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MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL DESULFURIZATION DISCLAIMER

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MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION

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ABSTRACT

MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION

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Fresh isolates of bacteria presumptively identified as *R. erythropolis* unequivocally have a DbtS^+ phenotype. The production of OH-BP from either DBT or DBTO_2 was confirmed by difference spectroscopy, HPLC, and mass spectrometry. The temperature, pH, and means of supplying the thiophenic or sulfonic sole source of sulfur were optimized. The maximal rate of growth of the organism, its affinity for sulfone, and the extent to which substrate was converted to product were determined by using batch, fed batch, and continuous cultures. For strain N1-36, the maximum specific growth rate was 0.235 hr^{-1} which corresponds to a minimal generation time of 2.95 hr. The K_s was estimated to be $0.39 \mu\text{M}$. With $100 \mu\text{M}$ DBT as the sole sulfur source, approximately $40 \mu\text{M}$ OH-BP are produced (after 40 hr of growth); with $100 \mu\text{M}$ DBTO_2 as the sole sulfur source, approximately $70 \mu\text{M}$ OH-BP are produced (after 40 hr of growth). The desulfurization activity is repressed by $\text{SO}_4^{=}$. OH-BP does not serve as a carbon source.

The DbtS^+ phenotype of the *R. erythropolis* isolates is stable and discrete. The isolates selectively remove sulfur from DBT, a compound which models a refractory form of organic sulfur in compounds characteristic of fossil fuels. The desulfurization occurs with no oxidation of carbon-carbon bonds. The stability and specificity (along with genetic regulation) indicate that microbial desulfurization is a real phenomenon in which a noxious element is removed without significantly affecting the calorific value of the substrate. Additional characterization (and optimization) would provide the basis of a very important form of fossil fuel beneficiation.

The existence (and instability of) a DbtS^+ phenotype in *Pseudomonas* spp. is also reported along with a rationale for using a broad-host-range plasmid, R68.45, to transmit the determinants of the DbtS^+ phenotype among gram negative bacteria. The structure of the plasmid (R68.45) and the genomes of two potential recipients, *Thiobacillus novellus* and *Thiobacillus acidophilus*, have been partially characterized by pulsed-field gel electrophoresis.

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MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL DESULFURIZATION

Isolation and characterization of strains of *Rhodococcus erythropolis* with a DbtS⁺ phenotype

I. INTRODUCTION

The value of bacterial desulfurization of fossil fuels is that the process is metabolically specific, ecologically benign, and economically attractive.

During the period from 1986 through 1993 the prospect of bacterial desulfurization of fossil fuel was transformed from a theoretically appealing concept to a demonstrable laboratory phenomenon. Results from several laboratories confirmed that there was not one but, rather, several metabolic bases of selectively removing sulfur from the carbon frame of sulfur-containing organic compounds characteristic of fossil fuels. The results in this report relate solely to the so-called "4S" pathway (named for the four sulfur-containing compounds in the sequence: {1} dibenzothiophene [DBT] → {2} dibenzothiophene sulfoxide [DBTO] → {3} dibenzosulfone [DBTO₂] → {4} dibenzosulfonate → monohydroxybiphenyl [OH-BP] + SO₄⁼. [An additional desulfurized product, biphenyl, has been hypothesized and another, o,o'-biphenyl, observed.]) (See Figure 1 and cf. 1).

The "4S" pathway is a metabolic capacity. The biological information conferring this capacity is presumed to be genetic. The mnemonic or rubric specifying this phenotype is: DbtS⁺.

In this report, the following subjects are discussed:

- isolating bacteria with a DbtS⁺ phenotype;
- confirming the production of a desulfurized product;
- determining the identity of the isolates;
- determining the growth characteristics of the isolates in batch and continuous cultures;
- determining the kinetics and yields of product in batch and continuous cultures.

In appendices, the following subjects are discussed:

- the occurrence of a similar phenotype in some *Pseudomonas* spp.;
- the genetic transfer of the DbtS⁺ phenotype between *Pseudomonas* spp. using a broad-host-range plasmid, R68.45;

FIGURE 1 The "4S" pathway. Progressive oxidation of sulfur is emphasized. The origin of oxygen (*i. e.*, the substrate that provides oxygen) is unknown. Three potential products are shown. The prominent crosses (or X's) on the arrows leading from the final products indicate that further metabolism of the organic products does not occur.

- the use of pulsed-field gel electrophoresis (PFGE) to confirm the physical map of the R68.45;
- the instability of the Dbts⁺ phenotype in *Pseudomonas* spp.;
- the use of PFGE to assess the sizes of the genomes in two species of *Thiobacillus*.

II. ISOLATING STRAINS WITH A Dbts⁺ PHENOTYPE

A. Sulfur bioavailability technique

While several means exist for isolating strains with a Dbts⁺ phenotype, the most effective is the "sulfur bio-availability" method. In this process, environmental samples thought to be a source of the desired organism are introduced into a growth medium lacking any source of sulfur other than DBT. The rationale is that no growth whatsoever will occur unless the sample contains an organism with the capacity to use the sulfur in the model compound. Since thiophene is a highly unusual form of organic sulfur (that is, not characteristic of the forms of organic sulfur ordinarily seen in living material), the expectation is that the organic sulfur will be transformed to some more conventional form of sulfur, even inorganic SO₄⁼, before being used by the bacteria.

Isolating bacteria with the ability to use DBT as sole sulfur source was an explicit and specific goal of the contracted research. A more refined goal is to isolate bacteria which perform the desulfurization rapidly. To this end, bacteria which grew in batch cultures with DBT as the sole sulfur source were grown in continuous cultures with DBTO₂ as sole sulfur source. (DBTO₂ rather than DBT was used in continuous culture because the solubility of the sulfone is greater than that of the thiophene.) During continuous growth, the dilution rate was successively increased from 0.1 to 0.15 and then to 0.2 reciprocal hours. Each of these successive rates might constitute a more stringent state of selection; bacteria whose growth rate (with the available sulfur source[s]) did not exceed the dilution rate would be "washed out."

A limitation of the sulfur bioavailability assay is that the utilizable sulfur source (e. g., $\text{SO}_4^{=}$) is likely to be used by other organisms present in the environmental sample. Thus, the growth which occurs in the medium inoculated with the sample is not confined to the organism with the DbtS^+ phenotype. Accordingly, subsequent to growth (in either batch or continuous culture), a "screening procedure" is needed to distinguish organisms which grew and possess the relevant phenotype from organisms which grew and lack the relevant phenotype (2).

B. Screening technique

Samples from the sulfur bioavailability assay were streaked on solid media; the resultant colonies were considered to be clones. Individual clones were picked and assayed for their ability to produce a fluorescent product and a phenolic product. The former capacity is assessed with the "fluorescent spray plate technique" and the latter with a chromogenic assay.

1. The fluorescent spray plate technique

In the fluorescent spray plate technique, the clones to be tested are distributed on the surface of an agar plate, allowed to grow, and then sprayed with an aerosol containing DBT in a highly volatile solvent. The solvent evaporates and the DBT remains as a condensate on the surface. When organisms which have the capacity to oxidize DBT to DBTO_2 or OH-BP are illuminated with "short" ultraviolet light, the colonies are surrounded by a purplish fluorescent ring or halo (1).

One limitation of the technique (at least in theory) is that the assay appears not to distinguish between organisms that can oxidize DBT but not produce a desulfurized product and organisms that can oxidize DBT and produce a desulfurized product. (This limitation is less problematic in practice than in theory. The intensity of fluorescence seen with DBTO_2 is much less than that seen from OH-BP; accordingly, the probabilities of picking organisms which are oxidizing DBT but not producing a desulfurized product are correspondingly small.) The presence of some desulfurized products, viz., OH-BP or o, o'-biphenyl, can be directly detected with a chromogenic assay for phenolic moieties (2).

