# THERMOSTABILIZATION OF DESULFURIZATION ENZYMES FROM *RHODOCOCCUS* sp. IGTS8

FINAL TECHNICAL REPORT

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## EXECUTIVE SUMMARY

The objective of this project was to develop thermophilic cultures capable of expressing the desulfurization (*dsz*) operon of *Rhodococcus* sp. IGTS8.

The approaches taken in this project included the development of plasmid and integrative expression vectors that function well in *Thermus thermophilus*, the cloning of *Rhodococcus dsz* genes in *Thermus* expression vectors, and the isolation of bacterial cultures that express the *dsz* operon at thermophilic temperatures.

This project has resulted in the development of plasmid and integrative expression vectors for use in *T. thermophilus*. The *dsz* genes have been expressed at moderately thermophilic temperatures ($52^0$C) in *Mycobacterium phlei* and at temperatures as high as $72^0$C in *T. thermophilus*. The tools and methods developed in this project will be generally useful for the expression of heterologous genes in Thermus.

Key developments in the project have been the isolation of a *Mycobacterium phlei* culture capable of expressing the desulfurization operon at $52^0$C, development of plasmid and integrative expression vectors for *Thermus thermophilus*, and the development of a host-vector system based on the malate dehydrogenase gene that allows plasmids to be stably maintained in *T. thermophilus* and provides a convenient reporter gene for the accurate quantification of gene expression. Publications have been prepared regarding each of these topics, copies of which are included in this report.

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## INTRODUCTION

### Significance

The quality of the nation's and the world's petroleum reserves is deteriorating and will continue to deteriorate. The increasing concentration of sulfur in increasingly heavy/viscous crude oil requires new desulfurization techniques to avoid environmental pollution resulting from petroleum combustion. Biodesulfurization, particularly biodesulfurization employing thermophilic cultures, can overcome limitations of existing technology and can provide an efficient and effective pollution prevention approach which is compatible with normal processes within the petroleum industry.

Heavy crude oils and residuum constitute a significant, and constantly increasing portion of U.S. petroleum reserves. These heavy oils possess high calorific content yet have comparatively low market values chiefly because of high sulfur and metals content and high viscosity/molecular weight. The sulfur content is of environmental concern due to potential sulfurous emissions from petroleum combustion. Metals present in heavy crude oils can poison catalysts used in hydrodesulfurization limiting the effectiveness of current technologies to remove sulfur from these oils. The high viscosity/high molecular weight of these oils limits the amount of higher value petroleum byproducts such as gasoline, aviation fuel, and diesel fuel that can be obtained as well as causes increased operating costs. These problems associated with heavy oils have prompted the preferential utilization of light crude oils. As light crude oils are consumed at a disproportional/high rate the amount of heavy oil as a percentage of remaining U.S. (or world) petroleum reserves continues to escalate.

New technologies capable of dealing with heavy oils to mitigate environmental concerns and increase byproduct yields in a cost effective manner are needed: biodesulfurization is one such new technology. Microorganisms routinely synthesize and degrade complex organic structures, and it is well known that biological reactions are capable of selectivity that greatly exceeds strictly chemical reactions. A microorganism, *Rhodococcus* sp. IGTS8, has been shown

to be capable of selectively cleaving carbon-sulfur bonds in petroleum, coal, and a wide range of model compounds resulting in the removal of sulfur and the retention of carbon and calorific value. Energy BioSystems Corporation (EBC) has conducted a comprehensive evaluation, particularly as regards petroleum, of the biodesulfurization technology originally developed by the Institute of Gas Technology (IGT) under a program funded by the U.S. Department of Energy/Pittsburgh Energy Technology Center. Encouraged by experimental results and feedback from the petroleum industry, EBC has licensed the technology, assembled a team of executives, engineers, and scientists from the petroleum industry, and is committed to the commercialization of biodesulfurization technology.

It is believed that biodesulfurization can be particularly useful in the treatment of heavy oils as the technology can remove sulfur and simultaneously reduce the viscosity/molecular weight of oil as a consequence of carbon-sulfur bond cleavage, and can tolerate heavy metal and salt concentrations typically found in heavy oils and produced/formation water. EBC's current efforts are focused on the biodesulfurization of diesel oil at mesophilic temperatures (25° to 40°C). While the potential of biodesulfurization has been demonstrated, more development is needed before the technology can be successfully commercialized for the treatment of heavy oils. Specific development needs include: developing cultures capable of performing biodesulfurization at thermophilic temperatures (60° to 100°C), developing cultures with higher levels of desulfurization activity, and process development research for the biodesulfurization of heavy oils.

## Background

### Economic and Environmental Concerns

Effective technologies for the treatment of heavy oils have been and continue to be a topic of keen interest. Literally hundreds of processes relevant to the desulfurization of heavy oils have been described in the patent literature, and the interest in such processes has steadily increased.(8, 10, 22) The consequence of standard practices within the petroleum industry over decades is that the need for new technologies capable of treating heavy oils and residuum will progressively intensify. The sulfur content and the average molecular weight of available petroleum in the U. S. and in the world has increased significantly in recent years and is expected to continue to increase.(22) Multiple factors such as physics, chemistry, environmental concerns,

and market forces contribute to this trend of increasing sulfur and molecular weight of available petroleum; however, the bottom line is that light crude oil is more readily recovered than heavy oil. Consequently deposits of light, low sulfur oil are preferentially brought into full production while known deposits of heavy/high sulfur petroleum are produced at less than full capacity or may even be idle. Moreover, as a deposit of light oil is harvested the lighter fractions are preferentially removed such that after primary or secondary production at a site that was originally characterized as containing light oil only heavy oil remains. **Because of this irreversible trend the time when available petroleum is predominantly or exclusively heavy high sulfur petroleum is not far off.**

In North America over 3 trillion barrels of known petroleum reserves are largely untapped or underutilized because of their high sulfur content and attendant viscosity problems.(22) It is well known in petroleum chemistry that sulfur and heavy metals are preferentially associated with the higher molecular weight fractions of oil.(5) The consequence of this is that not only is light oil easier to produce/harvest than is heavy oil because of physical properties, but also light oils contain significantly lower concentrations of undesirable impurities in comparison with heavy oils. Sulfur and heavy metal impurities of petroleum are of environmental concern due to the formation of acid rain as a consequence of sulfur emissions from the combustion of petroleum(16) and potential health effects of heavy metals present at high concentrations on fly ash resulting from the combustion of petroleum. Some sulfur heterocycles are suspected carcinogens and sulfur compounds in oil have been implicated in pipeline corrosion. Heavy metal contents in petroleum, chiefly nickel and vanadium, contribute to the poisoning of catalysts used in hydrodesulfurization or in catalytic cracking operations in petroleum refineries.(8) In addition to catalyst poisoning by heavy metals, sulfur itself in heterocyclic compounds is capable of poisoning catalysts by causing electronic modifications in Pd, Pt, Ni, and Ru compounds.(8) The poisoning of catalysts exasperates the problems associated with the processing of heavy oils and residuum by interfering with the methods employed to remediate the problems of sulfur content and molecular weight.

Various health concerns as well as the economic factors due to the presence of heavy metals and sulfur in heavy oils and residuum have made it such that light oils and low sulfur content oils are preferentially utilized. Environmental concerns prompt petroleum producing countries to consume the best oils, meaning the lightest and lowest sulfur content oils that are

locally available, while preferentially exporting heavier oils so that those oils available on the open market are predicted to decline in quality. Energy statistics predict that the U.S. will be importing from 65% to 70% of its oil demand by the year 2000.(10) Another factor which affects the quantity of heavy oils that are utilized and the importance of technologies capable of dealing with heavy oils and residuum is the increased demand for transportation fuels and other low molecular weight products.(22) This increased demand for lower molecular weight petroleum products is incompatible with the use of heavy oils as primary feedstocks because heavy feedstocks containing metals and sulfur increase the production of coke and gas and accelerates catalyst deactivation. But at the same time, the desire to obtain greater quantities of gasoline and other transportation fuels from each barrel of oil demands increased attempts to further process residuum. Therefore, not only will the petroleum industry be forced to process increasing quantities of heavy crude oil, increased processing of heavy residuum will occur. These heavy oils have increased levels of environmental contaminants and are less efficiently treated by existing technologies.

Hydrodesulfurization can be used to desulfurize heavy oils and residuum, but doesn't lead to significant decreases in molecular weight. The predominant method of upgrading heavy oil as regards decreasing molecular weight and increasing the yield of transportation fuels is the use of fluid catalytic cracking (FCC).(5) However, FCC cannot achieve desulfurization.(5) Moreover hydrodesulfurization and FCC catalysts are poisoned in the process of treating heavy oils because of the presence of sulfur heterocycles and heavy metal contaminants.(22) Therefore, a combination of technologies is needed to address both the removal of sulfur and the decrease in molecular weight of heavy oils and residuums in order to mitigate environmental problems and to get the greatest yield of value added products from petroleum.

The environmental concerns and the processing problems associated with the utilization of heavy oils and residuum have put into play powerful forces that have contributed and continue to contribute to the dynamics of the utilization of heavy oil. Both market pressure to prevent the utilization of heavy oil reserves and the creation of an incentive for the generation of new technologies capable of dealing with heavy oils and residuum are present because of the price differential between low and high sulfur petroleum. In 1990 the cost differential in the U.S. between low and high sulfur oil was about $3.60 a barrel. This price differential will cause the production of some high sulfur reserves to slow or stop which will result in the loss of some jobs.

