MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL DESULFURIZATION
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Cloning and Expression of the Sulfoxide/Sulfone/Sulfonate/Sulfate
Genes in *Pseudomonads* and *Thiobacillae*

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I. INTRODUCTION

This department of energy-sponsored mission-oriented research project currently has three emphases. They are:

- (i) determining the kinetics of monohydroxybiphenyl (OH-BP) production in batch culture by N1-36, an unidentified soil isolate, when the organism is presented with dibenzothiophene (DBT) or dibenzosulfone (DBTO₂);
- (ii) establishing reliable methods for physically characterizing R68.45, a broad host range plasmid;
- (iii) attempting to elicit a physiologically consistent and genetically stable ability of some gram negative soil isolates to convert DBT to o,o'-biphenol.

Each of these goals has subsidiary components. For example, in addition to establishing kinetics of formation of OH-BP by N1-36, analyses have been or will be performed to determine whether the catalytic activity can be achieved with irradiated cells, spheroplasts, and cell extracts. This report presents information on progress towards fulfilling both the principal goals listed above and some relevant ancillary activities.

II. PRODUCTION OF OH-BP BY N1-36

a. Detection of substrates and products

1. Mass spectrometry

A ²⁵²Cf mass spectrometer was used to establish that isolates N1-36, N1-43, and Q1a-22 produced OH-BP from both DBT and DBTO₂. All samples had a peak at 170. Pure OH-BP was used as a positive standard. N1-36 grown with MgSO₄ as sulfur source did not produce a peak attributable to OH-BP.

2. Fluorescence

The presence of DBT, DBTO₂, and OH-BP may be quantitatively monitored through fluorescence. In preparation for such analyses, the emission spectra of these compounds was measured when they were excited with 254 nm and 289 nm light from a 200 W Hg lamp. The data are summarized below:

LIGHT SOURCE	DBT (334-364 nm)	DBTO ₂ (404-435 nm)	OH-BP (422-432 nm)
-----area under peak in emission profile-----			
Ex: 254 nm	4063	137,568	137,895
Ex: 289 nm	8713	206,879	122,017

These data indicate that a simple spectral distinction between DBTO₂ and OH-BP is not possible. However, simultaneous equations may allow adequate discrimination.

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3. UV absorption

The spectrum of UV absorbance by OH-BP was measured. Subsequently, identical spectra were obtained from (i) uninoculated media containing OH-BP or (ii) media containing DBTO₂ which had supported growth of N1-36. The identity suggests that UV absorbance is yet another way to monitor the production of OH-BP. This technique substantiates earlier observations with fluorescence, colorimetry, HPLC, and GC/mass spectroscopy.

B. Kinetic analysis of OH-BP production by N1-36

One of the principal goals during the final year of contractual research is kinetic analysis of OH-BP production by the gram positive isolate, N1-36. Accordingly, this yet-to-be identified isolate was grown in batch culture in a New Brunswick Scientific BioFlo III "fermentor" equipped with dissolved oxygen and pH probes. The sole source of sulfur was 100 μ M DBTO₂. The data collected are depicted in Figure 1 on page 9. Several aspects of the data merit note. A high yield of cells was achieved. OH-BP production lagged behind growth allowing, once again, the interpretation that OH-BP production may be related to phase of growth. The abundance of OH-BP was stable during stationary phase indicating that, under these conditions, OH-BP was not consumed. Further observations suggested that OH-BP may be consumed by N1-36 when the concentration of glucose, the principal carbon source, declines (see below). The yield of OH-BP was estimated to be approximately 60 μ M in one experiment and 72 μ M in another; thus, the extent of DBTO₂ utilization is high. The pH of the medium dropped during the growth of the bacteria, a condition commonly seen with batch cultures; the decline in pH did not interfere with the production or continued presence of OH-BP. The addition of anti-foam correlated with a change in the measured amount of dissolved O₂; whether the anti-foam affected the accessibility of O₂ to the probe or affected the probe itself is not known. Monitoring of the culture was suspended when contaminating organisms were detected.

C. Consumption of OH-BP in shake flask cultures

In shake flasks, OH-BP appears to be used as a carbon source. The consumption of OH-BP can be retarded by inclusion of higher concentrations of glucose in the growth medium; hence, OH-BP appears to be used after consumption of glucose.