2. The phenol assay

Aliquots of cultures to be tested for the presence of phenolic compounds are mixed with equal volumes of two solutions: 5% $K_3Fe(CN)_6$ in an alkaline buffer and 0.25% 4-aminoantipyrene, the chromogenic reagent. The samples are incubated overnight; the presence of a red color the following day indicates the presence of a stable phenolic compound (2).

3. Performing the fluorescent assay and phenol assay in microtiter plates

Samples from the bioavailability cultures were streaked on solid agar; inocula from the resulting colonies were introduced into miniature (0.2 ml) cultures in the wells of microtiter plates containing 0.1 mM $DBTO_2$ as the sole sulfur source. After two days of incubation, the wells were scanned with "short" ultraviolet light (2). (As indicated earlier, 0.1 mM $DBTO_2$ does not, in practice, produce notable fluorescence. Also, see Figure 2.)

An aliquot of each of the cultures was also tested for the presence of OH-BP or o, o' biphenol (or similar phenolic moieties). Figure 2 presents results from microtiter assays; panel A shows the pattern of fluorescence while panel B shows the chromogenic responses. The combination of these two assays provides direct, positive, straightforward, presumptive evidence that clones produce a desulfurized product from DBT or ($DBTO_2$).

C. Designation of the isolates

A total of 21 isolates were obtained through the techniques described above. From the 21 isolates, three (N1-36, N1-43, and Q1a-22) were chosen for further study (2).

D. Conclusion

The sulfur bioavailability technique generated a variety of isolates; the abundance of the rapidly-growing strains with the relevant phenotype may have been increased in continuous cultures with increasing dilution rates. Screening for fluorescent products (with short ultraviolet light serving as the excitatory wavelength) and phenolic compounds (recognized with a chromogenic assay) allowed identification of isolates with a presumptive $DbtS^+$ phenotype.

FIGURE 2 The fluorescent and chromogenic assays performed in a microtiter plate. Miniature cultures having DBTO_2 as the sole sulfur source were exposed to ultraviolet light (upper panel); the presence of OH-BP or *o,o'*-biphenol produces fluorescence. Because other compounds also produce fluorescence, parallel cultures were treated with reagents which produce a colored response (lower panel) if phenolic moieties are present.

III. ESTABLISHING (RIGOROUSLY) THAT THE ISOLATES POSSESSED THE DbtS^+ PHENOTYPE

The selection and screen procedures strongly suggest that the isolates possess the desired phenotype. To establish definitively that they do, three exacting techniques were employed: HPLC, UV spectroscopy, and mass spectrometry. (Data were collected from all three isolates studied in detail; the data presented in this report were obtained principally from N1-36.)

A. HPLC

High Performance Liquid Chromatography (HPLC) was used to establish that the desulfurization product produced from DBT or DBTO_2 was indistinguishable from OH-BP. For this demonstration, the product produced by the isolate was compared with pure OH-BP and with the product mixed with pure OH-BP. The relevant data appear in Figure 3. The profile of OH-BP (obtained from Aldrich Chemical Company and having a purity in excess of 99%) appears in panel A; the profile of the product generated by N1-36 from DBTO_2 (extracted from the culture with an equal volume of acetonitrile and passed through a $0.2 \mu\text{m}$ filter) appear in panel B; the profile of the product of the bacterial isolate "spiked" with the pure standard appears in panel C. The equivalent retention times of the standard and the bacterial product (on a Brownlee [Spheri-5 RP-18 5 micron, $100 \times 4.6 \text{ mm}$] column and the single, symmetrical peak produced by the natural product mixed with standard indicate that the natural product and OH-BP are indistinguishable by HPLC (2, 3).

B. UV difference spectroscopy

UV difference spectra further substantiate that the product which accumulates in a medium containing DBTO_2 as sole sulfur source is OH-BP. This conclusion was unequivocally established through six spectra (see Figure 4). The first two comparisons, DBTO_2 vs. CHCl_3 and OH-BP vs. CHCl_3 , establish that the profiles of the substrate and product bear little resemblance (see panels A and B of Figure 4). The third spectrum, pure OH-BP added to CHCl_3 extract of a culture supernatant (which had supported growth of N1-43 with MgSO_4^- as sole sulfur source) vs. a CHCl_3

FIGURE 3 HPLC spectra for OH-BP and product produced by N1-36 from DBTO₂. Panel A: pure OH-BP solution. Panel B: culture filtrate. Panel C: culture filtrate "spiked" with pure OH-BP. Retention times for DBT and DBTO₂ are, respectively, approximately 16 minutes and 2.5 minutes. Negative controls had no detectable peak after the initial peak at ≈ 0.6 min.

FIGURE 4 UV difference spectra for pure DBTO₂, pure OH-BP, and cultures of N1-36 having DBTO₂ as the sole sulfur source. (In the following listing, the experimental sample is listed first and the reference sample is listed second). Panel A: pure DBTO₂ vs. CHCl₃. Panel B: pure OH-BP vs. CHCl₃. Panel C: pure OH-BP added to a CHCl₃ extract of culture supernatant having MgSO₄ as sole sulfur source vs. CHCl₃ extract with no additions. Panel D: supernatant from culture having DBTO₂ as sole sulfur source vs. supernatant after extraction with CHCl₃. Panel E: CHCl₃ extract of culture supernatant from DBTO₂ medium vs. CHCl₃ extract of culture supernatant from MgSO₄ medium. Panel F: pure OH-BP added to culture supernatant of DBTO₂ medium which had product removed by CHCl₃ extraction vs. culture supernatant extracted with CHCl₃.

extract of the same supernatant but without addition of any standard (*i. e.*, OH-BP) shows that the extraction process does not produce any materials which would obscure recognition of OH-BP (see panel C of Figure 4). The fourth spectrum, supernatant from a culture which had DBTO_2 as sole sulfur source *vs.* supernatant after extraction with CHCl_3 , shows that (i) OH-BP is present as a product, (ii) that the product can be clearly recognized among all other components in the medium, and (iii) that the OH-BP spectrum with an aqueous solvent is similar to but distinct from the OH-BP in an organic solvent. The fifth spectrum (see panel E of Figure 4) is a CHCl_3 extract of a culture supernatant having 0.1 mM DBTO_2 as sole sulfur source *vs.* a CHCl_3 extract of a culture supernatant having $\text{MgSO}_4^=$ as sole sulfur source; this key spectrum clearly shows a profile indistinguishable from that of pure OH-BP in CHCl_3 (*cf.* panel B). Finally, the last spectrum (pure OH-BP added to the supernatant of a culture which had 0.1 mM DBTO_2 as sole sulfur source *and which had been extracted with CHCl₃ vs.* a similar culture supernatant which had been extracted with CHCl_3) shows that the profile of pure OH-BP added to an aqueous medium which had been depleted of product generated from DBTO_2 is identical to the product produced from DBTO_2 (*cf.* panel D).

The difference spectra themselves and comparisons among the difference spectra (though sometimes challenging to understand completely) unequivocally establish that the product which accumulates in a medium inoculated with N1-43 and containing DBTO_2 as sole sulfur source is indistinguishable from pure OH-BP. The near identity of the profiles suggests that no significant amounts of additional organic products are produced from DBTO_2 . Furthermore, the product is, at least, vastly less abundant or, more likely, absent in cultures which have $\text{MgSO}_4^=$ as the sole sulfur source (2, 3).

