TECHNICAL REPORT
March 1 1989 through May 31, 1990

Project Title: Desulfurization of Coal by Genetically Modified Bacteria
ICCI Project Number: 89/1.3B-3
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ABSTRACT

We will isolate and characterize bacteria which have the ability to carry out each of the presumed four main steps required for the conversion of organic sulfur at the thiophene/sulfide level of oxidation to the soluble end product, sulfate. Eventually the genes for each step will be cloned into plasmids and analyzed genetically. They will then be assembled to give a genetic module which codes for the whole desulfurization pathway. The final organism will be tested for its removal of sulfur from finely ground high-sulfur Illinois coal.

In this reporting period we have screened a variety of clones which confer the ability to degrade sulfonic acids upon an E. coli host strain. Unfortunately all of these are unstable or detrimental to growth. We have obtained low-copy plasmids and will re-clone these genes. A clone bank covering 97% of the genome of strain DBTS2 has been constructed and is ready for screening. Mutants of strain BSA56 which show improved degradation of sulfones were isolated by resistance to dapsone. Growth conditions for desulfurization of model compounds by BSA56 and DBTS-series strains have been optimized. A new soil isolate which releases sulfur from DBT as hydrogen sulfide is being characterized.
EXECUTIVE SUMMARY

The ultimate purpose of this project is to provide a biotechnological alternative to the chemical and physical methods of coal purification. The vast reserves of Midwestern coal with a high sulfur content need to be desulfurized to avoid environmental pollution, in particular acid rain. Physical and chemical approaches to coal desulfurization tend to share the drawback of being rather expensive. Biological technology is still in its infancy and is the outsider in the race. However, using bacteria to clean coal should be substantially cheaper if a process can be developed. Although much needs to be done, the preliminary work on bacterial desulfurization has been promising and the possibility of an eventual biotechnological process has become a realistic alternative.

Our previous work has provided us with a series of different bacterial strains, mostly *Pseudomonas*, which show partial degradative ability towards a wide range of model organic sulfur compounds. Our overall strategy is to improve and combine the properties of several diverse bacterial strains in order to construct an efficient desulfurizing bacterium.

In the last reporting period we have examined the desulfurization ability of our best strains with regard to inoculum size, aeration, pH, and substrate concentration. Optimization of these parameters substantially increases the percentage sulfur release observed and this now approaches 90-100% for many model compounds over a seven day period. New natural isolates which degrade dibenzothiophene and release sulfur in the form of $\text{H}_2\text{S}$ rather than sulfate have been isolated and their characterization is in progress.

Several genes conferring the ability to degrade various aromatic sulfonic acids have been cloned from strain BSA56. However, when present in *E. coli* in high copy number they proved to be deleterious to growth. We are presently re-cloning these genes in medium to low copy number plasmids in the hope of achieving stable and healthy clones. A clone bank of the DBT sulfoxide degrader DBTS2 has been constructed in a wide host range cosmid, and will be screened in the near future. Mutants of BSA56 showing greatly improved growth on a range of sulfones have been isolated by selecting for resistance to dapsone, an antibiotic with a sulfone structure.
OBJECTIVES

The overall goal is to construct bacteria capable of degrading the organic sulfur compounds found in coal so releasing sulfate. Specifically:

1. Improvement of the degradative abilities of bacteria isolated previously and which can degrade such model compounds as dibenzothiophene, DBT sulfone and benzene sulfonic acid.

2. Characterization and cloning of genes for (a) the release of sulfate from sulfonates and (b) conversion of sulfones to sulfonates in the first year. Cloning of genes for other steps of the pathway is projected for the second year.

3. Optimizing gene expression and

4. Assembling the cloned genes are projected for the second year.

This year's objectives involve the further characterization and improvement of selected bacteria which have been previously isolated from the wild. Before actually cloning genes, it helps to know the detailed physiological properties they confer. Furthermore, it is necessary to gain some insight into how the genes of interest are regulated. A gene is of no practical value unless we know how to induce it so that it expresses itself. Finally, we hope to clone genes involved in the last half of the desulfurization pathway i.e:

Sulfone → Sulfonate → Sulfate

we will use DBT sulfone and phenyl sulfone as starting materials and hope to produce an efficient system for their desulfurization.