D. Method of delivering sulfur source affects reaction

The role of EtOH as substrate, solvent, or membrane permeabilizer is being assessed. The following three observations merit note:

- The presence of EtOH allows N1-36 to respond to SO₄⁼ more rapidly than if SO₄⁼ is introduced (i) in the absence of EtOH, (ii) on a nylon disk, or (iii) in acetonitrile.
- DBT as sulfur source more effectively supports growth of N1-36 if the DBT is introduced in EtOH rather than addition in a powder or enmeshed in nylon filters.
- The presence of EtOH allows N1-36 to respond to DBTO₂ more rapidly than if DBTO₂ is introduced as a powder.

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E. Dependence of organic sulfur oxidation on the viability of N1-36

An additional aspect of the kinetic analysis is to determine whether the N1-36 cells need be alive for the oxidation of DBTO₂ to OH-BP. For this analysis, UV-irradiated cells will be used. The viability of the cells, the physiological capacity to exhibit some standard metabolic activity (as yet not determined), and the ability to oxidize DBTO₂ will be compared. Accordingly, a UV killing curve was constructed; the data are presented in Figure 2 on page 10. The striking "shoulder" is notable and is reminiscent of that seen with *Deinococcus radiodurans*. The level of energy used in the irradiation is being calculated.

F. Site of oxidation of organic sulfur

Yet another aspect of the oxidation of DBTO₂ to OH-BP which is being investigated is whether the activity is associated with the cells *per se* or whether the activity occurs in the medium in which cells were grown. Aliquots of cultures containing cells or aliquots of the culture medium (from which the cells had been removed by centrifugation or filtration) were added to buffer containing DBTO₂. The presence of DBTO₂ and OH-BP were monitored by HPLC. The data indicated that the cell free samples showed no increased amounts of OH-BP though the level of DBTO₂ may have decreased. Samples of cultures including cells did show both decreased amounts of DBTO₂ and increased amounts of OH-BP. Furthermore, a 44 hour-old culture had more activity than did a 27 hour-old culture. This preliminary experiment was conducted, in part, to determine optimal times for analysis and is being repeated to obtain quantitative data.

G. Relevant miscellaneous observation

Dissolution of 1 mg dibenzosulfone per ml of glycerol was achieved by heating the glycerol moderately. The sulfone did not subsequently precipitate. This observation may be important for sulfone-limited growth of N1-36 in a chemostat.

III. R68.45: A VEHICLE FOR MOVING GENES

In the original conception of the contracted research, *in vivo* cloning was envisioned as a means of moving the genetic determinants for the Dbts⁺ phenotype from soil isolates to a variety of recipients including *Pseudomonas* spp. and *Thiobacillus* spp. The vehicle designated for such intra- and interspecific transmission is R68.45. But, as has been noted in numerous monthly reports, R68.45 is a difficult plasmid with which to work. Yields tend to be very low and the isolated plasmid often degrades rapidly during storage. Further, the plasmid is large and, thus, does not readily enter agarose gels used in electrophoretic analysis. Despite these difficulties, one of the students working on the project, Ms. Patricia A. McQueney, now routinely performs the required matings and reliably isolates the plasmid. The combination of genetic and physical evidence is important: interpretations based on genetic traits can be substantiated through physical characterizations.

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A. Pulsed-field gel electrophoresis of R68.45 and restriction fragments produced from R68.45

The principal source of difficulty when working with R68.45 is its size. The plasmid is approximately 60 kb and products of restriction endonuclease digestion of this large plasmid are themselves large. The large intact plasmid or the large restriction fragments resolve poorly by conventional agarose gel electrophoresis. However, the problem can be surmounted by use of pulsed-field gel electrophoresis. The conditions which are now used are: 1% agarose gel, 100 volt potential, 1-10 second ramp, 18 hr run. Digestions have been achieved with the following enzymes: *HindIII*, *EcoRI*, *SalI*. Double digestions have been performed with all possible pairs of enzymes. Figure 3 on page 11 presents some representative results. The contents of the lanes are as follows:

lane A:	lambda digested with <i>HindIII</i>
lane B:	undigested lambda; upper band 90 kb, lower band 45 kb
lane C:	5 kb ladder
lane D:	R68.45 linearized with <i>HindIII</i> ; 60 kb
lane E:	R68.45 linearized with <i>EcoRI</i> ; 60 kb
lane F:	R68.45 digested with <i>SalI</i> ; upper band 38 kb, lower band 19 kb
lane G:	R68.45 digested with <i>EcoRI</i> and <i>SalI</i> ; upper band 25 kb, mid-band 19 kb, lower band 14 kb
lane H:	R68.45 digested with <i>HindIII</i> and <i>SalI</i> ; upper band 30 kb, mid-band 19 kb, lower band 6-7 kb
lane I:	R68.45 digested with <i>EcoRI</i> and <i>HindIII</i> ; upper band 35-40 kb, lower band 20 kb