C. Mass spectroscopy

Within the scientific community, mass spectroscopy has achieved the status of "gold standard" among techniques for establishing the identity of organic compounds; accordingly, mass

FIGURE 5 Mass spectroscopy of standards, controls, and products produced by N1-36 in cultures having DBT as the sole source of sulfur. Standards were dissolved in CHCl_3 ; controls and experimental samples were extracted in CHCl_3 . Aliquots of the CHCl_3 solutions or extracts were dropped onto nitrocellulose disks; the solvent was allowed to evaporate; samples were then placed in the MS chamber. The amount of sample was $\approx 20 \mu\text{l}$ and collection time of positive ions ranged from 678 - 698 seconds. Accelerating potential was 10,000 volts. Panel A: pure OH-BP. Panel B: N1-36 grown in medium having MgSO_4 as sole sulfur source. Panel C: spectrum from a nitrocellulose disk. Panel D: N1-36 grown in medium having DBT as sole sulfur source.

spectroscopy was used to determine if OH-BP was produced from the desulfurization of DBT (or DBTO₂). Mass spectrometry was performed using a BIOION 20 mass spectrometer. (The BIOION 20 is a "fission fragment" spectrometer which measures secondary ions produced from the impact of the fission products of 252Cf. The fission fragments were estimated to have energies between 50 and 100 Mev.) Using this technology, a prominent component was observed in the spectra having a molecular weight of 170 daltons, the formula weight of OH-BP. Such a component is present in the profiles prepared with pure OH-BP of CHCl₃ extracts of medium which were inoculated with N1-36, N1-43, or Q1a-22 and which had 0.1 mM DBTO₂ as sole sulfur source (see Figure 5, panels A and B). Controls established that the peak was absent when (i) the solvent alone was analyzed (panel C of Figure 5) or when (ii) the solvent was used to extract media which were inoculated with any one of the isolates and which had MgSO₄⁼ as the sole sulfur source (data not shown; cf. 2). Thus, the mass spectroscopical experimental (and control) data substantiate that OH-BP is a prominent product of cultures inoculated with the isolates and having DBTO₂ as sole sulfur source (2, 3).

D. OH-BP is not a carbon source

The DbtS⁺ phenotype is defined as the capacity to *selectively* remove sulfur from DBT (split infinitives notwithstanding). The production of OH-BP (or o,o'-biphenyl or biphenyl) establishes this selectivity. Furthermore, the presence of the product indicates that no carbon-carbon bonds of the substrate are disrupted. Also, only one carbon atom is oxidized. These observations suggest that OH-BP cannot serve as a carbon source; this possibility was tested experimentally. N1-36 was inoculated onto solid media containing (i) no carbon source, (ii) OH-BP, glucose, or ethanol, (iii) ethanol plus each of the other carbon sources, and (iv) all three carbon sources. Growth was evident within two days on those media which contained ethanol and within four days in the medium having glucose alone. No colonies were observed on media having OH-BP as the sole carbon source. The observations were extended to liquid cultures. No growth was observed with media lacking a carbon source or having OH-BP as sole carbon source; liquid media containing

0.1% or 1.0% ethanol (with or without OH-BP) showed rapid and extensive growth (2, 3).

E. Conclusion

Three "state of the art" chemical techniques, HPLC, UV difference spectroscopy, and mass spectroscopy, indicate that OH-BP is produced by the new isolates when incubated in a medium containing DBT (or, more commonly, DBTO_2) as the sole sulfur source. Microbiological assays indicate that the substrates are used exclusively as a sulfur source and not as a carbon source.

IV. GENUS AND SPECIES-SPECIFIC IDENTITY OF ISOLATES

Determining the genus and species-specific identities of the isolates is a formidable task. Seventy-five to eighty percent of the Earth's extant biomass is microbiological and, of this, the vast majority is bacterial. In the (non-bacterial) world, there are hundreds of thousands, if not millions, of species. Yet in the more abundant bacterial world, there are many less than ten thousand (recognized) species. Significant questions arise. Does simplicity of form equal less diversity? Or does simplicity of form allow less (human) discrimination?

The identities of the Dbts^+ isolates were assessed using both contemporary, commercially-available technologies and established procedures. The former pertained to (i) the distinctive fatty acid compositions of bacterial species or (ii) the distinctive nutritional profiles; the latter pertained to cellular morphology and gross cultural characteristics.

A. Fatty acid composition

More than 300 fatty acids and related compounds have been found in bacteria. Information about the identity of organisms can be obtained from the presences and abundances of the compounds by comparing such fatty acid profiles with established libraries (2).

The Dbts^+ isolates were grown on trypticase soy agar and shipped to Microbial ID, Inc. (MIDI) in Newark, Delaware where the fatty acids were extracted and methylated. The identities and abundances of the fatty acids were determined by gas chromatography. The resulting chromatographic spectrum for each strain was digitized and, then, electronically compared with a database of $\geq 60,000$ spectra (generated from more than 600 aerobic bacteri-

al species.) The comparisons were used to assess "similarity" (which is the cumulative fractional equivalence of the abundance of individual fatty acids in the test organism divided by the mean of the abundance of the same fatty acid in aggregate populations of established species). Similarities equal to or greater than 0.5 are scored as a "good" match. The results of this analysis appear in Table 1. The species having the highest similarity may correspond to the true identity of the isolate; a more cautious conclusion is that the technique merely identifies some (reasonable) possibilities (2, 3).

TABLE 1 Species identification determined by fatty acid composition

Isolate	Species identity	Similarity
N1-36	Nocardia globerula	0.734
	Rhodococcus erythropolis	0.619
	Arthrobacter paraffineus	0.565
Q1a-22	Nocardia globerula	0.735
	Rhodococcus erythropolis	0.707
	Arthrobacter paraffineus	0.612
N1-43	No match	-

B. Nutritional profiles

BIOLOG, a commercial company, markets devices which reveal the ability of microorganisms to use 95 different carbon sources. The "device" is a standard 96 well microtiter plate; one well has no carbon source while the remaining 95 wells each have some distinct carbon source. All wells also contain TTC (2,3,5-triphenyltetrazolium chloride), a water-soluble, colorless dye which, upon reduction, becomes a water-insoluble, purple formazan. Finally, all wells contain a proprietary mix of growth factors and substrates; the mix allows growth of microorganisms having various growth requirements; but the concentrations and combinations of components in the mix (evidently) do not allow false positive results (as confirmed by the control well). BIOLOG markets plates for gram negative organisms and gram positive organisms (along with specialty plates and empty plates); in the project described here, plates suitable for the identification of gram positive organisms (see below) were used.

In practice, pure cultures of the test organism are grown to a standard extent. Then 0.5 ml aliquots of the culture are introduced into each of the wells. The rationale is that if the organism can utilize the substrate in the well, the organism will grow and produce an excess of "reducing power"; the reducing power will reduce the TTC to a purple precipitate; the utilization of substrate can readily be scored by noting the coordinates of the purple wells in the 8 x 12 microtiter plate matrix. The data are "entered" into a program which uses proprietary software to determine "per cent similarity" (an estimate of the relatedness of the experimental pattern with an averaged pattern observed among strains of the a reference species), "distance" (an estimate of how far the data are from being a perfect match with the average for a given species), and "acceptability of identification." When similarities are achieved equal to or greater than 0.5 with species in the database, identifications designated "acceptable" are given by the program.

Identifications of the DbtS⁺ isolates were performed in duplicate. For the first set of results, quantitative optical density data (at 590 nm) for each well were sent to BIOLOG where specialists interpreted the results. For the second set of

determinations, qualitative data were analyzed at Lehigh on a Zenith 386 ([IBM] PC compatible) microcomputer using BIOLOG's "Microlog I" software. In all instances, the identification achieved for the isolates was *R. erythropolis*. Unfortunately, the BIOLOG database does not include a nutritional profile for *Nocardia globerula* (2, 3). (See Table 2.)

TABLE 2 Species identification determined by nutritional profile

Isolate	Experiment	Species identity	Similarity	Distance
N1-36	1	Rhodococcus erythropolis	0.830	2.80
	2	Rhodococcus erythropolis	0.607	6.35
N1-43	1	Rhodococcus erythropolis	0.799	3.51
	2	Rhodococcus erythropolis	0.716	4.57
Q1a-22	1	Rhodococcus erythropolis	0.667	4.97
	2	Rhodococcus erythropolis	0.747	3.89

C. Cell and colony morphology

Cell morphology and gross cultural characteristics (including colony morphology) are established means of determining the identities of bacterial isolates.