Although, the same economic forces provide increased incentive for the development of alternative desulfurization technologies. In the years 1972 through 1985 the U.S. petroleum industry spent approximately $1.4 billion in capital and operating expenditures for dealing with pollution abatement. The National Petroleum Refineries Association has estimated the cost of meeting Clean Air Act regulations requiring a sulfur content of 0.05% for diesel fuel by 1994 has cost about $3.3 billion in capital expenditures and $1.2 billion in annual operating costs.(10) Similar figures for the treatment of heavy crude oils or residuum are not available, however, diesel fuel is far easier to desulfurize and handle than are heavy oils. So one would predict that the costs associated with upgrading heavy oils and residuums would be correspondingly higher. Because of the quantity of petroleum consumed in the U.S. and the price differential between high and low sulfur fuels, it is estimated that the value of an alternative desulfurization technology, such as biodesulfurization, which is capable of upgrading existing production by reducing sulfur content and the opening or reopening of markets for high sulfur petroleum reserves is in excess of $10 billion annually in the U.S.(10) Clearly then, there is a need for alternative technologies to deal with heavy oils and residuum and there is ample economic incentive for the development of appropriate technologies that are immune from the technical problems that limit existing technologies. Biodesulfurization is one such technology.

Biodesulfurization

Researchers have been examining the possibility of biodesulfurization of petroleum or other fossil fuels for over 4 decades.(3, 17) There are no commercial operations now for biodesulfurization of fossil fuels, however, there are economic studies that indicate the favorable prospect of developing such a process,(7, 24) and EBC is in the early stages of developing a commercial process for the biodesulfurization of diesel fuel. In summary, what occurred in biodesulfurization research until very recent times is that microorganisms capable of metabolizing organosulfur structures did so without selectivity or specificity. Carbon and fuel value were lost as a consequence of microbial desulfurization using previously exported microorganisms.(9, 17-19, 25, 26) What is wanted is selective recognition and cleavage of carbon sulfur bonds so that the sulfur is removed yet the carbon and fuel value remains intact.

The goal of isolating a culture that has that desired selectivity for the recognition of carbon sulfur bonds has been achieved and the culture *Rhodococcus* sp., IGTS8, has been

demonstrated to cleave carbon sulfur bonds in a wide range of compounds: thiophenes, sulfoxide, sulfones sulfides, disulfides, and mercaptans.(11) More importantly, IGTS8 has also been shown to desulfurize petroleum and coal.(12-15) *Rhodococcus* sp. IGTS8, is a gram positive gram variable microorganism that has no fastidious growth requirements.

Biodesulfurization could fit well with current practices in the petroleum industry if performed in conjunction with desalting and dewatering operations. However, this requires thermophilic cultures.

IGTS8 grows best at a pH of 7 and at 30°C. It will however, tolerate pHs of about 5.5 to 8 and temperatures of about 15°C to 34°C(11, 12). These ranges of pH and temperature are approximately the same for growing cells and for biocatalysts; although, some desulfurization activity can be obtained at temperatures as high as 40°C using biocatalysts. Importantly the biocatalysts tolerate sodium chloride concentrations of 6% or even higher, (12)such that the salt concentration that may be found in the co-produced water in petroleum production would not inhibit the desulfurization activity of biocatalysts. Therefore a potentially attractive means of implementing a petroleum biodesulfurization process could be to treat heavy oil on site in the production field prior to the initial separation of petroleum from produced water. Since biodesulfurization will require a water wash/separation step to remove biocatalysts and liberated sulfur from treated petroleum and because a water separation step is a normal component of petroleum production, performing biodesulfurization on produced oil would minimize the required processing steps and the produced water could be reinjected on site for secondary recovery/water flooding operations and could therefore eliminate the need for wastewater treatment. The reinjection of sulfur-laden water into a petroleum field generates concerns about the potential souring of wells, but strategies for the prevention and control of $H_2S$ formation are routinely used in existing petroleum production operations and could be employed to deal with water resulting from biodesulfurization treatments.

This scenario could greatly improve the economics of a biodesulfurization process and could integrate into existing petroleum production procedures with a minimum of modification. This approach would require biodesulfurization reactors to be present at petroleum production

sites; however, it is possible that conventional petroleum storage tanks can be used or modified for use in biodesulfurization treatments. The chief concern about this biodesulfurization approach would be the temperature of the produced oil and the thermal tolerance of biocatalysts. *Rhodococcus erethropolis* IGTS8 biocatalysts do not exhibit activity at temperatures exceeding 40°C;(11) however, the goal of this project was to improve thermotolerance of desulfurization-competent bacterial cultures.

Desalting and dewatering processes for petroleum are normally performed at temperatures of from 60°C to 100°C(5), and the use of elevated temperatures will be increasingly important in the treatment of heavy crude oil and residuum to deal with the viscosity of these heavy oils. If thermophilic cultures could be used to desulfurize heavy oils in conjunction with desalting and dewatering processes then with little change in oil industry practice the viscosity and sulfur content of heavy oils would be simultaneously reduced allowing more manageable oils, more closely resembling light crude oils, to be sent to refineries for subsequent processing. If the viscosity and sulfur content of heavy oils were to be reduced prior to being sent to the refinery this would subsequently improve the ability of current refinery processes like hydrodesulfurization and FCC to treat these heavy oils. Thus biodesulfurization of heavy crude oil would fit extremely well within current practices of the petroleum industry, but the biodesulfurization must be performed at thermophilic temperatures (60°C to 100°C). A microbial culture that can selectively desulfurize petroleum has already been identified, and this project will develop similar cultures which will desulfurize petroleum at thermophilic temperatures.

Target Genes

The three genes which encode the desulfurization pathway of *Rhodoccus* sp. IGTS8 (*dsz*A, *dsz*B, and *dsz*C) have been cloned and sequenced(4, 21, 27). These novel enzymes allow the selective cleavage of carbon-sulfur bonds in a range of organosulfur compounds and will specifically metabolize dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP) and sulfite. These three genes are expressed as an operon from a single promoter. The enzyme encoded by *dsz*C converts DBT directly to DBT-sulfone (dibenzothiophene-5, 5-dioxide) and the enzymes encoded by *dsz*A and *dsz*B act in concert, in intact cells, to convert DBT-sulfone to 2-HBP and sulfite. Biochemical analyses with purified enzymes have shown that the enzyme encoded by

*dsz*A promotes the conversion of DBT-sulfone to 2-hydroxybiphenyl-2-sulfinate and that the enzyme encoded by *dsz*B converts 2-hydroxybiphenyl-2-sulfinate to 2-HBP and sulfite. The cofactor $FMNH_2$ is required for the activity of *dsz*A and *dsz*C(6).

The biochemistry of biodesulfurization is known and assays are available for the desulfurization pathway and for each step within the desulfurization pathway. These convenient assays will assist in the development of cultures which contain thermotolerant derivatives of desulfurization enzymes, but a cell-free biodesulfurization process is impractical. The requirement of a three enzyme pathway along with cofactors prohibits the use of purified enzyme systems for a practical biodesulfurization process. Rather a practical biodesulfurization process will employ intact bacterial cells as biocatalysts. Because of this the use of immobilization or other post-production modification techniques to enhance the thermotrolerance of enzymes would not be practical for a biodesulfurization process.

Thermostabilization

Rational protein engineering studies have been a powerful tool, enabling the modification of some proteins to increase thermostability, shift pH optima, and alter substrate specificity (1, 2, 20, 23). However, in order to do this, a fair amount of information is required such as the amino acid sequence, the three dimensional structure, and the location of the active site of the protein. Even when all of this information is available, protein engineering is an uncertain undertaking and can be expensive and time consuming. It is more straight forward to use site specific mutagenesis to increase the hydrophobicity of proteins, however even this requires uncertain theoretical predictions. The amino acid sequences of the three desulfurization enzymes can be inferred from their DNA sequences; however, knowledge of the active sites or the three dimensional structures of these proteins is currently unavailable. To obtain this information would take a considerable amount of time and resources and is beyond the scope of this project. In addition, while there are some general rules/trends that have emerged regarding the thermostabilization of proteins (such as increasing the hydrophobicity of proteins placing proline residues at beta-turns in protein molecules and adding disulfide bonds) these are very general rules which often do not hold true and are difficult to implement. Other methods to increase the thermotolerance of enzymes such as immobilization or post-production modification techniques are not available since biodesulfurization uses three enzymes and associated cofactors. This

requires the need to use them in intact cells rather than immobilized enzyme systems.

Prior to this project the cloned desulfurization operon of *Rhodococcus* sp. IGTS8 has been transferred to and successfully expressed in several different bacterial species, particularly *Pseudomonas* species. Thermophilic cultures can be grown at temperatures as low as 40°C and vectors for the genetic modification of thermophiles have been developed. The approach taken in this project was to isolate desulfurization-competent thermophilic microorganisms using enrichment culture techniques and to transfer the *dsz* genes into the extreme thermophile *Thermus thermophilus*.

## RESULTS AND DISCUSSION

Key developments in the project have been the isolation of a *Mycobacterium phlei* culture capable of expressing the desulfurization operon at $52^0$C, development of plasmid and integrative expression vectors for *Thermus thermophilus*, and the development of a host-vector system based on the malate dehydrogenase gene that allows plasmids to be stably maintained in *T. thermophilus* and provides a convenient reporter gene for the accurate quantification of gene expression. Publications have been prepared regarding each of these topics, copies of which are included in this report as appendices. The three publications that have been prepared as a result of this project are "Isolation and Characterization of a Thermophilic Bacterium Capable of Dibenzothiophene Desulfurization" (Microbiology, submitted), "A New Host/Vector System for Thermus sp. Based on the Malate Dehydrogenase Gene" (Journal of Bacteriology, In Press), and "Inducible and Constitutive Expressions Using New Plasmid and Integrative Expression Vectors for Thermus sp." (Letters in Applied Microbiology, submitted) which are included in this report as appendices A, B, and C respectively.

