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

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

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ABSTRACT

The DbtS⁺ phenotype is defined as the selective ability to oxidize the sulfur in dibenzothiophene (DBT) successively to dibenzothiophene-5-oxide, dibenzosulfone, and, finally, either o,o'-biphenol or monohydroxybiphenyl. By using a fluorescent assay, many *Pseudomonas putida* isolates having a DbtS⁺ phenotype have been obtained. The ability of the isolates to generate o,o'-biphenol was confirmed with HPLC shortly after the time of isolation.

The broad-host-range plasmid, R68.45, was introduced from *P. putida* PRS 2003 into many soil isolates. The plasmid was able to mobilize the determinants for the DbtS⁺ phenotype. Accordingly, R68.45 and the determinants of the phenotype could be transferred simultaneously from soil isolates to *P. aeruginosa* 27853.

The DbtS⁺ phenotype in the isolates and in *P. aeruginosa* 27853 has proven to be unstable. Whether the instability is genetic, physiological, some combination of these two, or is founded on some other phenomenon is not known.

Fresh Gram-positive isolates with the DbtS⁺ phenotype have been isolated using the sulfur bioavailability assay. The DbtS⁺ phenotype in these isolates appears to be stable. The product of desulfurization of DBT of dibenzosulfone is monohydroxybiphenyl. The nature of the endproduct has been confirmed by HPLC, colorimetry, GC/mass spectroscopy, and UV absorption. The kinetics of monohydroxybiphenyl production are being studied in batch and continuous culture.

Study of the basis of cloning with R68.45 has continued. Data regarding *in vivo* cloning with R68.45 will be important when the genetic determinants for the DbtS⁺ phenotype must be moved from one species to another by "natural processes" rather than through methods of genetic engineering.

I. INTRODUCTION

The intent of the contracted research is to clone genes conferring the DbtS⁺ phenotype, a phenotype defined as the ability to oxidize selectively the sulfur in dibenzothiophene (DBT) and yield o,o'-biphenol (or monohydroxybiphenyl [OH-BP]). At Lehigh, a presumptive, fluorescent assay for the detection of organisms with the desired phenotype was designed. When applied, the screening regime yielded numerous isolates including a subset which produced o,o'-biphenol. The production of o,o'-biphenol was confirmed by HPLC. Subsequently, a broad-host-range plasmid, R68.45, was introduced into soil isolates exhibiting the DbtS⁺ phenotype. The broad-host-range plasmid was used, in turn, as an *in vivo* cloning vector; that is, the plasmid and the determinants were simultaneously transferred interspecifically to a fully characterized recipient bacterium.

The achievements summarized in the previous paragraph constitute significant accomplishments and were achieved by the conclusion of the first year of contractual research. The expectations for future research were (i) to extend the *in vivo* cloning to genetically marked members of the genus *Pseudomonas*; then (ii) to confirm the *in vivo* results with *in vitro* cloning in species of both the Pseudomonadaceae and the Enterobacteriaceae, and (iii) to return to *in vivo* cloning in order to transfer the determinants for the DbtS⁺ phenotype from soil isolates to members of the Thiobacillaceae.

This schedule, rational in conception, did not conform with a condition observed during experimentation. Inheritance of the acquired trait was unstable; the phenotype was invariably lost by the recipient during subsequent sub-cloning.

The problem with genetic stability was compounded by doubts which arose among other researchers. The conception of a DbtS⁺ phenotype asserts that DBT is oxidized first to dibenzothiophene-5-oxide which, in turn, is oxidized to dibenzosulfone (DBTO₂). The sulfone is then oxidized to either o,o'-biphenol or OH-BP (1). In the course of research, some investigators who initially observed the production of o,o'-biphenol saw the product less frequently; stated another way, the oxidations seemed to cease with the formation of the sulfone. Later yet, production of the sulfone became less demonstrable and formation of the sulfoxide was the fullest extent of oxidation of DBT which could be achieved. The question was then raised whether the formation of the sulfoxide was specific to the "4S" pathway or was merely a "side reaction" of some sort produced from "other" metabolic processes.