The nocardioform actinomycetes form a suprageneric group containing the following genera: *Corynebacterium*, *Gordona*, *Mycobacterium*, *Tsukamurella*, *Nocardia*, and *Rhodococcus* (4). The nocardioform actinomycetes are, in general, characterized as aerobic, gram-positive, catalase-positive actinomycetes that show a highly diverse morphology ranging from a branched mycelium (that fragments into rods or cocci) to aerial hypha; the hyphae may be barely visible or sufficiently copious to give the appearance of white down (5).

"Rhodococci have had a long and confused taxonomic pedigree" (6). Rhodococci were at, one time, assigned to a group of bacteria known as the "rhodochrous complex"; at other times, these bacteria have been assigned to the following genera: *Arthobacter*, *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, and *Nocardia* (6). Recently, the genus *Rhodococcus* was reintroduced and redefined by using primarily chemotaxonomy and numerical phenetic taxonomy. Currently, the genus *Rhodococcus* contains 13 species (4).

The isolates were gram positive, catalase positive, strictly aerobic, non-endospore forming, non-acid-fast rods. Cells from liquid cultures exist as individuals, or as pairs as in the letter "V," or, occasionally, as groups of 3 or 4 connected to form the shapes of the letters "N" and "W." Cells from young colonies are sometimes branched. The colonies are creamy, circular, convex or pulvinate, entire, smooth and butyrous (2).

The cell and colony morphology of the isolates show some differences from what is described in *Bergey's Manual of Systematic Bacteriology* for the *Rhodococcus* genus and more numerous differences from the descriptions for the *Nocardia* genus. To quote, the description for the *Rhodococcus* states, in part, that "In all strains, the morphogenetic cycle is initiated with the coccus or short rod stage... cocci may merely germinate into short rods, form filaments with side projections, show elementary branching or... produce extensively branched hyphae. The next generation of cocci or short rods are formed by the fragmentation of the rods, filaments and hyphae" (6). In *Nocardia*, "rudimentary to extensively branched vegetative hyphae [are seen] growing on the surface of, and penetrating, agar media often fragmenting in situ or on mechanical disruption into bacteroid, rod-shaped to

coccoid, nonmotile elements. Aerial hyphae... almost always form. Short to long chains of well to poorly formed conidia may occasionally be found on the aerial hyphae..." (7).

In this project, substrate mycelia, aerial mycelia, hyphae, conidia, filaments or cocci were never observed. On the basis of cell and colony morphology, the isolates appeared more closely related to *Rhodococcus* than to *Nocardia*.

D. Conclusion and recommendation

The identity of the isolates has been presumptively determined as *Rhodococcus erythropolis*. This identification was achieved using commercially available methods; the identification is not fully consistent with some results obtained using standard microscopical techniques. Thus, the strains may belong to a novel, undescribed species included in or related to the genus *Rhodococcus*.

In eukaryotic organisms, karyotyping has long been used to identify species. The technique is especially informative when banding patterns provide additional detail about the appearance of chromosomes. A molecular extension of characterization-by-chromosome-content is "DNA fingerprinting." Now, with prokaryotes, a counterpart to these techniques has become available, viz., Pulsed-Field Gel Electrophoresis (PFGE) of genomic "macro"-fragments created by digesting a bacterial genome with a rare-cutting restriction endonuclease. The resultant gels are stained and can be assessed qualitatively (by visual examination) or quantitatively (by laser scanning). Data acquired in this manner show distinct profiles for individual species. This analysis directly employs the genetic material of cells; accordingly, this technique may currently be the most definitive means of demonstrating the equivalence of a new isolate to a presently recognized bacterium. (Operationally, "presently recognized" would be a bacterium which has been deposited in a major repository and which has a name approved by the International Committee on Systematic Bacteriology.)

The identity of the isolates obtained and described in this project are known "presumptively." The formal process of identification has not been extended from the "presumptive" stage to the "confirmed" and "completed" stages. Accordingly, the rela-

tion of these isolates to other, seemingly similar isolates, e. g., IGTS8 isolated by John Kilbane of the Institute of Gas Technology and later identified as *Rhodococcus rhodochrous*, is not known.

The actual identity of DbtS⁺ isolates is important both for biological and for commercial reasons. The former would allow some understanding of the distribution of an atypical phenotype and, as the genetic basis of the phenotype becomes better understood, the means by which this phenotype was distributed among organisms. At the practical level, knowing that isolates used in a commercial (bio)process are identical or distinct will be important for legal reasons. These conditions establish that further characterization of the species, for example by PFGE, is essential.

V. DbtS⁺ PHENOTYPE IN BATCH AND FED-BATCH CULTURES

The preeminent feature of the DbtS⁺ phenotype is the ability to remove selectively the sulfur atom from DBT. The data presented in this section of the report pertain to this capacity in the *R. erythropolis* isolates. The consumption of substrate, appearance of product, and stability of product are correlated with growth phase. In addition, the optimal conditions for growth and production of product are presented. (See Figures 6, 7, 8, and 9.)

A. Growth in batch and fed-batch cultures

Both batch and fed-batch growth experiments with DBT as sole sulfur source were performed; DBTO₂, because of its greater solubility in aqueous media (compared to DBT), was used in five additional batch and fed-batch growth experiments. The disappearance of substrate (DBT or DBTO₂), the appearance of product (OH-BP), the concentration of dissolved oxygen (DO), the pH, and, in some instances, the concentration of glucose were monitored (2). The number of bacteria present is presented as colony forming units (CFUs).

Four representative cultures are shown: a batch culture with 100 μM DBT as sole sulfur source and lacking pH control; a fed-batch culture with 100 μM DBT as sole sulfur source and having pH control; a batch culture with 100 μM DBTO₂ as sole sulfur source and lacking pH control; and a fed-batch culture

with 100 μM DBTO₂ as sole sulfur source and having pH control. The experiments with DBT as sulfur source were performed in a New Brunswick C30 BioFlo reactor with a 355 ml working volume; the experiments with DBTO₂ as sulfur source were performed in a New Brunswick BioFlo III reactor with a 1.5 liter working volume (2).

All reactors were inoculated with a 5% inoculum. In all cultures, 0.3% glucose was the initial carbon source; in the fed-batch cultures, the concentration of glucose was restored to 0.3% in late exponential phase (2).

Two common features of the growth experiments are that the substrates, DBT or DBTO₂, disappear quickly and that the product, OH-BP, begins to appear in late exponential phase. Also, the abundance of the product declines during the prolonged exponential phase (2, 3, 8; and see below).

In the growth profiles, the transition from late exponential phase to stationary phase appeared to occur at approximately 40 hours. Measurable DBT had disappeared by 25 hours or so, and DBTO₂ by 16 hours. Appearance of OH-BP lagged behind cell growth but the maximum yield of the product appeared, typically, at approximately the same time as the transition from exponential to stationary phase. Notably, the yield of OH-BP was greater with DBTO₂ than with DBT; from 100 μM DBTO₂, the yield of OH-BP was typically 50-70 μM ; from 100 μM DBT, the yield was 40 μM .

As noted, the abundance of the product declines during exponential phase. One formal possibility was that the OH-BP was serving as a carbon source. (This possibility was tested directly and excluded [see section III. D].) The intent of the fed-batch cultures was to see if the introduction of a commonplace substrate, i. e., glucose, would stabilize the presence of OH-BP; the data indicate that the abundance of the product continued to decline even in the presence of additional carbon source.

(N. B.: At a later time in the project, ethanol was found to be a superior carbon source. Accordingly, repetition of the experiment with ethanol may be informative.)