## CONCLUSIONS

This project has resulted in the demonstration that the desulfurization genes originally isolated in *Rhodococcus erythropolis* IGTS8 reside on a conjugal plasmid, pSOX, that can be transferred to the moderate thermophile *Mycobacterium phlei* GTIS10. While *Rhodococcus erythropolis* IGTS8 is unable to grow at temperatures above $32^0$C, *Mycobacterium phlei* GTIS10 can grow at temperatures up to $52^0$C. The ability of the desulfurization enzymes to function at a given temperature was found to be more dependent upon the bacterial host than the intrinsic properties of the desulfurization enzymes. When studied as enzyme preparations *dszB* was found to be the most thermolabile in vitro and was rapidly inactivated at temperatures of $45^0$C and above, yet resting cells of *Rhodococcus erythropolis* IGTS8 exhibit measurable desulfurization activity at $45^0$C and resting cells of *Mycobacterium phlei* GTIS10 show desulfurization activity at temperatures as high as $57^0$C. The optimum desulfurization activity in each species corresponded to the optimum growth temperature for each species probably reflecting the importance of general cell metabolism of the host to supply the cofactors $FMNH_2$ and oxygen needed by the desulfurization pathway. These results suggested that the desulfurization enzymes may be capable of functioning at even higher temperatures in alternative hosts.

This project resulted in the development of tools and techniques for the genetic manipulation of *Thermus thermophilus* and Thermus species generally and has demonstrated that heterologous genes, including *dszC* can be expressed in *T. thermophilus*. Unfortunately it was not possible within this project to achieve expression of the complete desulfurization operon in *T. thermophilus*, but it is not known to what degree this is due to the thermolability of DSZ enzymes or to problems in obtaining simultaneous expression of all three *dsz* genes in Thermus.

This project demonstrated that biodesulfurization processes can indeed be designed to run at at least moderately thermophilic temperatures and future research should result in desulfurization-competent cultures that have even better thermotolerance. Perhaps the most valuable outcome of this project was the development of various integrative and plasmid vectors for the expression of genes in Thermus. Bioprocesses may benefit from faster reaction rates, greater solubility/lower viscosity of substrates, reduced operating costs and other benefits resulting from operating at thermophilic as opposed to mesophilic temperatures. The genetic tools for the expression of genes in Thermus may be of general use for the development of thermophilic bioprocesses for a variety of applications.

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# APPENDIX A:
# Isolation and Characterization of a Thermophilic Bacterium Capable of Dibenzothiophene Desulfurization

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## Abstract

An organism, identified as *Mycobacterium phlei* GTIS10, was isolated based on its ability to use dibenzothiophene (DBT) as a sole source of sulfur for growth at 45-52°C. Similar to other biodesulfurization competent organisms *M. phlei* GTIS10 converts DBT to 2-hydroxybiphenyl (2-HBP). The specific desulfurization activity of the 50°C *M. phlei* GTIS10 culture was determined to be on average 1.1 μmole 2-HBP/ min g-dry cell. The *dsz* operon of *M. phlei* GTIS10 was cloned and sequenced and was found to be identical to that of *Rhodococcus erythropolis* IGTS8. The presence of the 120-kb plasmid pSOX, which encodes the *dsz* operon, has been demonstrated in both *R. erythropolis* IGTS8 and *M. phlei* GTIS10. Even though identical *dsz* genes are contained in both cultures the temperature at which resting cells of *R. erythropolis* IGTS8 reach the highest rate of DBT metabolism is near 30°C whereas the temperature that shows the highest activity of resting cell cultures of *M. phlei* GTIS10 is near 45°C, and activity is detectable at temperatures as high as 57°C. In *M. phlei* GTIS10 the rate limiting step in vivo appears to be the conversion of DBT to dibenzothiophene sulfone catalyzed by the product of the *dsz*C gene, dibenzothiophene monooxygenase. The thermostability of individual desulfurization enzymes was determined and 2-hydroxybiphenyl-2-sulfinate sulfinolyase, encoded by *dsz*B, was found to be the most thermolabile. These results demonstrate that the thermostabilities of individual enzymes determined in vitro are not necessarily good predictors of the functional temperature range of enzymes in vivo.

## Introduction

Heavy crude oils and residuum constitute a significant and ever increasing portion of U.S. petroleum reserves. These heavy oils possess high calorific content yet have comparatively low market values chiefly because of high sulfur and metals content, high viscosity and molecular weight (14, 27). The sulfur content is of environmental concern due to potential sulfurous emissions from petroleum combustion. The problems associated with heavy oils have prompted the preferential utilization of light crude oils. As light crude oils are consumed at a disproportional rate, the amount of heavy oil as a percentage of remaining U.S. (or world) petroleum reserves continues to escalate (14).

New technologies capable of dealing with heavy oils to mitigate environmental concerns and to allow processing in conventional refineries in a cost-effective manner are needed: biodesulfurization is one such new technology. Recent publications have shown that several organisms possess the ability to selectively remove sulfur from DBT (4, 26, 29, 30, 35). The first and most extensively characterized is *R. erythropolis* IGTS8 (formerly *R. rhodochrous* IGTS8) (15). This bacterial culture contains the desulfurization operon (*dsz*) on a 120 kb plasmid (pSOX) (6) and has been shown to be capable of selectively cleaving carbon-sulfur bonds in petroleum, coal, and a wide range of model compounds resulting in the removal of sulfur and the retention of carbon and calorific value (12, 15, 18, 31). The *dsz*C gene encodes dibenzothiophene monooxygenase that catalyzes the conversion of DBT to dibenzothiophene sulfone (DBTS02). The *dsz*A gene encodes dibenzothiophene-5,5-dioxide monooxygenase that catalyzes the conversion of DBTS02 to 2-hydroxybiphenyl-2-sulfinate (HBPSi). The *dsz*B gene encodes 2-hydroxybiphenyl-2-sulfinate sulfinolyase that catalyzes the conversion of HBPSi to 2-hydroxybiphenyl (2HBP) (7). A fourth gene the *dsz*D gene encoding a NADH-FMN oxidoreductase is an accessory component of the desulfurization pathway and supplies cofactors needed for the monooxygenase reactions catalyzed by the products of *dsz*C and *dsz*A [Gray, 1996 #1536; Reichmuth, 2000 #89].

While the selective cleavage of carbon-sulfur bonds in model organosulfur compounds and the selective removal of sulfur from petroleum has been demonstrated by several microbial cultures this technology has not yet been successfully commercialized. Biocatalysts with

improved desulfurization rates and characteristics more favorable to an industrial-scale process are needed. Since petroleum is often produced, transported, and processed at elevated temperatures thermophilic microorganisms/enzymes which can function at temperatures ranging from 60 to 100°C may be the most appropriate microorganisms to examine for biorefining applications (8). Performing biorefining processes at higher temperatures is not only more compatible with existing industry practices, but may result in higher catalytic rates and the reduced viscosity of petroleum at higher temperatures will allow lower processing costs. Recently the isolation of the first desulfurization-competent thermophilic culture was reported (21). However, the specific desulfurization activity of this culture was quite low in comparison to previously characterized mesophilic cultures. The *dsz* operon DNA sequence from this *Paenibacillus* sp. was determined [Ishii, 2000 #1531] and significant homology to the *dsz*A, B and C genes of *Rhodococcus erythropolis* IGTS8 was found. The thermostability of various enzymes in the desulfurization pathway was not investigated although the entire pathway was cloned into *E. coli* and was expressed at 50°C. In this work the isolation and characterization of a new desulfurization-competent thermophilic culture is reported including an investigation of the thermostability of desulfurization enzymes in vivo and in vitro.

**Materials and Methods**

*Chemicals.* Antibiotics and chemicals were purchased from Aldrich/Sigma (Milwaukee, Wis.) and were of highest quality available. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4 ligase was purchased from Life Technologies (Gathersburg, MD). 2-hydroxybiphenyl-2-sulfinate to 2-hydroxybiphenyl (HBPSi) was synthesized by Dr. Christopher Oldfield, Napier University, Edinburg Scotland.

*Bacteria, culture isolation and growth conditions. R. erythropolis* IGTS8 (ATCC 53968) was isolated in our laboratory. *E. coli* strains DH5α and JM109 were purchased from Life Technologies. Five soil samples from coal processing or compost sites were used to screen for thermophilic desulfurizing organisms. Approximately 4 g of soil was added to a 1L water-jacketed vessel turbidostat apparatus. Sulfur-free medium BSM (18) was used for operation of the turbidostat and for growth of liquid cultures under sulfur-limited conditions (17). A mixed carbon source consisting of 5.0 g/L each of glucose, glycerol, and succinate was used. Water

flowed through a jacketed turbidostat apparatus at a temperature of 55°C to maintain thermophilic temperatures. DBT crystals were added as the sole sulfur source. The solubility of DBT at 30°C is near 5 μM, and the amount added in experiments ranged from 20μm to 200μm. The presence of DBT crystals causes minimal turbidity and does not interfere with the spectrophotometric determination of cell density for bacterial cultures growing at the expense of DBT. To ensure conditions to isolate cultures that selectively use DBT as a sole sulfur source the flow rate of the turbidostat was maintained at the fastest flowrate possible while avoiding washout. The cell density was maintained at about $10^4$-$10^5$ cells/ml. Colony forming units (CFU) were enumerated using Nutrient Agar (Difco). Cells from the turbidostat effluent were tested weekly at 30°C, 55°C, and 65°C for desulfurization competency by using the sulfur bioavailability assay (17) and by examining the reactor supernatant for presence of 2-HBP using the Gibbs assay (15).