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Fortunately, these concerns are directly addressable both experimentally and theoretically. Consider the former first. If isolation techniques had been truly sufficient in the past to isolate organisms that genuinely had a Dbts⁺ phenotype, then reapplication of those techniques should yield Dbts⁺ organisms anew. The current challenge would be to modify procedures so that stability was also achieved. The latter concern reveals that there are at least two forms of instability: genetic and physiological. The occurrence of these two forms of instability constitutes a conundrum. If the phenotype is lost genetically, then its physiological nature cannot be manifest; but if its physiological character cannot be elicited, whether the character is genetically present cannot be known.

The bulk of efforts during the second year of contractual research was spent surmounting some of the obstacles described in the previous paragraphs. This report presents how researchers at Lehigh succeeded in this enterprise.

PERSPECTIVE

The instability of the Dbts⁺ phenotype suggests that it is non-adaptive during serial transfer on complex laboratory media. The obverse of this proposition is that stability would be achieved in growth conditions in which the Dbts⁺ phenotype was adaptive. Growth of Dbts⁺ bacteria in a steady-state culture in which the extent of growth is limited by the effectiveness of the Dbts⁺ phenotype would provide *continuous selection* for the phenotype. Such dependence on the Dbts⁺ phenotype may be achieved in continuous culture with a growth medium which lacks inorganic sulfur and contains a unique and relevant organic sulfur source such as DBT, DBTO₂, or depyritized coal.

Bacteria are demonstrably responsive to adaptive regimes and steady-state growth may serve as an especially powerful setting for adaptation (2, 3). The constancy of the selective environment is, no doubt, important. Moreover, physiological states associated with low nutrient concentration and prolonged generation times may also be contributing factors (4). Recent, well-substantiated, and intriguing observations (5, 6) indicate that stress may both affect the expression of genes and the occurrence of "Cairnsian" mutations in genes whose products are needed in the selective environment. Finally, adaptive regimes based on steady state growth yield bacteria which are ever more fit for their environment; this long-established phenomenon has been designated periodic selection (7).

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RESULTS

Bearing in mind that fresh isolates display the relevant phenotype more robustly and that continuous selection may favor the DbtS^+ phenotype, new isolates were sought which could be studied in continuous culture.

A. New isolates

1. Enrichment

Six mixed cultures (L, M, N, O, P, Q) were selected on the basis of their ability to use DBTO_2 as a sole source of sulfur; specifically, organisms from soil samples were grown in a glucose salts medium, washed, inoculated into glucose salts medium lacking inorganic sulfur but containing DBTO_2 (= 's S-free glucose + DBTO_2), incubated for 2 days, placed in fresh S-free glucose + DBTO_2 medium, and monitored. Samples L, P, and Q grew somewhat better than controls and sample N1 grew much better. Eight samples of the various consortia were diluted 2.44×10^{-8} ; three continued to grow. The three cultures were grown and a total of eight replica cultures were then diluted 1.28×10^{-10} . All grew with DBTO_2 as the sole source of sulfur.

Q1a, Q1b, Q1c, and N1 were grown in glucose medium for 2 days. Inocula from these cultures were introduced into four media: $\text{SO}_4^{=}$ -free medium, $\text{SO}_4^{=}$ -free medium + 0.1 mM DBT, $\text{SO}_4^{=}$ -free medium + 0.1 mM DBTO_2 , and $\text{SO}_4^{=}$ -free medium + $\text{MgSO}_4^{=}$. The extents of growth of Q1a, Q1b, and Q1c, in media containing DBT or DBTO_2 were only slightly greater than in $\text{SO}_4^{=}$ -free medium. Growth in medium with $\text{MgSO}_4^{=}$ was clearly much stronger. The extent of growth of N1 in media containing DBT or DBTO_2 was nearly as great as growth in the presence of $\text{MgSO}_4^{=}$. Growth in $\text{SO}_4^{=}$ -free medium was much weaker.

2. Initial characterization and attempted identification

Three types of colonies dominated the chemostat culture: yellow colonies containing gram negative rods; white translucent colonies containing gram negative cocci or short rods; and small ivory colonies containing gram positive rods.

Only gram positive isolates were judged to be of potential interest. The relevant isolates were designated N1-36, N1-43, and Q1a-22. Additional staining showed that the organisms are nonacid fast and lack endospores. For the former result, *Mycobacterium smegmatis* was used as a positive control and *Escherichia coli* as a negative control; for the latter result, *Bacillus cereus* was used as a positive control and *E. coli* was used as a negative control.