A control experiment (data not shown) revealed that glucose consumption during stationary phase is dependent on pH control. In the absence of the pH control, consumption of glucose is slight whereas with pH control glucose consumption is nearly linear throughout a period of 170 hr.

FIGURE 6 Batch culture of *R. erythropolis* (N1-36) with DBT as sole sulfur source. Abundances of substrate (DBT), product (OH-BP), biomass (CFU), pH (x10) and dissolved oxygen (% saturation) are presented.

FIGURE 7 Fed-batch culture of *R. erythropolis* (N1-36) with DBT as sole sulfur source. Abundances of substrate (DBT), product (OH-BP), biomass (CFU), pH (x10) and dissolved oxygen (% saturation) are presented.

FIGURE 8 Batch culture of *R. erythropolis* (N1-36) with DBTO₂ as sole sulfur source. Abundances of substrate (DBTO₂), product (OH-BP), biomass (CFU), pH (x10) and dissolved oxygen (% saturation) are presented.

FIGURE 9 Fed-batch culture of *R. erythropolis* (N1-36) with DBTO₂ as sole sulfur source. Abundances of substrate (DBTO₂), product (OH-BP), biomass (CFU), pH (x10) and dissolved oxygen (% saturation) are presented.

1. Optimal pH for growth of *R. erythropolis* isolates and expression of the *Dbs⁺* phenotype

Cultures of N1-36 were grown at pH's 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; N1-43 and Q1a-22 were grown at the five higher pH's. DBTO₂ was the sole sulfur source in the media. The rates and extents of growth were monitored. In addition, the abundance of OH-BP was monitored (2).

The most rapid growth for strain N1-36 was at pH 5.5; a slightly less rapid growth was observed at pH 5.0. At both these pH's, the extents of growth were reduced an order of magnitude or more (in comparison to the other pH's). Rates of growth at the other pH's were marginally less than at the two low pH's. Higher pH's had minimal effects on the rates and extents of growth of all isolates. (Q1a-22 had lower growth rates at all pH's than did either N1-36 or N1-43 [2].)

For all cultures, the rate of OH-BP production was greater at pH 6.0 than at 6.5, 7.0, 7.5, or 8.0. For N1-36, the rate of OH-BP production was yet faster at pH 5.5; no OH-BP was produced at pH 5.0 (2).

The rate of desulfurization (OH-BP production per unit time with DBT or DBTO₂ as substrate) was determined for a 115 hour old culture of N1-36. The highest rate of desulfurization activity per 1 ml sample occurred at pH 6.0; the highest rate of desulfurization activity per cell occurred at pH 5.5. Because the cell yield was so adversely affected by the lower pH, pH 6.0 was used in all growth experiments (2).

2. The effect of ethanol

In the typical experiment, the DBT or DBTO₂ introduced into a culture was dissolved in ethanol. Accordingly, knowing the effect(s) of ethanol became important. To test the effects of the ethanol, sulfur sources (MgSO₄⁼, DBT, and DBTO₂) were introduced into sulfur-free media with or without addition of ethanol; when DBT and DBTO₂ were introduced without ethanol, they were added as powder or enmeshed in nylon filters. Growth of N1-36 was monitored spectrophotometrically. The results showed that the presence of ethanol allowed N1-36 to respond more rapidly to all sulfur sources (including SO₄⁼) (2).

Further analysis showed that ethanol at concentrations below 1.0% was a very good carbon source. 0.1% ethanol, 0.1% ethanol + 0.3% glucose, and 1.0% ethanol + 0.3% glucose showed rapid and extensive growth. 1.0% ethanol alone (possibly) showed a very slightly diminished growth rate. 0.3% glucose alone was a poor substrate: all of the cultures with any amount of ethanol showed no lag; the culture with glucose alone had a lag or lethargic growth extending for 60 or more hours (2).

Higher concentrations of ethanol (1.5%, 2.0%, 2.5%, and 3.0%) had increasingly adverse effects on the rate of growth (2).

3. The effects of DBT and DBTO₂ as substrate
(and potential inhibitor) and the effect of OH-BP
as a potential inhibitor

The possibility that DBT, DBTO₂, and/or OH-BP are inhibitors of growth of N1-36 was tested. Nine media, containing 50, 100, or 200 μ Moles of each of the compounds were constructed; growth was monitored spectrophotometrically. Neither DBT nor DBTO₂ had an evident effect on growth rate; OH-BP may have had a subtle effect. Generally, the concentrations of these reactants need not be considered a variable of any significance when comparing experiments (2, 8).

B. Conclusion

A 1:20 inoculum of *R. erythropolis* N1-36 (or N1-43 or Q1a-22) grows moderately rapidly in optimal conditions in batch or fed-batch cultures having 100 μ M DBT or 100 μ M DBTO₂ as sole sulfur source. A twenty fold increase (an extent of growth that would generate in the culture a cell density equal to that of the full grown inoculum) would occur in 4.32 generations. At minimum generation time (2.95 hr [see below]), exponential growth could be completed in approximately 13 hours. In practice, the transition to early stationary phase occurred after about 40 hours.

The optimal pH for production of OH-BP (per cell) was 6.0. Ethanol served as an effective vehicle for the introduction of DBT or DBTO₂, both of which have limited aqueous solubilities. Neither DBT nor DBTO₂ were inhibitory at concentrations through 200 μ M; OH-BP had a minor or no effect. The optimal temperature for growth (see VI. D.) was determined to be 30° C.

In batch or fed-batch cultures, substrate disappeared rapidly. DBT was depleted to undetectable concentrations in approximately 25 hours while DBTO_2 was diminished to undetectable amounts in approximately 16 hours. The appearance of OH-BP lagged behind cell growth but the maximum yield of the product appeared, typically, at approximately the same time as the transition from exponential to stationary phase. Notably, the yield of OH-BP was greater with DBTO_2 than with DBT; from 100 μM DBTO_2 , the yield of OH-BP was typically 50-70 μM ; from 100 μM DBT, the yield was 40 μM . Continuous selection may yield sub-strains with more rapid rates of growth or greater extents of growth and with more extensive conversion of substrate to product.

VI. GROWTH IN CONTINUOUS CULTURE

The behavior of N1-36 in batch and fed-batch cultures was described in the previous section. Other data, such as maximum specific growth rate, biomass yield at the expense of specific substrates, and the temperature dependence of the reaction rate are more readily obtained in continuous cultures. Accordingly, in this section, the relevant data are presented.

A. Experimental methods

A New Brunswick BioFlo C30 bioreactor was used for continuous cultures. The carbon source was glucose; the concentration of this substrate was "not limiting" (i. e., it was "in excess"); specifically, the concentration of glucose was 6 g/l. The growth limiting nutrient was sulfur; this element was present in the medium in the form of DBTO_2 at a concentration of 50 μM . In an early experiment, DBTO_2 at 50 μM was found precipitated from the medium in the nutrient reservoir; stirring and mild heating of the substrate reservoir appeared to alleviate the problem; that is to say, no visible precipitate was evident during a seven day period and analyses showed that DBTO_2 concentrations stayed constant. Consequently, during experimentation, the substrate reservoir (10 liters of medium in a glass carboy) was always stirred and mildly heated (2).

The dilution rates of continuous cultures (in reciprocal hr) were calculated by dividing the nutrient flow rate (ml/hr) by the

total working volume (355 ml) of the reactor. Steady states were obtained by passing four working volumes of medium through the reactor under one set of experimental conditions (i. e., the same temperature and/or dilution rates). Samples were taken after steady states were established. During steady state growth, concentrations of substrate, product, and biomass were measured. (2).

The maximum specific growth rate ($\mu_{\max}$) of N1-36 was obtained in continuous culture by using a wash-out technique. In this procedure, steady state is established and the dilution rate is then abruptly increased to a value well above $\mu_{\max}$. The specific conditions for the experiments described in this report are that steady state was achieved at dilution rate (D) 0.193 hr^{-1} . D was then abruptly increased to 0.399 hr^{-1} . Samples were taken and biomass was measured at the initial steady state and at 0.5 to 3 hr intervals during wash-out (2, 8).