*Species identification.* The identification of the species of the bacterial isolate obtained was done by determining the DNA sequence of a 500 bp portion of the 16S-rRNA gene and comparison with a library of known DNA sequences (MIDI Labs, Newark, Delaware).

*Preparation of cell free extracts.* *R. erythropolis* IGTS8 cells or *M. phlei* GTIS10 were grown in BSM with dibenzothiophene sulfone (DBTSO2) as the sole sulfur source until mid-log phase. The cells were harvested by centrifugation at 10,000 x g for 10 minutes, washed with 50mM HEPES, pH 7.5 and resuspended in the same buffer, containing 1mM dithiotheitol (DTT) and 30ug/ml phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted with a sonicator (Branson Sonic Power Company, Cell Disrupter 350), pulsed on ice using 20-second intervals with a 20-second resting period between each interval for a total of 2 minutes. The sonicator was set on a 70% duty cycle and with the output set at 7.0. Cell debris was removed by centrifugation at 9000 x g for 10 minutes. Protein was quantified using the Pierce Biochemical (Rockford, IL ) BCA Protein Kit. Cell free extracts were examined by 6% polyacrylamide-bis-acrylamide-SDS gel electrophoresis crosslinked with Temed according to standard procedures (36). A low molecular weight calibration kit for SDS electrophoresis (Amersham Pharamacia Biotech) was used as the protein standard to estimate protein molecular weight.

*Biochemical Assays and Analytical Techniques.* FMN oxidoreductase (*dsz*D) activity was measured by the oxidation of NADH monitored at 340 nm (9). Biodesulfurization competency was quantified by measuring the amount of 2-hydroxybiphenyl in culture supernatants as well as by making specific activity determinations. The sample preparation and operating procedures described by Oldfield et al.(28) were employed for analyses employing HPLC while some analyses employed the Gibbs assay. GC-MS and/or thin layer chromatography (TLC) analyses were performed on ethyl acetate extracts derived from growing cell cultures, resting cell cultures, and cell free extracts exposed to various organosulfur model compounds. *Mycobacterium phlei* GTIS10 resting cells were prepared by growing the culture on thiophene or dibenzothiophene sulfone as the sole sulfur source until cells were in late logarithmic phase (approximately 48 hours). Cells were harvested by centrifugation, washed twice with 50mM hepes pH 7.5, and resuspended in the same buffer. Dry cell weight was determined and recorded. These resting cell suspensions were then incubated at room temperature, 30, 37, 45, 52, 57, 62, 65 and 75 °C with 200 μM DBT or DBTSO2. Samples were removed at various intervals over a 24 hour period of time and specific activity was determined based on the quantity of 2-HBP produced per minute per gram dry cell weight (μmole 2-HBP/ min g-dry cell). When cell free extracts were used the extracts were divided into portions and pre-incubated at 30, 37, 45, 52, 65, and 72°C for 30, 60, and 120 minutes. At each time interval, 500 μl of sample was removed and residual activity of the *dsz* enzymes was determined by the addition of 10 μM of appropriate organosulfur substrates, 4mM NADH, and 10μM FMNH. The samples were then incubated at 30°C for 60 min and were assayed by GC-MS, TLC, or HPLC.

For analysis of the extracts by GC-MS a Hewlett Packard 5971 mass selective detector and 5890 series II GC with HP 7673 auto sampler tower and a 30 meter Rezteck XTI-5 column was used. The final oven temperature was maintained at 300°C. Mass spectrographs were compared with various libraries of mass spectrograph data prepared from known standard compounds. Several chromatograph libraries were consulted to determine the identity of metabolites. TLC was performed by spotting 30 μl of an ethyl acetate extracted sample on Whatman Silica PE Sil G/UV plates. The running phase solvents used were chloroform and toluene in the ratio of 1:3. These plates were later observed under UV light.

*Plasmids and recombinant DNA techniques.* Recombinant techniques were standard (36) unless otherwise noted. Plasmid pGEM T-easy was purchased from Promega (Madison, WI). The

expression vector pFLAG-CTC, which contains the *tac* promoter, was purchased from Sigma Chemical Co., (St. Louis, MO). Southern blots and hybridization were conducted according to Sambrook *et al.* (36) using 65°C hybridization temperature. The probe used in hybridization studies was a 3.7 Kb DNA fragment that contained the entire desulfurization operon from *R. erythropolis* IGTS8 and was $\alpha^{32}$P-CTP (Amersham) labeled by nick translation. *M. phlei* GTIS10 total DNA was isolated according to Pelicic *et al.* (32). To construct a *M. phlei* GTIS10 genomic DNA library partially digested *Sau*3A fragments were cloned into the unique *Bam*HI cloning site of the phagemid derivative pMYF131. The average DNA insert size was about 15 kb (M. Foustein, personal communication). The DNA was packaged into Promega (Madison, WI) packaging extracts according to the manufacturers guidelines. The primary library was amplified using *E. coli* LE392 as the host strain and subjected to screening by southern blot hybridization.

*Desulfurization gene cloning.* Genomic DNA from *M. phlei* GTIS10 was used as the template for the amplification of the *dsz*A, *dsz*B and *dsz*C genes using PCR. Restriction sites were added to both 5′ and 3′ ends on each amplified product to facilitate subsequent in-phase cloning into the pFLAG-CTC expression vector. The PCR primers used were:

*dsz*A sense: 5′-GAATTCGATGAGAATAATGACTCAACAACGACAAATGCATC-3′
***dsz*A antisense: 5′-AGATCTTCATGAAGGTTGTCCTTGCAGTTGT-3′**
*dsz*B sense: 5′-GAATTCGATGAGAATAATGACAAGCCGCGTCGACCCCGCAAAC-3′
*dsz*B antisense: 5′-AGATCTCTATCGGTGGCGATTGAGGCTGTTGTTCAGA-3′
*dsz*C sense:5′-GAATTCGATGAGAATAATGACACTGTCACCTGAAAAGCAGC-3′
*dsz*C antisense: 5′-AGATCTTCAGGAGGTGAAGCCGGGAATCGGG-3′
*dsz*D sense: 5′-GAATTCGATGAGAATAATGTCTGACAAGCCGAATGCCGTTT-3′
*dsz*D antisense: 5′-AGATCTCTATTGACCTAACGGAGTCGGGCGG-3′

The underlined sequences indicate restriction enzyme recognition sites.
The DNA sequences of the *dsz* operon and the pSOX replication genes used to design PCR primers were from Genebank Accession numbers L37363 and AF059700 respectively. The PCR primers used to isolate the pSOX replication genes were :

pSOX sense: 5′- CGCTCAGCACTGGGTGGAAA-3′ and pSOX antisense 5′-TCCGCCGCGTTAGAGATGGA-3′

*DNA sequencing and analysis.* PCR-generated and restriction enzyme generated DNA fragments were cloned into pFLAG-CTC and sequences were determined on both strands by primer walking by the University of Iowa DNA facility (Iowa City, Iowa). Sequence analysis was performed with the BLAST programs of the National Center for Biotechnology Information (Bethesda, Md.).

## RESULTS AND DISCUSSION

**Isolation and characterization of a thermophilic desulfurization competent culture.**

A turbidostat was operated at 55°C using sulfur-free mineral salts medium and dibenzothiophene (DBT) as the sole source of sulfur. The turbidostat was inoculated with samples obtained from various locations where thermophilic conditions and/or chronic hydrocarbon contamination exist. After approximately four months a culture was recovered from the turbidostat that could grow well in a sulfur bioavailability assay using DBT at 45°C. Initially, a mixed culture was obtained, however, individual colonies were isolated by streaking the mixed culture onto nutrient agar plates. These individual colonies were subsequently used to inoculate sulfur bioavailablity assays and in this way a pure culture capable of utilizing DBT as a sole source of sulfur was obtained. Although the culture was originally isolated from a turbidostat operated at 55°C the culture was unable to grow at temperatures above 52°C. Attachment of the culture to the soil/sediment matrix in the slurry may provide protection from slightly higher temperatures and there may have been some cooling in the turbidostat below the set point of the circulating water used to maintain temperature. The pure culture was identified as *M. phlei* based on a determination of the DNA sequence of a 500 bp portion of its 16S-RNA gene and was designated as *M. phlei* GTIS10.

The growth of *M. phlei* GTIS10 at 50°C using DBT as the sole sulfur source is illustrated in Figure 1. The growth of the culture was monitored spectrophotometrically by recording the change in optical density $A_{600\ nm}$ over time. *M. phlei* GTIS10 converted DBT to 2-hydroxybiphenyl (2-HBP) which accumulated in the culture supernatant and was quantified to serve as an indication of the rate and extent of DBT metabolism. The accumulation of 2-HBP in the supernatant of the growing culture was quantified using HPLC and the results are included in Figure 1. The generation time of *M. phlei* GTIS10 growing at 50°C in defined salts media with DBT as the sole sulfur source is approximately 8 hours, which is equivalent to the growth rate of this culture using sulfate under similar conditions. The accumulation of 2-HBP closely paralleled the growth of the culture and indicated that stationary phase cells do not continue to metabolize DBT. A modest decrease in 2-HBP concentration was seen in stationary phase

cultures that is most probably due to the sorption of 2-HBP to biomass as has been previously observed in studies of DBT metabolism (28).