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Identification of subcultures of these organisms was attempted on the basis of coincidence of gas chromatographic profiles of their fatty acids and those of known bacteria. (These analyses were performed by Microbial ID, Inc. of Newark, DL.) For an identification, a score generated by a matching algorithm should ≥ 0.5 . No identification was achieved for N1-43. N1-36 and Q1a-22 had matching indices of 0.218 and 0.286, respectively, with *Rhodococcus rhodochrous*. However, the cell and colony morphologies of the isolates do not correspond with those of *Rhodococcus* spp.

B. Techniques used to demonstrate production of monohydroxybiphenyl

1. HPLC

Samples were taken for HPLC analysis from the last culture inoculated. N1 is estimated to have produced 20-30 μM OH-BP from the 100 μM substrate during two days of incubation. The product generated by N1 from DBTO₂ (panel B in the figure on page 7) has an equivalent retention time as 25 μg of commercially available OH-BP (panel A). (The retention time is 3.46 ± 0.08 min [$N = 6$].) When the product from N1 is "spiked" with OH-BP, the metabolic product and the commercial product comigrate and elute from the column in a symmetrical peak (panel C). Similar results were obtained with DBT (though the peaks were somewhat smaller).

A confounding factor when working with DBTO₂ in an aqueous medium is that it appears to partition into cells. Acetonitrile extraction DBTO₂ from a suspension of cells versus extraction of DBTO₂ from a similar medium from which the cells had been sedimented produces different yields of DBTO₂. Specifically, the former yields greater amounts of the product.

2. Colorimetric analysis

Additional demonstration that the product contained a phenolic component was achieved with a colorimetric reaction. "Phenol was measured by [a] colorimetric method based on the rapid condensation of phenol with 4-aminoantipyrine followed by oxidation with potassium ferricyanide under alkaline conditions to give a red colored product" (21). In particular, 4 ml samples having a concentration of phenol of $\leq 100 \mu\text{g L}^{-1}$ were mixed with 0.1 ml of 0.1 M glycine buffer (pH 9.7, adjusted with NaOH) containing 5.0% K₃Fe(CN)₆ and subsequently mixed with 1 ml of 0.1 M glycine buffer (pH = 9.7) containing 0.25% 4-aminoantipyrine; absorbance was measured at 505 nm. Using this assay, DBT, dibenzothiophene-5-oxide, DBTO₂, and biphenyl generate no color; by contrast, both OH-BP and o,o'-biphenol do yield color with that from the latter compound being greater than that from the former.

OH-BP production in BATCH culture

3. GC/mass spectroscopy

CHCl_3 extraction was shown to be adequate for extracting OH-BP from growth medium. Quantitative analysis with HPLC indicated that 93% of the OH-BP in culture medium was extracted.

N1-43, N1-36, and Q1-22a were inoculated into glucose-salts- $\text{SO}_4^{=}$ -free medium supplemented with 0.1 mM DBT and glucose-salts- $\text{SO}_4^{=}$ -free medium supplemented with 0.1 mM DBTO_2 . Cultures were incubated for 5 days, centrifuged, and the supernatant was extracted with 1/20 volume of CHCl_3 . The extract was stored at -20°C for one day and then analyzed by GC/MS. All samples had a 170 molecular weight peak but had as well components with lower molecular weights, e. g., 115 and 83, which could not be readily identified. (The profiles are presented in a figure on page 9; the upper panel depicts the profile produced from pure monohydroxybiphenyl; the central panel depicts the profile produced when strain N1-43 was supplied with DBT as the sole sulfur source; the bottom panel depicts the profile produced when strain N1-36 was supplied with DBT as the sole sulfur source. The pertinent peak in each panel is highlighted with an arrow.)

4. UV analysis

UV spectroscopy can also be used to analyze the presence of OH-BP in CHCl_3 extracts of cultures in which DBTO_2 served as the sole sulfur source for strain N1-36.^A

C. Kinetics of monohydroxybiphenyl production in batch culture

N1-36, N1-43, and Q1a-22 were inoculated from slants into multiple samples of glucose- $\text{SO}_4^{=}$ -free medium supplemented with DBTO_2 ; the pH's of the various samples of media had been adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0. The growth of Q1a-22 was largely equivalent at the various pH's though there may have been a marginally faster rate of growth at pH 6.5. The rates of growth for N1-36 and N1-43 were also slightly greater at pH 6.5. Notably, for both isolates the extent of growth was markedly reduced at pH 6.0. OH-BP production began in early exponential phase for all cultures. The rate and extent of OH-BP production for both N1-36 and N1-43 were greatest at pH 6.0. The extent of OH-BP production increased with the passage of time suggesting that OH-BP may be an end product of a metabolic process. The final concentrations of OH-BP produced at pH 6.0 from 100 μM DBTO_2 by Q1a-22, N1-36, and N1-43, respectively, were approximately 21, 34, and 38 μM .