INTRODUCTION AND BACKGROUND

The utilization of coal within the U.S. has increased dramatically since the early 1970s. Coal production is expected to double between 1978 and 1990, with Appalachian and Illinoisian fields as the largest sources of this coal. One problem associated with using such coal is the emission of SO2 during combustion. Physical and chemical
methods of coal desulfurization are either expensive or result in a loss of fine coal particles. The sulfur content of Appalachian and Illinoisian coal varies from 3.0 to 5.5% and consists of various thiol, sulfide, disulfide, and thiophene groups. At present, both Thiobacillus and Sulfolobus have been shown capable of converting the inorganic sulfur in coal into soluble sulfate, which can be washed out. Our intent is to complement this by developing bacteria capable of degrading the organic sulfur into sulfate.

**EXPERIMENTAL PROCEDURES**

The techniques and procedures used have mostly been described in our previous technical reports.

**Characterization of Current Soil Isolates:**

Employing the sulfur-deficient mineral salts medium described in previous progress reports, the soil isolates were evaluated with the appropriate model compound for growth rates, substrate specificity, substrate concentration, and inoculum size. Growth rates, sulfate-sulfur, and model compound concentration were determined as previously described.

**Isolation of New Strains for Thiophene Desulfurization.**

Several new strains (from a total of 33) were evaluated for the effect of temperature, sulfate concentration, and yeast extract concentration on DBT hydrolysis. For these experiments, the growth medium pH was 6.0, and cultures were incubated for 21 days. For the latter two experiments, the incubation temperature was 28°C. Sulfate-sulfur concentration varied from 0.1 to 10mM as Na₂SO₄, while yeast extract varied from 0.003 to 0.2%. DBT was sprayed onto solid media employing a 0.5% stock solution in thyl ether; while broth cultures employed a 0.05% concentration of DBT.

One soil isolate (T) was chosen for further study and evaluated for its ability for growth and hydrolysis on carbazole, dibenzofuran, dibenzothiophene sulphone, flourene, and thianthrene. The growth medium contained 0.005% yeast extract, and the pH was adjusted to 6.0. Incubation time was for 21 days at 28°C. The test model compounds were prepared as 0.5% stock solutions in ethyl ether or benzene, and sprayed on the surface of the plates.
RESULTS AND DISCUSSION

Characterization of Current Soil Isolates.

Table 1 summarizes the generation times and growth rates for strains Bwt, DBTS-2 19, 22, and 26, and BSA-56. As previously reported the generation time for most of these strains varied from 40.5 to 47.7 hours. However, the actual population increase per hour was 1.59% for strain Bwt, 0.60 to 0.77% for the DBTS strains, and 0.18% for strain BSA-56. These results imply that the slow growth rates of these bacteria will result in slow desulfurization rates unless higher inoculum rates are used, or that the genes involved in the desulfurization step are amplified by cloning.

Figures 1 through 6 illustrate the ability of all 6 strains to grow on DBT, DBTS, and BSA. Except for strain BSA-56, each strain showed a preference for the model compound that was employed for their initial isolation. Some growth was noted by strain Bwt on DBTS and BSA, and by the DBTS strains on DBT and BSA. Higher growth rates were observed with strain BSA-56 on DBT and DBTS. Theses results indicate that these strains are capable of growth on different forms of organic sulfur other than the initial model compound used for isolation. However, due to the different forms of S associated with thiophenes, growth does not continue with zero to low desulfurization rates (0-3.2%).

Figures 7 through 11 show the effect of substrate concentration (0.02%, 0.2%, and 2%) on the growth of strains BWT, DBTS-2, 19, 22, and 26. Figure 12 summarizes the effect of BSA concentration (0.02%, 0.088%, 0.2%, 0.88%, and 8.8%) on strain BSA-56. Strain Bwt showed the greatest amount of growth on 0.2% DBT, while all of the DBTS strains showed the highest level of growth with 2% DBTS. It should be noted that strains DBTS-2 and 26 had a shorter lag phase (48 hours) than strains DBTS-19 and 22 (96 hours). For strain BSA-56, the greatest amount of growth occured with 0.088% BSA, while similar growth patterns were observed with 0.02, 0.2, and 0.88% BSA. No growth was observed for 2% (data not shown) or 8.8% BSA. The lack of growth can not be attributed to low pH, since the final pH in the growth medium for all concentrations of BSA was adjusted to 6.8. The data suggests that high concentrations of sulfonic acids may be toxic and inhibit growth. The limited ability of strains Bwt and DBTS-2, 19, 22, and 26 to grow on BSA (Figures 1 through 6) may be related to a sulfoinc acid toxicity.
Figure 13 summarizes the percent desulfurization rates of strains DBTS-2, 19, 22 and 26, and BSA-56 as a function of substrate concentration. In all cases, as the substrate concentration increases, the percent desulfurization (after 7 days of incubation) decreases. A substrate concentration of 0.02% BSA resulted in 100% desulfurization by strain BSA-56, while a concentration of 0.2% DBTS resulted in a desulfurization rate of 50 to 80% by the DBTS strains. These data suggest that it may be easier to desulfurize higher concentrations of sulfones than sulfonic acids, and again implies the possible toxicity of higher concentrations of BSA. The lack of an inhibitory effect by 0.2% DBTS may suggest an alternative desulfurization pathway used by the DBTS strains versus that of strain BSA-56.