To determine the saturation constant for sulfur sources, continuous cultures of N1-36 at 30°C were established at various dilution rates. During steady states ($\mu = D$), biomass and substrate concentrations were measured at each dilution rate. The saturation constant (K_s) was calculated by the Monod equation (Monod, 1942) with known $\mu_{\max}$, μ and S (steady-state substrate concentration). The yield coefficient ($Y_{x/s}$) was calculated with known biomass (X) and substrate concentrations (S).

To determine the temperature dependence of growth of the *R. erythropolis* isolates, continuous cultures at a constant dilution rate, $D = 0.12 \text{ hr}^{-1}$, were grown at temperatures ranging from 21°C to 35°C . At each temperature, aliquots of culture were sampled before and after one-hundredth volume of 10 mM DBTO_2 solution was injected into the steady-state culture. Biomass, substrate, and product concentrations were measured. From these data, Arrhenius plots were constructed and temperature characteristics were calculated.

**B. Experimental determinations of $\mu_{\max}$ and t_d
(doubling time): ("Wash-out technique")**

Preliminary data indicated that $\mu_{\max}$ is approximately 0.2 hr^{-1} . To wash (growing) cells out of the reactor, the dilution

rate must be larger than $\mu_{\max}$. Accordingly, a steady state ($\mu = D = 0.193 \text{ hr}^{-1}$, which is close to but less than $\mu_{\max}$) was chosen as the starting point for the wash-out experiment, and, after steady-state was achieved, the dilution rate, D , was abruptly increased to 0.399 hr^{-1} (2, 8).

Figure 10 presents the data from the wash-out experiment. The dilution rate was changed at time zero. The differences between the slope at any point of the wash-out curve and the dilution rate is the specific growth rate (μ) at that point. Accordingly:

$$D - \mu_{\max} = \frac{d \ln X}{dt}$$

The experimental data show that $\ln X$ decreased with time. After ten hours at $D = 0.399 \text{ hr}^{-1}$ (i. e., ≥ 4 working volumes of medium having passed through the reactor), the cells in the reactor were (presumed to be) growing at $\mu = \max$; stated another way, μ was presumed to have become stable. The previous equation was rearranged to yield $\mu_{\max}$:

$$\mu_{\max} = D - \frac{d \ln X}{(-dt)}$$

From these calculations, the maximum specific growth rate of N1-36 was established as 0.235 reciprocal hour (i. e., $\mu_{\max} = 0.235 \text{ hr}^{-1}$) (2, 8).

$\mu_{\max}$ can be converted to a minimum doubling time, $t_{d,\min}$ (or minimum generation time, G), according to the following equation:

$$\ln \frac{X}{X_0} = \mu t$$

When $X = 2X_0$, $t = t_d$:

and
$$t_d = \frac{\ln 2}{\mu}$$

$$t_{d,min} = \frac{\ln 2}{\mu_{max}}$$

The minimum doubling time (or generation time) obtained in this way for N1-36 is 2.95 hours (2, 8).

C. Experimental determinations of $Y_{x/s}$ and K_s

Continuous culture was also used to determine the "yield coefficient", $Y_{x/s}$, and the "saturation constant", K_s of *R. erythropolis* for DBT or DBTO₂. $Y_{x/s}$ is defined as:

$$Y_{x/s} = \frac{X - X_0}{S_0 - S}$$

where X is the steady state cell concentration and X_0 is the cell concentration at a specific growth rate of zero (i. e., when no substrate is used to support growth). S is the concentration of substrate in the bioreactor; S_0 is the concentration of substrate in the substrate reservoir. K_s is defined as the concentration of substrate which gives half maximal growth rate. K_s is a constant in the equation (of a rectangular hyperbola) which defines the relation between growth rate and substrate concentration, viz.,:

$$\mu = \frac{\mu_{max} S}{K_s + S}$$

The previous equation can be rearranged for the direct calculation of K_s . Specifically,

$$K_s = S \cdot \left(\frac{\mu_{max}}{\mu} - 1 \right)$$

To determine these values, the dilution rate of a bioreactor was sequentially set at different levels. After steady state was achieved at a given dilution rate, samples were taken; biomass and concentrations of substrate and product were measured.

FIGURE 10 "Wash out" curve for calculation of $\mu_{\max}$. (See text for experimental rationale.)

Figure 11 presents relevant data; the substrate was DBTO₂; OH-BP was the product; biomass (dry weight) was estimated from cell concentration. X was estimated by extending the biomass curve to the Y axis (yielding a value of 450 mg biomass). This value was then divided by the amount of substrate consumed. Since no substrate was detectable when the bioreactor was operated at low dilution rates, S was set at zero. Accordingly, with DBTO₂ at 50 μM , $Y_{X/S} = 9 \text{ mg}/\mu\text{M}$

The same sort of data were used to estimate K_S . A K_S equal to 0.39 μM DBTO₂ was achieved by averaging several measurements at dilution rates that were sufficiently high to produce credible data. Because some substrate was likely to be sequestered in the cells and thus escape measurement, the calculated K_S should be considered an underestimate. Though the consumption of DBT or DBTO₂ and production of OH-BP appear to be genuine physiological activities (desulfurization requires the presence of an inducer and the absence of $\text{SO}_4^{=}$), the affinity of the bacterium for the substrate appears low. The basis of this low affinity (poor transportation, unfavorable thermodynamics) needs to be carefully investigated. Such studies might indicate whether bacterial desulfurization of fossil fuels may be mediated more effectively by intact bacteria or, alternatively, cell free systems.

D. Experimental determination of Arrhenius equation

The Arrhenius equation (for bacteria) relates the growth rate, k , to the inverse of absolute temperature, T ; specifically

$$k = Ae^{-\frac{E_a}{RT}}$$

(k is the rate constant for growth (i.e., μ_{max}) or death (i.e., δ_{max}), T is absolute temperature, E_a is the activation energy for the process, and R is the gas constant (2).

For steady state growth at any temperature, K_S , S , and $\ln X$ are constant and $\ln X \gg \ln X_0$; thus,

$$\ln X \propto \mu \propto \mu_{\text{max}}$$

FIGURE 11 The relationship of specific growth rate ($\mu [= D]$) to observed biomass, (theoretical biomass), steady-state substrate (DBTO₂) concentration, and steady-state product (OH-BP) concentration.

Accordingly,

$$\ln X = B e^{-\frac{E_a}{RT}}$$

which can be rearranged to produce:

$$\ln(\ln X) = \ln B \left(-\frac{E_a}{R \cdot T} \right)$$

Figure 12 shows $\ln(\ln X)$ and [OH-BP] vs. temperatures in continuous culture. Both growth rate and OH-BP concentration achieve maximum values at 30° C (2).

E_a for growth between 24° C and 30° C, and between 21° C and 24° were calculated from the slopes in the Arrhenius plot as $E_{aG1} = 0.8366$ kcal/mol and $E_{aG2} = 8.155$ kcal/mol, respectively. E_a for death (between 30° C and 33° C) was calculated in a similar manner as $E_{aD} = -14.90$ kcal/mol (2).

E. Conclusions

Strain N1-36 of *R. erythropolis* has a maximum specific growth rate of 0.235 hr^{-1} which corresponds to a minimum doubling time of 2.95 hr. K_s for DBTO_2 was estimated to be $\approx 0.39 \text{ } \mu\text{M}$. The optimal temperature for growth and desulfurization was 30° C.