B. Matings

For our analyses, R68.45 has been moved among the following organisms: *Pseudomonas putida* PRS 2003 and 12633, *P. aeruginosa* 27853 and several derived forms of this strain, *Escherichia coli* HB101, and *E. coli* B WU 3+10.

1. Genetic evidence for the formation of R primes

Mating protocols have been designed to generate R primes which complement methionine auxotrophs of *P. aeruginosa* 27853. The particulars are as follows: *P. putida* was "filter mated" with *P. aeruginosa* 27853-1S. The donor is unable to grow on glucose at 42° C, or benzoate at 32° C, or if kanamycin and carbenicillin are included in the medium. The recipient is unable to grow unless methionine is included in the medium. The species and genus identities of the donor and recipient were confirmed with BioLog plates. Transconjugants were identified by their ability to grow at 42° C on agar plates containing antibiotics, benzoic acid and methionine. The presence of a specific type of R' in transconjugants was recognized by the ability of the transconjugant to grow in the absence of methionine. The frequency of R68.45 transfer (per donor) was estimated to be 1.1×10^{-1} . The frequency of R'met formation and transfer was estimated to be 9.9×10^{-7} .

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2. Genetic stability of R primes

The stabilities of the R primes in *P. aeruginosa* was tested by extensive growth on a non-selective medium and then "stippling" 50 colonies from each isolate onto: (1) plates of nutrient agar (to test for viability), (2) plates of itaconic acid + methionine + antibiotics (to test for the presence of R68.45 in *P. aeruginosa*, and (3) plates of benzoic acid plus antibiotics (to test for the continued presence of R'met. Six of the original R primes appear stable.

C. Physical evidence for the formation of R primes

The evidence presented above is genetic. The interpretation that R primes have been produced needs to be substantiated with physical evidence. Two approaches have been used. One uses "mini-preps" to detect quickly a difference in the size of putative R primes and R68.45. The second is to purify the putative R prime, perform restriction endonuclease digestions, and compare the restriction fragments from the R prime with those from R68.45.

1. Mini-preps

Twenty colonies suspected of containing an R'met were examined by gel electrophoresis. All gave identical patterns; the identity suggests that an R' may have formed early and been transferred vertically and possibly horizontally. The pattern showed a doublet with sizes approximating that of R68.45. Further, there was a smaller band which can be seen when plasmids are isolated from *P. aeruginosa* 27853.1S which has not been mated.

2. Purified primes

Physical characterization of R primes purified in CsCl gradients was attempted, but difficulties with isolation procedures were encountered because the host organism produced copious alginate which interferes with extraction. Accordingly, the plasmid was moved into a more tractable system. The R68.45 in the *P. aeruginosa* 27853.1S transconjugant was passed into *E. coli* B. The plasmid has been isolated from this recipient and purified by CsCl centrifugation. Restriction endonuclease analyses of several putative R primes are now being performed.

D. Significance

Data regarding R prime formation and stabilization of derivatives will be important when the genetic determinants for the Dbts⁺ must be moved from one species to another by "natural processes" rather than through methods of genetic engineering.

IV. CONTINUED CHARACTERIZATION OF FRESH DBTS⁺ SOIL ISOLATES.

Sixteen isolates have been newly obtained from the coal storage sites where earlier strains which had appeared to hold so much promise had been obtained.

A. Partial characterization of the isolates

All of the fresh isolates produce fluorescence when exposed to DBT by the spray plate technique. The isolates are strictly aerobic; none exhibits an ability to function as a facultative anaerobe. Microscopic analysis has shown that all of the isolates are rod-shaped. Fourteen were gram negative while

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two were ambiguous. *Escherichia coli* and *Bacillus subtilis* served, respectively, as the gram negative and gram positive controls.