Figures 14 through 19 illustrate the effect of inoculum size of all 6 strains on 2% substrate concentration. For strain Bwt, the inoculation rate of 8.12 X 10^7 cells/ml was the only treatment that sustained growth for the entire duration of the experiment (240 hours). Growth was observed for the inoculation rate of 8.12 X 10^5 cells/ml until 180 hours followed by cell death. Strains DBTS-2, 19, and 22 all showed the highest growth rate with the highest inoculation rate (10^8 cells/ml) accompanied by the shortest lag period (56 hours). The inoculation rate of 10^6 cells/ml showed increased growth with time following a 96 hour lag period. However, strain DBTS-26 with the inoculation rates of 9.24 X 10^7 cell/ml, and 9.24 X 10^5 cells/ml showed equivalent rates of growth following 96 hours of growth. The former inoculation rate had a lag period of only 20 hours, while the latter rate had a lag period of 66 hours. The lowest inoculation rate also showed an increase in growth after 160 hours. These results suggest that strain DBTS-26 should be used in preference to the other DBTS strains for future coal desulfurization studies. Strain BSA-56, like strain Bwt, only showed sustained growth at the highest inoculation rate (4.96 X 10^9 cells/ml). All of these results again indicate the need to use high rates of inoculation or amplification of the desulfurization genes if coal desulfurization is to be successful.

Currently, the concentrations of sulfate-sulfur and the model compounds used in these studies are being determined. Sulfur balance sheets will be summarized in our next quarterly report.

Isolation of New Strains for Thiophene Desulfurization.

As previously described in our last quarterly report, 33 new soil
isolates have been cultured that have the ability to hydrolyze DBT. The optimum pH for growth and DBT hydrolysis is 6.0, with H₂S as the desulfurization product. Since these organisms reduce S, all cultures are grown statically, and appear to require the same growth conditions.

Table 2 summarizes the effect of temperature on soil isolate T. Optimum growth and DBT hydrolysis occurred at a temperature of 30°C. In another study (Table 3) isolate T was evaluated for its ability to grow and hydrolyze carbazole, dibenzofuran, dibenzothiophene sulfone, fluorene, and thianthrene. No growth and hydrolysis was observed with carbazole, dibenzofuran, fluorene and thianthrene, while poor growth and hydrolysis was detected with dibenzothiophene sulfone. These results show that isolate T (and probably the other soil isolates) has the same temperature requirement as our 6 current strains previously mentioned in this report, and similarly shows specificity to the original model compound used for isolation. These results also imply that for any given thiophene compound a specific organism may exist for desulfurization.

Table 4 summarizes the effect of sulfate concentration on the growth of soil isolates T, DD, HH and LL, and on DBT hydrolysis. For all strains tested, no inhibition of growth or DBT hydrolysis was observed. These results suggest that the desulfurization genes are probably regulated by a metabolite or end product of DBT utilization.

Figure 20 summarizes the effect of yeast extract concentration on DBT hydrolysis. The optimum concentration of yeast extract is approximately 0.05%. These results indicate that isolate T (and possibly our other isolates) require low concentrations of growth factors for optimal DBT hydrolysis. Higher concentrations of yeast extract are inhibitory since this growth supplement can act as an alternate carbon source for growth. We will continue to evaluate the growth requirement of our cultures to optimize the desulfurization of the tested model compounds.

Construction of Genomic Library of DBTS2.