A. This observation was made soon after the completion of the second year of contractual research and during the time of preparation of this report. Hence, it along with more detailed description properly belongs in the final report for the project.

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D. Growth in a chemostat

A chemostat inoculated with the "mixed culture" of N1 was grown on $\text{SO}_4^{=}$ -free glucose medium supplemented with 0.1 mM DBTO_2 . Subsequent to heat sterilization, the DBTO_2 was in solution; upon cooling, some precipitate was seen. When the dilution rate was set to 0.1 hr^{-1} (which corresponds to a generation time [G] of approximately 7 hr), nearly all DBTO_2 (which elutes at -2.38 min) was converted to OH-BP (see panel A of the figure on page 11). When the dilution rate was increased to 0.2 hr^{-1} ($G \approx 3.5 \text{ hr}$), the cell concentration in the chemostat decreased and the amount of DBTO_2 in the effluent increased. Panel B shows the presence of OH-BP in the liquid effluent 7 hr after the steady-state dilution rate was increased from 0.1 hr^{-1} to 0.2 hr^{-1} . Panel C shows the greatly diminished presence of OH-BP and the presence of DBTO_2 . The production of OH-BP was monitored by HPLC; coincidence of the product produced in by the N1 culture and pure OH-BP was demonstrated by "spiking" the culture product with the pure compound and observing a single symmetrical peak.

The estimated steady-state number of cells ml^{-1} at $D = 0.1 \text{ hr}^{-1}$ was 1.4×10^9 ; 7 hr after D was increased to 0.2 hr^{-1} , the cell number ml^{-1} was 6.6×10^8 ; 22 hr after the change in dilution rate, the number of cells ml^{-1} was 2.0×10^8 .

IV. FUTURE RESEARCH

Dbts^+ soil isolates will be grown in both *genuine* chemostats and *modified* chemostats called "bioreactors". All media components in a genuine chemostat are introduced and expelled at a constant rate. In the modified chemostat, the DBT (enmeshed in nylon filters) or coals are present continuously (though, one hopes, in decreasing abundance). Because of the presence of a particulate rather than a soluble substrate, some researchers favor calling a modified chemostat a "bioreactor." When DBTO_2 is the sole source of sulfur, the Dbts^+ bacteria will be grown in chemostats (as described in this report). When Dbts^+ bacteria are grown in the presence of DBT or coal, bioreactors will be used. A key feature of the bioreactor experiments which will be conducted in the immediate future is that the initial extent of growth will be restricted by provision of very low concentrations of $\text{SO}_4^{=}$; simultaneously, DBT or depyritized Louisiana Tech coal will be present as an alternative abundant sulfur source. The bioreactor will favor organisms which have become fully adapted for desulfurization of organic sulfur in DBT or coal. Rapid adaptation is expected for two reasons: the Dbts^+ phenotype is expressed when the opportunity for growth is constrained and mutation to more effective desulfurization will be advantageous. Both of these circumstances favoring

OH-BP production in CONTINUOUS culture

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adaptation are enhanced in the bioreactor and will be readily manifest through increased yield (Y) of bacteria, a modified K_s value of DbtS^+ bacteria, and desulfurization of the insoluble organic sulfur source.

Optimization of the DbtS^+ phenotype may be achieved through continuous selection in steady-state culture. Such adaptation to DBT and subsequently to a depyritized coal will provide quantitative data demonstrating the potential of DbtS^+ bacteria to beneficiate coal.