The desulfurization activity of cells harvested at various phases of growth of *M. phlei* GTIS10 were determined and the best desulfurization activity was observed in resting cells obtained from mid to late log phase cultures (data not shown). The specific desulfurization activity of the 50°C *M. phlei* GTIS10 culture was determined to be on average 1.1 μmole 2-HBP/ min g-dry cell when resting cells were switched to DBT. We show a higher specific activity for *Rhodococcus erythropolis* IGTS8 than previous studies where other researchers activity values ranged from approximately 0.6 (28) to .275 μmole 2-HBP/ min g-dry cell (21). The thermophilic desulfurization competent *Paenibacillus* sp strain highest specific activity was approximately 0.08 μmole 2-HBP/ min g-dry cell (21).

The range of organosulfur substrates that can be metabolized by *M. phlei* GTIS10 was investigated using various compounds as the sole source of sulfur in growth assays at 45°C, and the results are shown in Table 1. Test compounds were added at a concentration of 1 mM and the results reported in Table 1 are derived from a minimum of two independent growth tests for each compound. The range of substrates utilized as sulfur sources by *M. phlei* GTIS10 is quite broad and essentially the same as results reported for *R. erythropolis* IGTS8 (15). It is important to note that *M. phlei* GTIS10 grew well with thiophene as the sole sulfur source while R. *erythropolis* IGTS8 has been reported to be unable to utilize thiophene[Oldfield, 1998 #1533].

**Characterization of the desulfurization genes from *M. phlei* GTIS10.**

*M. phlei* GTIS10 appears to be highly similar to *R. erythropolis* IGTS8 as regards biodesulfurization except that the maximum growth temperature for *R. erythropolis* IGTS8 is about 33°C whereas *M. phlei* GTIS10 can grow up to temperatures of 52°C. DNA hybridization experiments were performed to investigate the relatedness of desulfurization genes from *R. erythropolis* IGTS8 and *M. phlei* GTIS10. Genomic DNA was prepared from both cultures, digested with several restriction enzymes, and used in Southern blotting experiments employing a DNA probe containing the entire *dsz* operon from *R. erythropolis* IGTS8. Since these two

cultures show such different temperature ranges for growth it was anticipated that the sequences of the *dsz* genes in *M. phlei* GTIS10 versus *R. erythropolis* IGTS8 would be different or contain mutations conferring thermostability, but the results of Southern hybridization experiments showed there was no size differences in restriction enzyme digest patterns of the *dsz* genes in these two microorganisms (data not shown). The individual *dsz* genes of *M. phlei* GTIS10 were amplified by PCR using primers based on the *R. erythropolis* IGTS8 *dsz* operon, cloned into a pFLAG-CTC expression vector and sequenced. Surprisingly, all three of the genes in the desulfurization operon (*dsz* A, B, and C) appear to be identical in *M. phlei* GTIS10 and *R. erythropolis* IGTS8. The finding that no differences in the DNA sequences of any of the desulfurization genes of *M. phlei* GTIS10 versus *R. erythropolis* IGTS8 was unexpected and raised the concern of possible sample contamination during PCR. To address this concern a *M. phlei* GTIS10 DNA phagemid library was constructed. The average *M. phlei* GTIS10 chromosomal insert in the phagemid library was 15 kb. The library was transferred onto nylon membranes and hybridized to a DNA probe containing the entire *dsz* operon from *R. erythropolis* IGTS8. Several positive plaques were isolated and submitted for sequencing of the entire *dsz* operon. Again, we observed 100% homology of the *M. phlei* GTIS10 *dsz* genes to the *R. erythropolis* IGTS8 desulfurization operon. Moreover, this second cloning of desulfurization genes from *M. phlei* GTIS10 was performed in a laboratory that has never worked with *R. erythropolis* IGTS8 or any other desulfurization-competent culture, so sample contamination was not a concern. The phagemid insert, containing the *dsz* operon derived from *M. phlei* GTIS10 was sequenced, in both the five and three prime directions about 1.5 kb beyond the boundaries of the *dsz* genes. Identical sequences were observed in both the three prime *dsz* promoter region and five prime region of sequences derived from *Mycobaterium phlei* GTIS10 in comparison with sequences derived from *Rhodoccus erythropolis* IGTS8.

Because of the presence of identical *dsz* operons and flanking regions in both *R. erythropolis* IGTS8 and *M. phlei* GTIS10 we examined *M. phlei* GTIS10 total DNA for the presence of the 120 Kb plasmid pSOX replicon (5). *M. phlei* GTIS10 DNA was examined in PCR experiments using primers targeting an 860 bp sequence of the replication region of the pSOX replicon. The presence of the pSOX plasmid replicon was confirmed (data not shown). The pSOX plasmid replication genes are related to those from *Mycobacterium* replicons so it is quite reasonable that this plasmid should function in *M. phlei* GTIS10. The *dsz*D gene of *R. erythropolis* IGTS8 was

used as a probe in hybridization experiments with *M. phlei* GTIS10 DNA. *M.phlei* GTIS10 contains sequences homologous with *dsz*D but the location of *dsz*D in *M. phlei* GTIS10 is unknown other than it is not within 1.5 kb of the other *dsz* genes. Downstream from the *dsz* operon of *R. erythropolis* IGTS8 and *M. phlei* GTIS10 there are two open reading frames (orf's) that have strong homology to a *Mycobacterium smegmatis* translocase and an IS6120 associated orf (Genebank Accession number M69064).

While there was no attempt to promote the conjugal transfer of the pSOX plasmid from *R. erythropolis* IGTS8 to other more thermotolerant bacteria, the simplest explanation for the presence of *dsz* operons with identical DNA sequence in both *R. erythropolis* IGTS8 and *M. phlei* GTIS10 is the transfer of the pSOX plasmid from *R. erythropolis* IGTS8, which was in use in our laboratory, to *M. phlei* GTIS10. This hypothesis is further supported by the detection of pSOX replication genes in *M. phlei* GTIS10.

**Thermotolerance of the desulfurization enzymes.**

To further investigate the thermotolerance of the desulfurization enzymes contained in *M. phlei* GTIS10 and *R. erythropolis* IGTS8 each culture was grown using DBTSO2 as a sole source of sulfur and logarithmic phase cells of each culture were obtained, washed, resuspended in minimal medium containing DBT, and incubated 24 hours at various temperatures. The quantity of 2-HBP produced by the conversion of DBT by each culture at each temperature was quantified by HPLC over time and the specific activity results are shown in Figure 2. Interestingly, despite the fact that DNA sequence analysis shows that both cultures contain identical *dsz* operons/enzymes, the specific activity varied with the incubation temperature. Figure 2 illustrates that *R. erythropolis* IGTS8 showed maximal activity at 30°C and a progressive loss of activity at 37°C and 45°C, while no activity is observed at temperatures of 52°C or above. These results correlate well with the range of temperatures known to support the growth of *R. erythropolis* IGTS8 which grows maximally at about 30°C and does not grow at temperatures above about 33°C (15). On the other hand, *M. phlei* GTIS10 exhibits activity over the temperature range of 25°C to 57°C with maximal activity at 45°C, and no activity at 62°C. These results also correspond well to the range of temperatures known to support the growth of *M. phlei* GTIS10 which grows maximally at about 45°C and does not grow at temperatures above about 52°C.

Taken altogether then, the results shown in Figure 2 plus the knowledge that DNA sequence analysis shows that both cultures contain identical *dsz* operons/enzymes indicate that the ability of the *dsz* enzymes to function is greatly influenced by the bacterial host strain. *R. erythropolis* IGTS8 exhibits maximal desulfurization activity corresponding to the optimum growth temperature of the culture and then desulfurization activity declines in concert with decreasing cell viability at higher temperatures. Figure 2 indicates that desulfurization activity in *M. phlei* GTIS10 also showed a maximum activity at about 45°C corresponding to the optimum temperature for this culture, and that desulfurization specific activity continues at temperatures up to 57°C which is beyond the growth range for this culture. The desulfurization pathway requires NADH, $FMNH_2$, and oxygen in order to complete the conversion of DBT to 2-HBP. The host must supply these factors so that the functional temperature range of the desulfurization pathway is seen to be different in two different bacterial hosts. The observation that the *dsz* operon has two apparent temperature maximums in two different bacterial hosts suggests that perhaps if the *dsz* operon could be expressed in a thermophilic bacterial host the desulfurization enzymes may function at even higher temperatures.

To further explore the thermostability of the desulfurization enzymes the *R. erythropolis* IGTS8 *dsz*A, *dsz*B, and *dsz*C *genes* were cloned individually and expressed in *E. coli* at high levels so that the thermostability of individual desulfurization enzymes could be determined. Each desulfurization gene was cloned into the *E. coli* expression vector pFLAG-CTC under control of the *tac* promoter. The genes were induced by the addition of IPTG, cell lysates were prepared and analyzed on polyacrylamide gels and with enzymatic assays. Polyacrylamide gel electrophoresis was performed to confirm that proteins of the correct molecular weight were present in the cell lysates (data not shown).