Chromosomal DNA of DBTS2 was partially digested with Xho 1 and ligated to the Xho 1 site in the cosmid vector pVK100. Recombinant cosmids carried DNA fragments sized from 19 kb to 29 kb.
The sum of 672 clones were collected. Assuming the genome size of strain DBTS2 to be 5,000 kb (e.g. *E. coli* is 4.200 kb), this clone bank covers more than 97% of the genome. Mutants which no longer utilize DBT-sulfone were isolated by EMS and NTG mutagenesis. These deficient mutants will be used as host strains in screening the cosmid bank. The clones which can restore the DBT-sulfone utilization (hydrolysis) will be selected and analyzed.

**Growth of Bwt strain on Dibenzothiophene**

Strain Bwt utilizes biphenyl and benzoate but cannot utilize naphthalene. Also, Bwt metabolized DBT to some extent, at a starting concentration of DBT less than 0.05% w/v [2.7 mM]. DBT inhibited the growth at 0.1% (5.4 mM). This strain excretes an orange pigment into the medium in the presence of biphenyl and DBT. This maybe a surfactant which emulsifies hydrophobic hydrocarbons.

Hydrophobic degradation intermediates of DBT were extracted by chioroform and the UV wavelength range (340nm - 200nm) was scanned by use of a Hitachi spectrometer. Results are listed on Table 1. At 48 hours, two peaks at 255nm appeared. The peak at 247nm kept growing while the height of the peak at 255nm decreased at 72 hours. Two new peaks emerged at 230nm and 204nm and disappeared by 120 hours. Further analysis will be completed by HPLC to identify the intermediates.

A variety of Bwt mutant strains were made. These mutants were selected on the basis of colony pigmentation and loss of ability to grow on biphenyl and benzoate. Currently, these mutants are being tested for DBT degradation.

**Cloning of Sulfonate Degradation Genes from BSA56.**

The overall procedure has been described previously and is summarized in Figure 1. The isolates we obtained consisted of plasmid pJOE810 carrying a fragment of chromosomal DNA from strain BSA56. These plasmids are now in host strain DC1090 which cannot normally grow without a supply of the sulfur containing amino acid cysteine. They confer the ability for DC1090 to grow using aromatic sulfonates instead of cysteine. Table 6 lists several such plasmid isolates and their ability in the degradation of benzene sulfonic acid (BSA), toluene sulfonic acid (TSA), pyridine-3-sulfonic acid (PSA) and naphthalene-1-sulfonic acid (NSA). Some isolates
were found which degrade each of these substrates, in several combinations. Presumably at least 3 or 4 separate genes must be involved.

Unfortunately these isolates were either very sick and slow growing or else they grew well but tended to lose the plasmid and hence their sulfonate degrading properties. We feel the reason for this is that plasmids derived from pJOE810 are present in 50 or more copies per cell and that the sulfonate degradation genes are therefore expressed at such high levels as to be toxic.

We will tackle this problem by changing to use plasmids which have only 5 to 10 copies per cell. We are presently carrying out preliminary experiments using two such plasmids, pMAK904 and pHSG575. Both are low copy plasmids which have the same polylinker cloning sites as pUC19 derived plasmids like pJOE810. Hence the same restriction enzymes can be used. Instead of ampicillin, plasmid pMAK904 is selected by kanamycin resistance and plasmid pHSG575 chloramphenicol.

We have made DNA preparations of both new plasmids. Because these plasmids are only present at 5-10 copies per cell, it is necessary to grow much larger cultures for DNA extraction and this part of the procedure therefore unfortunately takes longer. We will be ligating and selecting in the near future.