B. Derivative strains which are hyper-responsive to DBT

These isolates were grown in a liquid glucose medium lacking an identifiable sulfur source; microcolonies were created by placing approximately 6.5×10^5 of these cells on an agar medium containing glucose and no sulfur beyond the sulfur which contaminates agar. After one or two days of incubation, 5 μ l drops of 0.5% DBT in ethyl ether were applied to the microcolonies. After two and three weeks of incubation, the colonies were examined for the presence of papillae, color, and fluorescence when stimulated with UV light. On several colonies papillae were evident and on many colonies patches of growth emerged from the edge of colonies. Some of these areas of growth were brightly colored, some moderately colored, and some were faintly colored. Samples from the areas of growth were streaked onto fresh agar plates lacking exogenous $\text{SO}_4^{=}$. After overnight incubation, the plates were examined with the spray plate technique. A range of colors was seen; some colonies exhibited fluorescent rings. With M9 medium, the relevant result--fluorescence--was confounded by fluorescence which occurred in the minimal medium. Hence, analyses will need to be performed under different growth conditions.

C. Application of physiologic stress to new isolates

One of the sixteen new isolates, V19, and *P. putida* 12633 have been put through a regime in which the organisms grow in a liquid glucose salts medium lacking a known source of sulfur. When stationary phase is achieved, the cells are harvested by centrifugation and then washed three times in saline. The pH of the medium which supported growth is adjusted to neutrality, the supply of glucose is replenished and the medium is then filter sterilized. This medium is then reinoculated with $1/25^{\text{th}}$ of the cells which were harvested during the previous round of growth. The inoculated cultures are incubated and the turbidity is monitored. Two parallel sets of cultures are processed with the sole difference being that, in one, the liquid medium contains $\text{SO}_4^{=}$ and, in the other, DPT is present.

The growth of cells in medium with no sulfur source should deplete the medium of any trace of sulfur with unknown origin. However, after four rounds of transfer, no difference in growth in medium with $\text{SO}_4^{=}$ or without a known sulfur source has been observed for either *P. putida* 12633 or V19. Likewise, no stimulation in growth was noted in flasks containing nylon disks with DBT enmeshed among the fibers.

These experiments continue.

The susceptibility of V19 to dibenzosulfone was measured. The same rates and extents of growth were seen when the concentrations of dibenzosulfone were 0, 10, and 100 μ Molar.

V. MISCELLANEOUS ACTIVITIES

A. Use of Biolog "technology" for identification of isolates and donors, recipients and transconjugants functioning in genetic crosses

Data from more than 30 Biolog determinations have been entered into a database. The data will be used to assess variability in phenotype among the

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organisms we routinely use for genetic analyses. The data will be used to determine if the presence of a broad host range plasmid has an effect on phenotypic stability.

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

The chromosomes of *Thiobacillus novellus* and *Thiobacillus acidophilus* have been digested with rare cutting restriction endonucleases and subjected to pulsed-field gel electrophoresis. Progress is being made in identifying suitable enzymes and electrophoretic conditions.

VI. CONCLUSIONS

Substantial progress has been made in the kinetic analysis of OH-BP production by N1-36 in batch culture. Several sophisticated on-line and off-line methods are available to measure the disappearance of substrates and the appearance of products. In addition, other relevant measures of the culture, e. g., pH, dissolved O₂, and concentration of nutrient, are monitored. These preliminary efforts have been followed by several informative "runs" in which culture variables have been altered to determine their effect on the rate and extent of production of the desired product. Results of these runs (and, no doubt, additional data and interpretations) will be presented in the next quarterly report.

Reliable extraction of R68.45 and R primes and characterization by pulsed-field gel electrophoresis of these entities and fragments produced by digestion with restriction endonucleases have been achieved. Further, a model for the production, transmission, and physical characterization of R primes has been successfully studied. The model relies on the complementation of met⁻ auxotrophs by R primes which have presumably acquired genetic determinants which confer methionine prototrophy.

Recently acquired soil isolates have been physiologically challenged by successive transfers in the absence of an identifiable sulfur source. The organisms have subsequently been exposed to DBT and the response of subpopulations has been noted. Subpopulations which exhibit especially pronounced responses to DBT have become candidates for further characterization.

Progress on the mission-oriented project is good. Valuable data are being acquired with an organism whose identity is not yet known (N1-36) and intriguing observations are being made with organisms whose identity is more readily determined (*Pseudomonas* spp.)

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FIGURE 1

FIGURE 2

N1-36 Viability After UV Radiation

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FIGURE 3

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VII. MILESTONE SCHEDULE/STATUS REPORT

Milestones and schedules are being revised during the tenth quarter.

Budget removed.

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