Cell lysates containing *dsz*A, *dsz*B and *dsz*C protein were pre-incubated at 30°, 37°, 45°, 52°, 65°, and 72°C for 30, 60, and 120 minutes. The enzyme solutions were assayed by adding the appropriate substrate and/or co-factors, incubated at 30°C for an hour and then determining the amount of product formed by HPLC and/or TLC analysis. The results from both TLC and HPLC analyses indicate that *dsz*B exhibits activity (conversion of HBPsi to 2-HBP) at 30° and 37°C but little or no activity is seen at temperatures of 45°C or higher even with exposure times as brief as 30 minutes. The dibenzothiophene monooxygenase encoded by *dsz*C retains activity

even after 2 hours of exposure at 72°C, although at reduced levels compared to 30-45°C pre-incubation reactions. The formation of protein inclusion bodies prevented the production of a functional dibenzothiophene-5, 5'-dioxide monooxygenase from the *dsz*A gene cloned in the *E. coli* expression vector pFLAG-CTC despite repeated attempts.

The metabolites of dibenzothiophene produced by *M. phlei* GTIS10 and *R. erythropolis* IGTS8 were determined by GC-MS analysis of extracts of cultures grown at 45°C and 30°C respectively with DBT as the sole source of sulfur . Cultures were harvested in mid log phase of growth at an optical density of approximately 1.0 at $A_{600\ nm}$. The results are listed in Table 2 which shows that both cultures metabolized a similar amount of DBT, but the relative abundance of metabolites produced by each culture showed differences. Of all of the DBT metabolized by *R. erythropolis* IGTS8 95% was converted to 2-HBP; whereas, for *M. phlei* GTIS10 only 65% of the DBT metabolized was converted to 2-HBP. In particular *M. phlei* GTIS10 produced greater levels of dibenzothiophene sulfoxide and dihydroxybiphenyls than did *R. erythropolis* IGTS8. A significant difference between *M. phlei* GTIS10 and *R. erythropolis* IGTS8 illustrated in Table 2 are the results obtained for dihydroxybiphenyls. About 3.3% of the DBT metabolized by *M. phlei* GTIS10 was converted to dihydroxybiphenyls, while no dihydroxybiphenyls were observed in the metabolites produced by *R. erythropolis* IGTS8. While in the experiment reported in Table 2 no dihydroxybiphenyls were observed as metabolites produced by *R. erythropolis* IGTS8 occasionally trace amounts of dihydroxybiphenyls are observed as metabolites of DBT produced by *R. erythropolis* IGTS8 (data not shown) and this has been reported by others as well (9, 28).

Since *dsz*B catalyzes the last step in the desulfurization pathway and is responsible for the release of sulfur from DBT it must be functional in order to allow cultures to grow and utilize DBT as a sole sulfur source and it is required to allow the production of 2-HBP in the data reported in Figure 2. Therefore it is surprising that the *dsz*B enzyme appears to be thermally inactivated in vitro by exposure to temperatures as low as 45°C yet some activity is detected in whole cells at temperatures of 57°C. While the *dsz*B enzyme may be the most thermolabile enzyme in the desulfurization pathway thermal inactivation is not instantaneous and it apparently has enough residual activity even at 57°C to allow the accumulation of 2-HBP to be detected. Table 2 shows that dihydroxybiphenyls were observed in the supernatant of 50°C GTIS10

cultures. Oldfield *et al* [Oldfield, 1997 #1538] describes a minor desulfurization pathway in which the dibenzothiophene-5,5-dioxide monooxygenase (*dsz*A product) produces dihydroxybiphenyls from biphenylene sulfone. Biphenylene sulfone can be formed from non-enzymatic oxidation of 2-hydroxybiphenyl-2- sulfinate (HBPSi) . Perhaps at higher temperatures, the *dsz*B enzyme is inactive and the minor desulfurization pathway catalyzed by *dsz*A is active to provide sulfur to the cells.

The dibenzothiophene monooxygenase encoded by *dszC* is responsible for the conversion of DBT to dibenzothiophene sulfoxide and then to dibenzothiophene sulfone. The elevated levels of dibenzothiophene sulfoxide seen in *M. phlei* GTIS10, as compared with *R. erythropolis* IGTS8 (see Table 2), might suggest that the dibenzothiophene monooxygenase is temperature sensitive and works less efficiently at 45°C than at 30°C. However, the results of in vitro experiments do not support this conclusion, as dibenzothiophene monooxygenase exhibits good activity at 45°C while the 2-hydroxybiphenyl-2-sulfinate sulfinolyase encoded by *dsz*B is the most thermolabile desulfurization enzyme. The *dsz*C enzyme requires other cofactors/substrates for proper functioning: $FMNH_2$ and oxygen. The FMN oxidoreductase encoded by *dsz*D is responsible for providing $FMNH_2$ to enable the reactions catalyzed by *dsz*C to proceed. The accumulation of DBTSO in the supernatant of the GTIS10 45°C growing cell experiment might indicate that the FMN oxidoreductase was significantly inactivated in vivo at 45°C and the dszC gene did not go to completeion due to lack of cofactor. We examined the *M. phlei* GTIS10 *dsz*D for thermal inactivation. The *M. phlei* GTIS10 FMN oxidoreductase (*dsz*D) functioned with little inactivation up to 45°C but was progressively inactivated by exposure to higher temperatures. The half-life of the *dsz*D enzyme was determined to be about 17 minutes at 50°C; however, slight activity was detectable even after incubation at 72°C for 60 minutes.

These data demonstrate that the thermostability of enzymes determined in vitro is not necessarily a good predictor of the functional range of an enzyme in vivo, and that the same operon can yield metabolic pathways with different rate limiting steps and different yields of metabolites in different hosts.

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Table 1. Range of Organosulfur Substrates Utilized by *M. phlei* GTIS10

| *Substrate* | *Used as sole source of sulfur* |
|---|---|
| Dibenzothiophene | + |
| Dibenzothiophene sulfoxide | + |
| Dibenzothiophene sulfone | + |
| 4,8-dimethyl dibenzothiophene | + |
| 2-methyl dibenzothiophene | + |
| Dibenzothiophene-2,8-dicarbaldehyde | + |
| Trithiane | + |
| Thianthrene | + |
| Thianaphthene | - |
| Thiophene | + |
| 2-ethyl thiophene | + |
| 2-thiophene carboxylic acid | - |
| 2-thiophene carbonitrile | - |
| 3-thiophene carboxylic acid | - |
| Trans-1,2-dithiane-4,5-diol | + |
| 1,4-dithiane-2,5-diol | + |

| | |
|---|---|
| 1,3-dithiane | + |
| 1,4-dithiane | + |
| Trimethylsilyl-1,3-dithiane | + |
| Thioxanthrene-9-one | + |
| 1,4,7-trithiacyclononane | - |
| 1,4,7-trithiacyclodecane | - |
| Thiodiglycol | + |
| Thioxanthene | + |
| Phenyl sulfoxide | - |
| Thionin | - |
| Sulfanilamide | - |
| 2-nitrophenyl disulfide | + |
| 4-nitrophenyl disulfide | + |
| 3-nitrophenyl disulfide | - |
| 4-aminophenyl disulfide | - |
| Tolyl disulfide | - |
| Benzyl disulfide | + |
| Phenyl disulfide | - |
| Dimethylsulfoxide | + |
| 2,2'-thiobis(4,6-dichlorophenol) | - |
| Diphenylthiocarbazone | + |
| 5,5'-dithio-bis(2-nitrobenzoic acid) | - |
| 2(methylmercapto)benzimidazole | + |
| 2-methyl-b-naphthothiazole | - |
| 2-phenylbenzothiazole | - |
| Thiazole | + |
| Benzothiophene | + |

Table 2. The Relative Abundance of Metabolite of Dibenzothiophene Produced by *Mycobacterium phlei* IGTS10 Differs from *Rhodococcus erythropolis* IGTS8

| **Compound** | ***Rhodococcus erythropolis* IGTS8** | ***Mycobacterium phlei* IGTS10** |
|---|---|---|
| dibenzothiophene | 1590 [1] | 1580 |
| dibenzothiophene sulfoxide | 24 | 180 |
| dibenzothiophene sulfone | < 1.0 | 1.0 |
| hydroxybiphenyl sulfine | < 1.0 | < 1.0 |
| 2-hydroxy biphenyl | 481 | 382 |
| dihydroxy biphenyl #1 | < 1.0 | 5.0 |
| dihydroxy biphenyl #2 | < 1.0 | 115 |
| Total | 2095 | 2162 |

[1] = All concentrations are expressed as μg/ml of the ethyl acetate extract derived from each culture.