VII. REGULATION (AND SITE OF ACTIVITY)

A. Regulation of the DbtS^+ phenotype

The DbtS^+ phenotype is defined in terms of the ability to desulfurize DBT. However, the expression of this metabolic activity is not affected solely by the genetic information of the isolates; rather, environmental signals may be co-regulators of the genetic expression. A principal concern in determining the effect of environment was determination of the effect of $\text{SO}_4^{=}$.

To assess the effect of $\text{SO}_4^{=}$, five media were used: (i) $200 \text{ } \mu\text{M}$ $\text{MgSO}_4^{=}$ as sole sulfur source; (ii) $100 \text{ } \mu\text{M}$ DBT as sole sulfur source; (iii) $100 \text{ } \mu\text{M}$ DBTO_2 as sole sulfur source; (iv) $100 \text{ } \mu\text{M}$ DBT + $200 \text{ } \mu\text{M}$ $\text{MgSO}_4^{=}$ as sulfur sources; and (v) $100 \text{ } \mu\text{M}$ DBTO_2 + $200 \text{ } \mu\text{M}$ $\text{MgSO}_4^{=}$. N1-36 was grown in the media and the desulfurization activities with DBT and with DBTO_2 were measured; the experiment

FIGURE 12 Arrhenius profiles displaying temperature-dependent growth rates and temperature-dependent steady-state OH-BP concentrations.

was performed two times; in one instance the data were collected from cultures at 48.5 hr and 70 hr; in the other instance, data were collected at 21.5 hr, 43.7 hr, and 67.7 hr (2).

The observations can be summarized qualitatively as follows:

- when $\text{SO}_4^{=}$ was the sulfur source, no desulfurization activity was detectable with either DBT or DBTO_2 serving as substrate;
- when DBT was the sulfur source, desulfurization activity occurred with either DBT or DBTO_2 serving as substrate;
- when DBTO_2 was the sulfur source, desulfurization activity occurred with either DBT or DBTO_2 serving as substrate;
- when either $\text{DBT} + \text{SO}_4^{=}$ or $\text{DBTO}_2 + \text{SO}_4^{=}$ were the sulfur source, no desulfurization activity was detectable.

The qualitative observations show definitively that the presence of $\text{SO}_4^{=}$ precludes desulfurization activity. Further, the presence of DBT or DBTO_2 is needed for desulfurization activity. The data suggest that a thiophene or sulfone functions as an inducer and that sulfate may be a repressor (see below). (A lack of quantitative consistency among samples precludes a more detailed analysis. The quantitative differences may be attributable to nothing more than differences in growth condition.)

The desulfurization data do not distinguish between repression of genetic expression versus inhibition of enzyme activity. To distinguish between these possibilities, the following experiment was performed. N1-36 cells from two-day-old cultures were harvested and washed; the harvested cells had been growing in media with DBTO_2 as the sole sulfur source or with $\text{DBTO}_2 + \text{MgSO}_4^{=}$. The desulfurization activity of the cells grown on DBTO_2 alone was measured with and without the addition of $\text{MgSO}_4^{=}$; the desulfurization activity of the cells grown on $\text{DBTO}_2 + \text{MgSO}_4^{=}$ was

measured without additional MgSO_4 . The results showed that when MgSO_4 was present in the culture medium, no desulfurization activity was detectable. But when MgSO_4 was added to the desulfurization assay for cells grown in the absence of (exogenous) SO_4 , no appreciable effect was seen from the addition. Accordingly, the SO_4 appears to affect gene expression and not affect enzyme activity (2).

B. Site of activities

The sub-cellular site of desulfurization of DBTO_2 was studied. (These "studies," it must be emphasized, were very preliminary.) Desulfurization activity was studied with washed cells, culture filtrates, irradiated cells, membrane preparations, and cell extracts.

The intent of comparing culture filtrate versus washed cells was to determine if the desulfurization activity was confined to cells or whether the mediator of the activity was released into the culture medium. The data showed that cells were far more active with respect to both substrate disappearance and product appearance than were filtrates. The slight activity in filtrates may arise from cells lysed during growth or filtration. Boiled (denatured) filtrates showed no detectable desulfurization activity (2).

The intent of comparing irradiated cells versus washed cells was to determine if desulfurization activity requires viable cells. Cells were irradiated to an extent that destroyed their ability to replicate and form colonies on solid media. Qualitative results indicate that such cells have high activity; that is to say, approximately equivalent to that seen with washed cells. Irradiated cells that were harvested, washed, and suspended in buffer had somewhat reduced activity (2).

Cells were fractionated to determine if desulfurization activities were associated with a specific fraction. N1-36 cells were grown in DBTO_2 medium, harvested, washed, and resuspended in pH 6.0 phosphate buffer. The suspension was sonified for 5 min at setting "6.5" (Branson Cell Disrupter 200 Sonifier) in an ice water bath. The sonicate was centrifuged at $5000 \times g$ for 30 min

to pellet cell wall fragments and large membranous structures. The supernatant was incubated at 21° C for 30 min; 21° C is assumed to be a temperature above the phase transition temperature of the membrane lipid; accordingly, the incubation should allow tiny membrane fragments to fuse into larger fragments which could be sedimented. After incubation, the supernatant was centrifuged at 30,000 x g for 30 minutes. The data showed that entire cells had desulfurization activity, that fractions com.lh13

prised of walls or membranes had marginal activity, and that no activity was detectable in the cytoplasmic fraction (2).

C. Disappearance of substrate versus appearance of product

The concentrations of DBT and DBTO₂ decrease in media inoculated with *R. erythropolis* regardless of whether or not SO₄⁼ is present. Stated another way, the abundance of substrate decreases even under conditions when desulfurization activity has been repressed; i. e., when no product is generated. This observation suggests that uptake of substrate and initial oxidation may be phenomena separate from desulfurization (2).

The disappearance of DBT may reflect dissolution into the limiting membrane of the cell or association with some protein. In the latter instance, the association may be specific or non-specific.

D. Conclusion (and questions)

The foregoing observations and speculation indicate that the events of the "4S pathway" need to be more completely defined. Which activities are mediated by proteins? Which activities are "passive"? To what extent are the events metabolically coordinated? Where are the activities located in cells?

These questions underscore even more basic concerns. The sequence of oxidations in the "4S" pathway are defined. But the source of oxygen remains unspecified. Is the source molecular oxygen? ...peroxide? ...an organic peroxide? The most fundamental question regards the extent to which the "4S" pathway is "real." The possibility exists that the phenotype is recognized because of the way isolates are selected and subsequently ob-

served. Such a circumstance is wholly acceptable for the pragmatic goal of bacterial desulfurization of fossil fuels. Nonetheless, the prospect of an industrially successful bioprocess is greatly enhanced by achieving a consistent, coherent, comprehensive understanding of the phenomenon.

VIII. GENERAL CONCLUSION

Fresh isolates of bacteria presumptively identified as *R. erythropolis* unequivocally have a DbtS^+ phenotype. The production of OH-BP from either DBT or DBTO_2 was confirmed by difference spectroscopy, HPLC, and mass spectrometry. The temperature, pH, and means of supplying the thiophenic or sulfonic sole source of sulfur were optimized. The maximal rate of growth of the organism, its affinity for sulfone, and the extent to which substrate was converted to product were determined by using batch, fed batch, and continuous cultures. For strain N1-36, the maximum specific growth rate was 0.235 hr^{-1} which corresponds to a minimal generation time of 2.95 hr. The K_s was estimated to be $0.39 \mu\text{M}$. With $100 \mu\text{M}$ DBT as the sole sulfur source, approximately $40 \mu\text{M}$ OH-BP are produced (after 40 hr of growth); with $100 \mu\text{M}$ DBTO_2 as the sole sulfur source, approximately $70 \mu\text{M}$ OH-BP are produced (after 40 hr of growth). The desulfurization activity is repressed by $\text{SO}_4^{=}$. OH-BP does not serve as a carbon source.