**Sulfone Using Derivatives of BSA56.**

Strain BSA56 grows moderately well with a wide range of sulfonates but very poorly with sulfones (See our first Quarterly Report). We selected mutants of BSA56 which are resistant to the antibacterial agent dapsone. Dapsone is bis (aminophenyl) sulfone and is used in the treatment of leprosy and malaria as the drug of second choice. Strain BSA56 is highly susceptible to dapsone and is killed by 15 μg/ml in minimal medium. After mutagenesis with ethyl methane sulfonate, we plated aliquots of a BSA56 culture onto medium containing higher concentrations of dapsone. Several mutants were isolated. These were tested for resistance to dapsone [Table 7] and for their growth properties versus a variety of sulfones, both as sulfur source [Table 8] and carbon source [Table 9]. Strain BSA56 grows on sulfonic acids after 5-6 days, and shows very poor growth on sulfones. The dapsone resistant derivatives grow well on several sulfonic acids after only 3 days and also grow on both aliphatic and
aromatic sulfones. Most of the sulfonates and sulfones could be used both as sulfur and/or carbon source for growth. We also found that dapsone resistant mutants #1-3 grew poorly on dapsone itself as C-source and mutants #4-6 grew quite well on dapsone. Thus resistance is presumable due to the ability to efficiently degrade sulfone compounds as seen both for dapsone itself and for phenyl sulfone, DBT sulfone etc. Dapsone is not soluble above the concentrations used in Table 7, so it is not possible to test if higher levels could kill the mutants.
### Table 1. Generation Time and Growth Rates of Soil Isolates. *

<table>
<thead>
<tr>
<th>Strain</th>
<th>Model Compound</th>
<th>Generation Time (hr)</th>
<th>Growth Rate (% Increase/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bwt</td>
<td>DBT</td>
<td>46.1</td>
<td>1.59</td>
</tr>
<tr>
<td>DBTS-2</td>
<td>DBTS</td>
<td>40.5</td>
<td>0.77</td>
</tr>
<tr>
<td>DBTS-19</td>
<td>DBTS</td>
<td>47.7</td>
<td>0.60</td>
</tr>
<tr>
<td>DBTS-22</td>
<td>DBTS</td>
<td>45.3</td>
<td>0.60</td>
</tr>
<tr>
<td>DBTS-26</td>
<td>DBTS</td>
<td>46.2</td>
<td>0.77</td>
</tr>
<tr>
<td>BSA-56</td>
<td>DBTS</td>
<td>57.5</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* pH = 7.0, incubation temperature -28°C, substrate concentration was 0.05%.

### Table 2. Effect of Temperature on Growth and DBT hydrolysis by Isolate T. *

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Growth</th>
<th>DBT Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>±</td>
<td>-</td>
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<td>20</td>
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</tr>
<tr>
<td>25</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- no growth  ± weak growth  + growth  ++ good growth

* Growth medium contained 0.005% yeast extract; pH=6.0; incubation time was 21 days. Plates were sprayed with 0.5% DBT in ethyl ether.
Table 3. Evaluation of Isolate T for Growth on Aromatic Heterocyclic Compounds. *

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Growth</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbazole</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dibenzothiophene-sulfone</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Flourene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thianthrene</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- negative ± weak + positive

* Growth medium contained 0.005% yeast extract; pH = 6.0; incubation time = 21 days; temperature = 28°C. Plates were sprayed with 0.5% substrate in ethyl ether or benzene.

Table 4. Effect of Sulfate on the Growth of Isolates T, DD, HH and LL, and DBT Hydrolysis. *

<table>
<thead>
<tr>
<th>Sulfate Concentration, mM Na₂SO₄</th>
<th>Soil Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>G**</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
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<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

* Growth medium contained 0.005% yeast extract; pH = 6.0; incubation time = 21 days; temperature = 28°C. Plates were sprayed with 0.5% DBT in ethyl ether.
** G = growth; H = hydrolysis
Table 5. UV Wavelength Scan of DBT Degradation Intermediates of Bwt

<table>
<thead>
<tr>
<th></th>
<th>323,312, 285, 278*, 262 nm</th>
</tr>
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<tbody>
<tr>
<td>DBT</td>
<td>DBT-5-oxide</td>
</tr>
<tr>
<td>48 hrs extract</td>
<td>324, 313, 286, 278*, 262*, 255, 247 nm</td>
</tr>
<tr>
<td>72 hrs extract</td>
<td>324, 313, 286, 278*, 262*, 255*, 2457, 240*, 230, 208 nm</td>
</tr>
<tr>
<td>120 hrs extract</td>
<td>324, 313, 286, 278*, 255*, 247 nm</td>
</tr>
</tbody>
</table>

* Shoulder

Table 6. Cloning of Genes for Sulfonate Degradation

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Enzyme</th>
<th>BSA</th>
<th>TSA</th>
<th>PSA</th>
<th>NSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>XbaI</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>SphI</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>++</td>
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<tr>
<td>H</td>
<td>SphI</td>
<td>±</td>
<td>±</td>
<td>-</td>
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<tr>
<td>I</td>
<td>HindIII</td>
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<td>±</td>
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<tr>
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<td>SphI</td>
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<td>++</td>
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<tr>
<td>T</td>
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<tr>
<td>U</td>
<td>KpnI</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

BSA = benzene sulfonic acid; TSA = toluene sulfonic acid;
PSA = pyridine sulfonic acid; NSE = 1-naphthalene sulfonic acid.
Table 7. Resistance of NTG-mutagenised BSA-56 to Dapsone.