V. CONCLUSIONS

Gram positive isolates with a robust capacity to desulfurize DBT and to generate monohydroxybiphenyl have been isolated and partially characterized. The species and genus identities of the isolates are not known. The DbtS^+ phenotype of the fresh isolates appears to be stable. Growth in both batch and continuous culture has been demonstrated as has production of OH-BP in both growth conditions. Thorough characterization of this phenotype including the kinetics of the reactions of the "4S" pathway is presently being achieved. Through kinetic analyses, optimal conditions for the production of OH-BP from DBT and DBTO_2 will be established. Continuous culture of the isolates in the optimal conditions is expected to maximize the efficacy of the DbtS^+ phenotype.

VI. REFERENCES

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VII. APPENDIX: CLONING WITH R68.45

Refinements in procedures for transmitting, isolating, restricting, and visualizing R68.45 and primes derived from R68.45

R68.45 is a notoriously difficult plasmid with which to work. Yields are often low or even zero and when the plasmid is obtained it is often unstable. Thus, the ability to isolate R68.45 routinely, to distinguish it from R primes, and to detect products of restriction endonuclease digestion are notable accomplishments. These results were achieved by refining the isolation procedures and subsequent purification of the plasmid. The successful procedure for isolating R68.45 uses cells from a liter culture, exposes the cells to Pronase and sodium dodecylsulfate for 1 hour at 37° C, and achieves denaturation of nucleic acids through high alkalinity (12-12.5 using 3 M NaOH). The pH is returned to 8.5 with Tris, the nucleic acid is extracted with redistilled phenol, and the plasmid is purified by equilibrium centrifugation on CsCl gradients. Plasmids are recognized in the CsCl gradients by intercalation of ethidium bromide between bases of the DNA. Removal of ethidium bromide with butanol saturated with buffered EDTA and removal of CsCl through ethanol precipitations rather than dialysis proved to be procedurally important. Why any one procedure is clearly successful and another is wholly unsuccessful is, in most instances, presently a mystery of molecular biology. R68.45 isolated with these techniques gives very tight ("aesthetically pleasing") bands in agarose gel electrophoresis.

In addition, restriction endonuclease digestion of R68.45 with SalI, EcoRI, and HindIII, individually or in combination, have been successfully performed. The restriction fragments are sometimes large (e. g., 35-40 kb); these can be distinguished by pulsed-field gel electrophoresis. The specific conditions which are being used are: 1% agarose gel; 130 volt potential; 1 sec → 6 sec ramp; 17 hr run.

Formation of R primes from R68.45

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. *Thiobacillus* spp.

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Crosses have been performed between *Pseudomonas putida* PRS 2003/R68.45 and a methionine auxotroph, *P. aeruginosa* 27853 2A, to detect R primes which carry met determinants. The presumptive R primes cannot be distinguished from R68.45 on agarose gels. Restriction endonuclease digests of R primes and R68.45 are being compared to provide a more refined analysis of differences in composition. Both the mechanism of R prime formation and the organization of R primes are incompletely known. The restriction endonuclease analysis should indicate if the acquired genetic information in an R prime is always inserted into a single site in the original plasmid or into a variety of sites.

Significance of successful isolation of R68.45 and formation of R primes

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

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VIII. PERSONNEL

Diane L. Dutt

B. S. (biology) Moravian College 1977
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IX. COST MANAGEMENT REPORT

DATE RUN	TIME RUN	REPORT PAGE
07/13/81	20:58:57	37
LEHIGH UNIVERSITY	FINANCIAL RECORDS SYSTEM	PROGRAM TO FBM099
RESEARCH ACCOUNT SUMMARY IN WHOLE DOLLARS AS OF 06/30/91		ACCOUNT PAGE 1
START 06/30/89	EXP 06/29/92	AGENCY - DOE
EXP 06/29/92	EXP 06/29/92	BIOLOGY MWS HALL
PROJ-TO-DATE	CURRENT MONTH	BUDGET AVAILABLE
50,197	12,096	3,606
39,035	1,245	10,777
28,320	850	11,220
1,520	1,200	320
119,072	15,391	22,376
28,335	4,002	5,152
167,407	19,394	10,199
22,305	931	301-
765	5	364
7,068	125CR	855
2,430	25	2,153
27,160	11,726	15,434
10,760	235	235-
70,108	836	9,112
217,595	20,229	27,382
116,167	12,340	10,199
333,762	32,549	26,246
*** TOTAL DIRECT COSTS		36,445
INDIRECT COSTS		
**** GRAND TOTAL		48,258

(1) - NOT SUBJECT TO INDIRECT COST.

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