The DbtS^+ phenotype of the *R. erythropolis* isolates is stable and discrete. The isolates selectively remove sulfur from DBT, a compound which models a refractory form of organic sulfur in compounds characteristic of fossil fuels. The desulfurization occurs with no oxidation of carbon-carbon bonds. The stability and specificity (along with genetic regulation) indicate that microbial desulfurization is a real phenomenon in which a noxious element is removed without significantly affecting the calorific value of the substrate. Additional characterization (and optimization) would provide the basis of a very important form of fossil fuel beneficiation. The United States Department of Energy should be in the forefront of this research.

IX. APPENDICES**A. APPENDIX I: THE PRESENCE AND INSTABILITY OF THE DbtS⁺ PHENOTYPE IN *Pseudomonas* SPP.**

The original intent of the contracted research was characterization of the DbtS⁺ phenotype in *Pseudomonas* spp. Then, the genetic determinants for the well-characterized phenotype were to be mobilized with a broad-host-range plasmid, R68.45, and transmitted interspecifically among *Pseudomonas* recipients. A longer term goal was to use such "in vivo genetic engineering" to transmit the determinant intergenerically to *Thiobacillae* spp. capable of oxidizing the inorganic sulfur of coal.

Initially, this work progressed very well. Exacting protocols were used to demonstrate persuasively that the phenotype could be mobilized and that "transconjugants" were generated having the DbtS⁺ phenotype (from the donor), antibiotic resistances from R68.45, and, principally, the phenotype of the recipient to which the dbtS⁺ determinants had been transferred. The same rigor showed that the DbtS⁺ phenotype in the transconjugants was not stable. After isolation and sub-culturing and storage, many, at first, and then all of the transconjugants lacked the DbtS⁺ phenotype.

The collection of the dozens of original isolates having the DbtS⁺ phenotype was taken from storage and the individual isolates were re-examined. Again, the phenotype was, more often than not, diminished in its intensity or completely absent.

At this juncture, a decision was made to obtain fresh isolates from soil samples and to devise a continuous selection for the maintenance of the phenotype. Dr. Wang Ping undertook this work which produced not additional *Pseudomonas* spp. but *Rhodococcus erythropolis* which she used very effectively.

During the year that the contract was extended (without additional cost to the Department of Energy), Mr. Todd Davies isolated fresh *Pseudomonas* spp. with the DbtS⁺ phenotype. The newly isolated strains were maintained on a (standard) defined glucose-salts medium (containing an inorganic sulfur source,

viz., Mg_2SO_4^-); the isolates were transferred approximately once a month. Care was taken to be certain that the desulfurization product produced from DBT was a phenolic compound. Accordingly, the presence of a phenolic product was scored qualitatively by fluorescence and quantitatively by a chromogenic assay (see section II. B. 1 and II. B. 2 of the final report). (Failure to measure specifically the presence of a phenolic product can generate great confusion [cf. 9].) The intensities of the chromogenic response observed in three isolates over a 10.5 month period are presented in Figure 13.

The data unequivocally show the loss of the phenotype. Since these were isolates obtained from soil samples and not transconjugants, the instability cannot be attributed to the introduction of a broad-host-range plasmid (like R68.45).

Notably, this phenomenon, viz., isolation of *Pseudomonas* spp. with a DbtS^+ phenotype from a soil habitat, maintenance of the organism in the laboratory under standard conditions, and loss of the relevant phenotype, has occurred before; specifically, an organism CB1, the focus of much interest in the late 1980's, suffered the same fate as isolates in the Lehigh collection. Also, other investigators who had received isolates from Lehigh observed that the DbtS^+ phenotype disappeared progressively; first the organisms no longer produced o,o'-biphenol (the product generated by *Pseudomonas* spp. from DBT [see section I. A. for products of the "4S" pathway) and, later, loss of the ability to produce the sulfonate.

The recurrent loss of the DbtS^+ from *Pseudomonas* spp. is a practical problem. At the same time, it is a fascinating biological phenomenon. Why should a bacterium maintained under standard (favorable) conditions lose a phenotype? Could this loss be overcome by selection? Can the presence of the DbtS^+ phenotype be both optimized and stabilized? What would be learned about bacteria generally and microbial desulfurization specifically by such important investigations?

FIGURE 13 The temporal course of the decreasing ability of three *Pseudomonas* isolates to produce phenolic products from DBTO₂

B. APPENDIX II: THE STRUCTURE OF THE BROAD-HOST-RANGE PLASMID, R68.45, AND DERIVED R PRIMES AS DETERMINED BY PFGE

The broad-host-range plasmid, R68.45, was used successfully to transfer determinants for the DbtS⁺ phenotype from one *Pseudomonas* species to another. The mechanism for this transfer was not known. A likely means of transfer, though, was that the genetic determinants for the DbtS⁺ phenotype residing in a "donor" organism became incorporated into the R68.45 plasmid and were then transferred to a "recipient" organism. In such a mechanism, the R-plasmid which has acquired genetic information from the donor is said to be an R' (R-prime). If the R' lost none of its original genetic information or if the amount lost were different from the amount gained, R-plasmids and R-primes would be different in their size.

To determine if R68.45 and R-primes had different physical organizations, the plasmids and primes were isolated, purified, and digested with restriction endonucleases. The fragments produced were too large to be examined by conventional agarose gel electrophoresis. Accordingly, Pulsed-Field Gel Electrophoresis (PFGE) was used to resolve the macrofragments. The analysis produced two important results. First, the technique directly substantiated earlier (indirect) inferences about the size of R68.45; the data indicated that the broad-host-range plasmid is a structure with approximately 60 kb. Second, the analysis showed that R-primes were larger and that a specific fragment produced by restriction endonucleases, the one which contains repeats of an insertion sequence, IS21, was increased in size (10).

C. APPENDIX III: THE GENOMIC STRUCTURE OF *Thiobacillus acidophilus* AND *Thiobacillus novellus* AS DETERMINED BY PFGE

Determining the species specific and generic identities of bacteria is a challenging task (see section IV). Discriminating among phenotypes in bacteria is often a daunting, if not an impossible, task. As noted in the final report, PFGE of genomic macrofragments is emerging as the definitive means of identifying a bacterium. This methodology is relevant to the long term goal of complete microbial desulfurization of fossil fuels.

Organisms such as the *Rhodococcus* spp. isolated by this laboratory and members of other laboratories (e. g., individuals at the Institute of Gas Technology) are effective in removing organic sulfur from compounds that model especially refractory forms of sulfur in fossil fuels. Inorganic sulfur can be removed microbially by members of another genus, viz., *Thiobacillus*. Proponents of microbial desulfurization envision the transfer of the genetic determinants for desulfurization of organic sulfur to a *Thiobacillus* spp. and, in doing so, creating a "super bug" capable of selectively removing both organic and inorganic sulfur. (Hence, the interest noted in Appendix I of transferring the determinants for the Dbts^+ phenotype using a broad-host-range plasmid.)

For success in this long term goal, a means of identifying definitively the species receiving new genetic information is essential. PFGE of genomic macrofragments provides a means for such definitive identification. Genomic characterization of *Thiobacillus* species is an important pre-requisite to the construction of the desired "super-bug." During the time of contractual research, preliminary studies of this sort were performed with two species of *Thiobacillus*, viz., *T. novellus* and *T. acidophilus*. The chromosome of *T. novellus* was digested with *Dra*I and *Xba*I yielding 15 and 13 bands, respectively. Using these two enzymes, the chromosome size of *T. novellus* is estimated, preliminarily, to be 3.3 Mbp and 3.7 Mbp. The *T. acidophilus* genome was digested with *Spe*I and *Xba*I yielding, in each case, 15 fragments; the corresponding estimates of genome size are 3.5 Mbp and 3.3 Mbp (11). Current efforts to cure *T. acidophilus* of genetically cryptic but physically observable plasmids will differentiate between the contributions to genome size made by plasmids and the chromosome.

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