<table>
<thead>
<tr>
<th>Dapsone (µg/ml)</th>
<th>BSA-56 (parent)</th>
<th>Dapsone-resistant mutants 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1000</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1200</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Medium - M9 with 1 mM MgSO₄, 0.4% lactate
Incubation was done at 37°C for 3 days.
Table 8. Growth of Dapsone-resistant mutants on different aliphatic and aromatic sulfur compounds as a sole source of sulfur.

<table>
<thead>
<tr>
<th>S - Source</th>
<th>Parent Strain (BSA-56)</th>
<th>Dapsone - Resistant Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ethyl Sulfone</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>1 - NSA</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Phenyl sulfone</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>TSA #</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Methyl Sulfone</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>TMSO₂</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Sulfanilate</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>DBTSO₂</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

# Growth on TSA as S - source produced pinkish - yellow colonies, indicative of a colored intermediate in the aromatic degradation.

Medium:- M9 with 1 mM MgCl₂, 0.4% Glycerol and 0.1% S-source (= test compound). Cultures were grown at 37°C for 5 days.

* no growth but with spontaneous mutants.
Table 9. Growth of Dapsone resistant mutants on different aliphatic and aromatic sulfur compounds as a carbon source.

<table>
<thead>
<tr>
<th>C - Source</th>
<th>Parent Strain (BSA-56)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-(OH)2 Benzoate</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>TSA</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Sulfanilate</td>
<td>-</td>
<td>+/-</td>
<td>*-</td>
<td>*-</td>
<td>*-</td>
<td>*-</td>
<td>*-</td>
</tr>
<tr>
<td>TMSO₂</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Ethyl Sulfone</td>
<td>+/-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Methyl Sulfone</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Phenyl Sulfone</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DBT Sulfone</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>1 - NSA</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Ethane Sulfonate</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Butane Sulfonate</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Benzene Sulfonate</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* no growth, but with spontaneous mutants.

Medium: M9 with 1mM MgSO₄ and 0.1% of C - source.
Growth was at 37°C for 3 days.
Fig. 1. Substrate Specificity of Strain Bwt

Absorbance 600nm

Time, hours

--- DBTS  --- BSA  --- DBT
Fig. 2. Substrate Specificity for Strain DBTS-2

Absorbance 800nm

Time, hours

Substrate

--- DBTS

--- BSA

--- DBT
Fig. 3. Substrate Specificity for Strain DBTS-19

Absorbance 600nm

Substrate

--- DBTS  --- BSA  --- DBT

Time, hours
Fig. 4. Substrate Specificity for Strain DBTS-22

Absorbance
600 nm

Time, hours

Substrate

--- DBTS  --- BSA  --- DBT
Fig. 5. Substrate Specificity for Strain DBTS-26

Absorbance
600nm

0 15 30 45 60 75 90 105 120 135 150 165
Time, hours

Substrate

DBTS
BSA
DBT
Fig. 6. Substrate Specificity for Strain BSA-56

Absorbance at 600nm vs Time (hours)

Substrate:
- DBTS
- BSA
- DBT
Fig. 7.

Growth Response of Bwt to Substrate Concentration

\[ \text{Absorbance} \]

\begin{align*}
&0.5 \\
&0.4 \\
&0.3 \\
&0.2 \\
&0.1 \\
&0.0 \\
\end{align*}

\begin{align*}
\text{Time, hour} & = 0 \quad 24 \quad 48 \quad 72 \quad 96 \quad 120 \quad 144 \quad 168 \\
\end{align*}

- 0.02% DBT
- 0.2% DBT
- 2% DBT
Fig. 8. Growth Response of DBTS-2 to Substrate Concentration

Absorbance
600 nm

Time, hours

0  24  48  72  96  120  144  168

--- 0.02% DBTS  +  0.2% DBTS  ←  2% DBTS
Fig. 9.