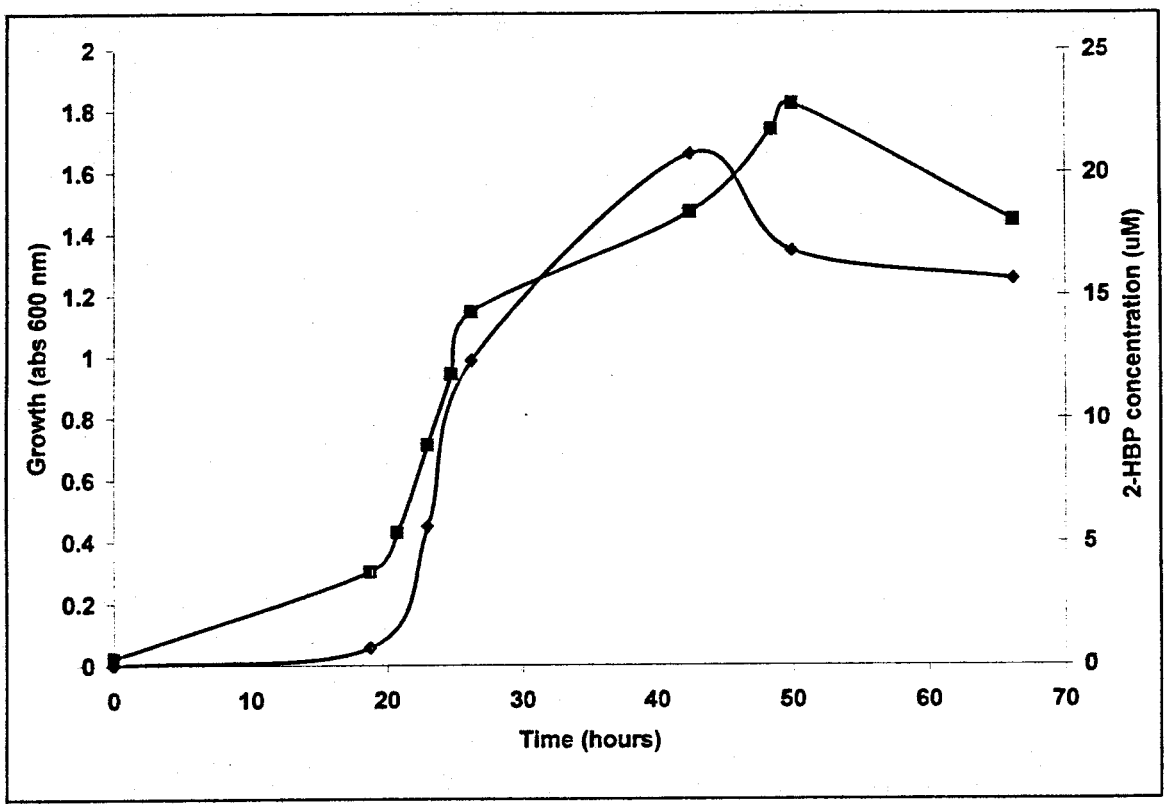


Figure 1. Growth of *M. phlei* GTIS10 at $50^0$C using DBT as the sole sulfur source. The accumulation of 2-hydroxybiphenyl (2-HBP) in the supernatant of the growing culture was examined by removing 1.0 ml aliquots at each data point and quantifying 2-HBP using HPLC. Each data point recorded are averages of three replicate samples from three separate experiments for a total of nine data points. Standard deviation is 5% or less for all data. ◆ , 2-HBP; ■ , growth

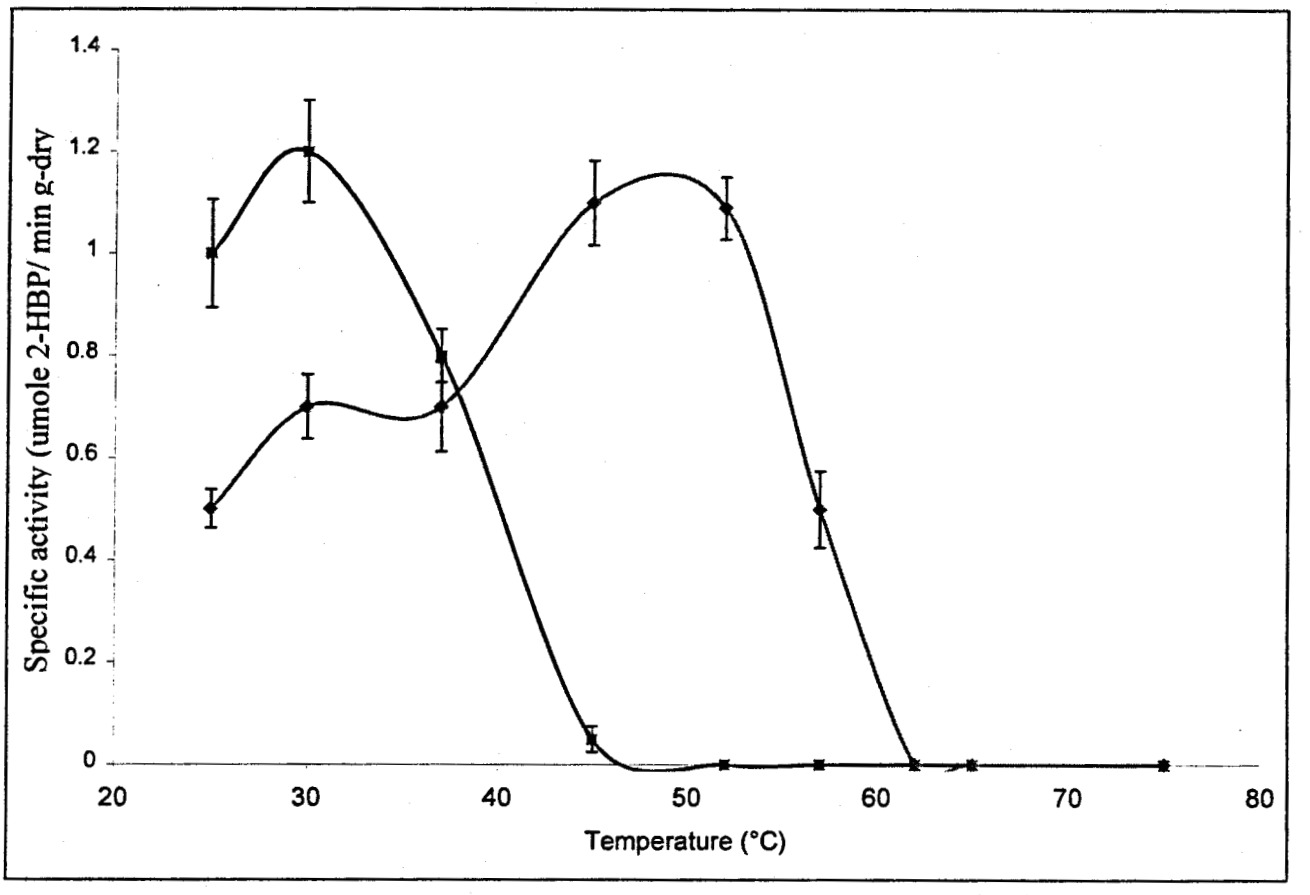


Figure 2. Resting cells of *M. phlei* GTIS10 exhibit specific desulfurization activity at higher temperatures than resting cells of *R. erythropolis* IGTS8. The amount of 2-HBP produced by the conversion of DBT by each culture after incubation for 24 hours at various temperatures was quantified by HPLC. Rate of change in 2-HBP concentration was calculated from the linear portion of the curve, generally the first 4 hours of the incubation. Specific desulfurization activity values recorded are averages of three replicate samples from three separate experiments for a total of nine data points. Standard deviation was less than 10 %. ◆, *M. phlei* GTIS10 ; ■, *R. erythropolis* IGTS8.

# APPENDIX B:
# A New Host/Vector System for Thermus sp. Based on the Malate Dehydrogenase Gene

**A new host/vector system for *Thermus* sp. based on the malate dehydrogenase gene.**

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Running Title: *Thermus* host vector system

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**Abstract**

A *Thermus thermophilus* HB27 strain was constructed in which the malate dehydrogenase *(mdh)* gene was deleted. The *Δmdh* colonies are recognized by a small colony phenotype. Wildtype phenotype is restored by transformation with *Thermus* plasmids or integration vector containing an intact *mdh* gene. The wildtype phenotype provides a positive selection tool for the introduction of plasmid DNA into *Thermus sp. and* because *mdh* levels can be readily quantified, this host/vector system is a convenient tool for monitoring gene expression.

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*Thermus* sp. are Gram negative thermophilic microorganisms that grow at temperatures between 50°C and 82°C *[Oshima, 1974 #1337; Brock, 1969 #1356].* Plasmid vectors that have been used in *Thermus* have previously been constructed that encode tryptophan, leucine, or pyrimidine synthesis genes to complement auxotrophic/deleted hosts, thus providing for a positive selection of transformants [Koyama, 1990 #919; Raven, 1995 #1357]. The host-vector system described here is an improvement on previously reported plasmid vectors for *Thermus* sp. because not only is the *E.coli-Thermus* shuttle vector easily selected and maintained in *Thermus,* but also malate dehydrogenase activity encoded by the *mdh* gene present in this vector can be readily and accurately quantified [Horinouchi, 1987 #1064].

