-----34-----35-----36

Excellent progress has occurred with this task.

Genus and species identification of N1-36 and other gram positive
isolates:

29-----30-----31-----32

Programs have been acquired but have not been functional. The problems prom-
ise to be minor and good progress is anticipated.

Pulsed-field gel electrophoresis of *Thiobacillus* spp. chromo-
somes:

28-----29-----30-----31-----32-----33-----34-----35-----36

Effective conditions for digestion and pulsed-field gel electrophoresis have
been achieved. All indications are that this work, too, will be a success.

Preparation of final report:

34-----35-----36

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COST MANAGEMENT REPORT

DATE RUN 12/31/91
TIME RUN 22:12:01
PROGRAM ID FBH099
ACCOUNT PAGE 1

REPORT PAGE 58
PROGRAM ID FBH099
ACCOUNT PAGE 1

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RESEARCH ACCOUNT SUMMARY IN WHOLE DOLLARS AS OF 12/31/91

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EXP. 06/29/92
ACT: 5-35074
DEPT: MOLECULAR BIOLOGY

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AGENCY - DOE
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BALANCE BEFORE
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DESCRIPTION	PROJ-TO-DATE BUDGET	CURRENT MONTH EXPENDITURES	PROJ-TO-DATE EXPENDITURES	BALANCE BEFORE COMMITMENTS	OPEN COMMITMENTS	BUDGET AVAILABLE
FACULTY - SUMMER	50,197		50,197	58		58
SUPPORT STAFF	39,035	1,318	36,168	2,867	7,910	5,043
RES ASST STIPEND	28,320	900	22,500	5,820	5,400	420
OTHER	1,520		1,200	320		320
* TOTAL SALARY/WAGE	119,072	2,218	110,007	9,065	13,310	4,245
E & B REIMB-SPONS RES	28,335	396	26,621	1,714	1,714	
** TOTAL COMPENSATION	147,407	2,614	136,628	10,779	15,024	4,245
SUPPLIES	27,835	912	28,812	977		977
COMMUNICATION/POSTAGE	765	9	457	308		308
DOMESTIC TRAVEL	7,068		6,602	466		466
PUBLICATION COSTS	2,430	116	409	2,021		2,021
EQUIPMENT (1)	27,160		11,726	15,434		15,434
OUTSIDE SERVICES			235	235		235
EQUIPMENT USAGE			130	130		130
OTHER	4,930		2,176	2,754		2,754
* TOT OTHER DIRECT COST	70,188	1,038	50,566	19,642		19,642
*** TOTAL DIRECT COSTS	217,595	3,452	187,174	30,421	15,024	15,397
INDIRECT COSTS	116,167	2,191	106,743	9,424	9,424	
**** GRAND TOTAL	333,762	5,643	293,917	39,845	24,448	15,397

(1) - NOT SUBJECT TO INDIRECT COST.

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