Growth Response of DBTS-19 to Substrate Concentration

Absorbance @ 600nm

Time, hours

- 0.02% DBTS  -+ 0.2% DBTS  - 2% DBTS
Fig. 10.  

Growth Response of DBTS-22 to Substrate Concentration

![Graph showing absorbance over time for different substrate concentrations: 0.02% DBTS, 0.2% DBTS, and 2% DBTS.](image-url)
Fig. 11.  Growth Response of DBTS-26 to Substrate Concentration
Fig. 12. Growth Response of BSA-56 to Substrate Concentration

Absorbance at 600nm

0 0.1 0.2 0.3 0.4 0.5

0 24 48 72 96 120 144 168

Time, hours

- 0.02% BSA
- 0.088% BSA
- 0.2% BSA
- 0.98% BSA
- 3.8% BSA
Effect of Substrate Concentration on Desulfurization Rates

Fig. 13.

![Graph showing the effect of substrate concentration on desulfurization rates. The x-axis represents substrate concentration in %, and the y-axis represents desulfurization rate. The graph includes lines for DBTS-26, DBTS-18, BB-56, and DBTS-22.]
Fig. 14. Effect of Inoculum Size on the Growth of Strain Bwt in the Presence of 2% DBT

Inoculum Size, cells

- $8.12 \times 10^7$ — $8.12 \times 10^6$ — $8.12 \times 10^4$
Fig. 15. Effect of Inoculum Size on the Growth of Strain DBTS-2 in the Presence of 2% DBTS

Absorbance

0 20 40 60 80 100 120 140 160 180
Time, hours

6.88x10^6
6.88x10^8
6.88x10^4
Fig. 16. Effect of Inoculum Size on the Growth of Strain DBTS-19 in the Presence of 2% DBTS
Fig. 17. Effect of Inoculum Size on the Growth of Strain DBTS22 in the Presence of 2% DBTS

Absorbance

Time, hours

0 20 40 60 80 100 120 140 160 180

0 0.5 1 1.5 2

--- 9.80x10^8 --- 8.80x10^6 ---* 8.80x10^4
Fig. 18. Effect of Inoculum Size on the Growth of Strain DBTS26 in the Presence of 2% DBTS

Absorbance
600nm

Time, hours

Inoculum Size, cells

--- 9.24x10^7
--- 9.24x10^6
--- 9.24x10^4
Fig. 19. Effect of Inoculum Size on the Growth of Strain BSA56 in the Presence of 0.2% BSA
FIG. 20. Effect of Yeast Extract on DPT Hydropytosis by Soil Isolate 1.
DNA from BSA56
PLASMID pJOE801 (Black Colonies)

Cut with:
Hind III Bam HI Pst I
Xho I EcoRI

LIGATE

TRANSFORM INTO DC1090 hsd cysA

SELECT
a) Ampicillin Resistance
b) Use of Sulfonates as S-source
c) White colonies (insert)

Fig. 21
CONCLUSIONS AND RECOMMENDATIONS

Overall, we have achieved most of our proposed objectives. We had projected the cloning of genes for two steps of the 4S-desulfurization pathway in this years work. In fact we have the genes for only one step but we do have a clone bank and several suspects for the second step. Thus, we are on course, but the genetics has been a little slower than hoped. In brief then we have demonstrated:

1) Our natural bacterial isolates can in fact remove almost 100% of the organic sulfur from DBT-sulfone and aromatic sulfonic acids provided that culture conditions are improved by increasing aeration and keeping the pH neutral.

2) A new series of natural isolates has been found which release organic sulfur as hydrogen sulfide. These strains presumably use different pathways and provide a reservoir of genes for future bacterial strain improvements and genetic manipulations.

3) Starting from BSA56, the most efficient degrader of aryl sulfonic acids we have selected mutants which are more resistant to chemical toxicity and can degrade a much wider range of organic sulfur compounds.

4) Genes for the release of inorganic sulfur from aryl sulfonic acids have been cloned into Escherichia coli. The plasmid carrying E. coli strains have gained the ability to use aryl sulfonates as a sulfur source.

5) A gene library has been made from DNA of strain DBTS2 which degrades DBT-sulfone. The library is being screened for the genes involved in sulfone breakdown.

Our recommendations are that we should continue as previously planned. We hope to clone further genes of the 4S pathway and continue strain improvement. Improved strains will be tested versus high sulfur coal samples.