We describe the construction of a *T. thermophilus* Δ*mdh* strain containing a deletion of the entire DNA sequence encoding the *mdh* gene and its use as a host for *Thermus* plasmids expressing an intact *mdh* gene. The use of this host/vector system approach was demonstrated in both *T. thermophilus* and *T. flavus* and should be useful in *Thermus* sp. generally. The TCA cycle in *Thermus* sp., like most microorganisms, plays a central role in metabolism. Malate dehydrogenase (MDH) catalyses the dehydrogenation of malate to oxaloacetate using $NAD^+$ as a cofactor and is a key enzyme in the TCA cycle. The *T. thermophilus* Δ*mdh* host/expression vector system offers a strong positive selection tool for the introduction of mdh-containing plasmid DNA into *Thermus* sp.. The growth rate advantage of $mdh^+$ versus *Δmdh* cells enriches for cells that retain mdh-containing plasmids which has the effect of stabilizing these plasmids in *Δmdh* hosts. Additionally mdh can be used as a reporter gene to quantify promoter strength in *T. thermophilus.*

To create the Δ*mdh* strain of *T. thermophilus* HB27 we constructed an integration vector designated pUC-S KmA. The construction is detailed in Figure 1. A 2.3 kb PCR fragment containing a region spanning three separate genes: succinate--CoA ligase (*scsA*), malate dehydrogenase (*mdh*) and purine phosphoribosyltransferase (*gpt*) gene was PCR amplified from the *Thermus flavus* chromosome and cloned into pUC 18. A 10 ul PCR amplification reaction was conducted in an Idaho Technologies Rapid Air Thermo-Cycler in the presence of 8% glycerol and 1% dimethyl sulfoxide (DMSO). The PCR amplification cycle was ran for 40 cycles at 94°C, 55°C and 1 min holding at 72°C. The 984 bp *mdh* gene is located near the center of this 2.3 kb fragment so that the chromosomal regions that flank the *mdh* are 780 bp five prime and 560 bp three prime. The entire coding sequence of the *mdh* gene was removed by restriction enzymes and replaced with a thermotolerant kanamycin resistance cassette *($Km^R$)*[Matsumura, 1985 #1358]. This vector designated pUC-SKmA can replicate in *E. coli* but not in *Thermus*. However, homologous recombination between the *scs*A and *gpt* gene sequences allows pUC-SKmA to integrate into the chromosome when it was used to transform *T. thermophilus* HB27 [Koyama-Y, 1986 #2159]. Transformants were screened at 55°C on TT rich medium [Lasa, 1992 #772] supplemented with kanamycin (40 μg/ml). Approximately 1 x $10^4$ kanamycin resistant transformants per μg DNA were observed. Two distinct colony types arose after 5 days incubation. The majority of the colonies were very small (0.1 to 0.5 mM) even after 5 days. A few colonies (60 - 100 CFU) were much larger (1.5 to 2.8 mM), the same size as wild type *T. thermophilus* HB27 colonies. The two colonies types were sub-cultured and total DNA (plasmid and chromosomal) was dot blotted onto a nylon membrane. The dot blot was probed with a Digoxigenin-11-dUTP (DIG) labeled *T. flavus mdh* gene. DNA prepared from the smaller colonies did not hybridize to the *T. flavus mdh* gene whereas the DNA harvested from the larger colonies hybridized to the *mdh* probe. The membrane was also probed with a DIG labeled $Km^R$ cassette, both small and large colony types hybridized to the $Km^R$ cassette. Because MDH is a key enzyme in the TCA cycle the colonies resulting from double crossover integration events *(Δmdh)*, are recognized by this small colony phenotype. The larger $Km^R$ colonies were single crossover integration events where the chromosomal *mdh* is intact and the entire plasmid is in the chromosome. The *T. thermophilus Δmdh* $Km^R$ mutant strain was designated MM8-5. *T. thermophilus Δmdh* $Km^R$ MM8-5 was used as a recipient in further transformation experiments.

A derivative of *T. flavus* containing a deletion of the mdh gene was also constructed using the approach described above. This strain was designated TfMM-1.

Construction of *Thermus* vectors containing *mdh* as a reporter gene.

The malate dehydrogenase *(mdh)* gene from *T. flavus* was amplified by PCR and cloned into a *Thermus* and *E. coli* expression vector pTEXI. The expression vector pTEXI is capable of replication in both *Thermus* sp. and *E. coli,* and the promoter (J17) employed in this expression vector functions in both bacterial hosts. J17 is a constitutive promoter isolated in our lab from *T. thermophilus* chromosomal DNA. The expression vector containing the *mdh* gene, designated pTEXI-mdh, is diagramed in Figure 2 A.

The J17 promoter from pTEXI-J17 mdh was replaced by two constitutive *Thermus* promoters isolated in our laboratory (D50-3 and P2-100). These promoters have low and medium levels of expression in *Thermus* relative to J17. The resulting plasmids were designated pTEX1- D50-3 and pTEX1-P2-100. An integrative vector was constructed to examine the expression of the *mdh* gene under control of the J17 promoter present as a single integrated copy. This construct was designated pSJ17mdhA and is shown in Figure 2B. pSJ17mdhA contains pUC19 sequences and can replicate in *E. coli.* pSJl7mdhA does not replicate in *Thermus* as a plasmid but integrates into the chromosome by a double crossover event. pSJ17mdhA has the J17 promoter-*mdh* gene cassette flanked by the *scsA* and *gpt* chromosomal regions. A transcriptional termination sequence from the *T. flavus* phenylalanyl tRNA synthetase operon [Keller, 1992 #801] was cloned upstream of the cassette to prevent transcription read through from the native succinate--CoA ligase/malate dehydrogenase operon promoter.

Plasmids pTEXl-mdh, pTEXI-D50-3 and pTEXl-P2_100 and the integrative vector pSJl7mdhA were transformed into MM8-5. Transformants were easily detected by the restoration of cultures to the wildtype or larger and faster growing colonies by the expression of the malate dehydrogenase gene located on these expression vectors. Typically *T. thermophilus* strain MM8-5 takes 4 to 5 days to form small visible colonies at 55°C in TT supplemented with 40μg/ml kanamycin. *T. thermophilus* MM8-5 transformants that received an expression vector encoding the mdh gene yielded larger colonies in 2 to 3 days. The presence of pTEXI-*mdh* and the related

*mdh* expression vectors pTEXI-D50-3 and pTEXI-P2-100 in MM8-5 was confirmed by harvesting plasmid DNA from *Thermus* colonies which was subsequently visualized on an electrophoresis gel and used to transform the plasmid back into *E. coli* (data not shown). In contrast, and as expected, plasmid DNA could not be detected/recovered from *T. thermophilus* MM8-5 transformants that received the integrative expression vector pSJ17mdhA.

Expression vector pTEXI-mdh and the alternative promoter pTEX derivatives are very stable in both *T. thermophilus* MM8-5 and *T. flavus* TfMM-1 respectively. After greater than 20 generations of growth under non-selective conditions pTEX plasmids were detected in all of the colonies examined (100 for each species). This result is expected because those *T. thermophilus* MM8-5 or *T. flavus* TflVIM-1, cells that possess expression vectors containing the *mdh* gene grow more rapidly than plasmid-free cells that lack a functional *mdh* gene.

Malate dehydrogenase activity of *Thermus* constructs

The levels of malate dehydrogenase (MDH) [Horinouchi, 1987 #1064] being produced by plasmid and integrative expression vectors were evaluated in both *T. thermophilus* HB27 and MM8-5. Crude lysates prepared from each culture were assayed for MDH activity at two temperatures (25°C and 50°C) and results are shown in Table 1. *T. thermophilus* MM8-5 had slight to no MDH activity confirming the complete deletion of the *mdh* gene from the chromosome of this strain. The activity observed in assays performed at 50°C reflect a slight amount of background due to the conversion of NADH to NAD by unidentified components of cell lysates rather than the MDH-dependent conversion of oxaloacetate and NADH to malate and NAD.

The MDH activity of crude extracts assayed at 50°C is on average 9 times higher than the activity levels measured at 25°C. The data in Table 1 clearly indicate that promoters D50-3, P2-300, and J17 have different strengths resulting in MDH levels in MM8-5 strains that are 0.28, 1.16, and 1.65 times the MDH level in wild type *T. thermophilus* HB27 respectively. Since each promoter is evaluated here in identical genetic constructs that differ only by virtue of the promoter driving the expression of the *mdh* gene these MDH levels should serve to accurately quantify the strength of these promoters. Other strains whose MDH activity is listed in Table 1

all use the same promoter, J17, to express the *mdh* gene in various backgrounds. MM8-5/pS-J17mdh-A contains a single copy of the *mdh* gene integrated into the chromosome under the control of the J17 promoter. MM8-5/pTEXI-mdh contains the *mdh* gene under the control of the J17 promoter on a plasmid vector and the HB27/pTEXI-mdh contains two separate sources of the *mdh* gene, a wild type *mdh* gene on the chromosome as well as the *mdh* gene under the control of the J17 promoter on a plasmid vector. Since MM8-5/pTEXI-mdh yields 32.6 units of MDH/mg protein it is unexpected that HB27/pTEXI-mdh that contains two separate copies *mdh* gene, a wild type *mdh* gene on the chromosome as well as the *mdh* gene on a plasmid vector, shows nearly the same MDH activity (29.6 units/mg). There may be some plasmid instability contributed to homologous recombination between the two *mdh* gene copies. Although there is no apparent difference in the copy number of the plasmid in these two strains as observed in plasmid DNA samples prepared from equal amounts of cells from both cultures (data not shown). Perhaps there are currently unknown factors that limit the amount of MDH protein that can be made in *T. thermophilus,* but this would require further investigation.

The addition of a functioning malate dehydrogenase gene to *Thermus* expression vectors provides a plasmid stabilizing mechanism in *Δmdh* hosts, without the addition of antibiotics. This *Thermus* malate dehydrogenase based host-vector system should prove to be useful in future genetic studies with *Thermus* sp. particularly because malate dehydrogenase activity can be conveniently and accurately quantified using a simple spectrophotometric assay and can be used as reporter gene to evaluate promoter strength and gene expression. The results reported here demonstrate the use of this host/vector system to examine constitutive promoters of various strengths. This *Thermus* host/vector system can also be useful in the investigation of genetic expression in *Thermus sp.*

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Table 1. Malate dehydrogenase activity of *Thermus* vector constructs.

| **Culture and Condition** | **Promoter expressing *mdh*** | **MDH activity at 25°C (Units MDH/mg)** | **MDH activity at 50°C (Units MDH/mg)** |
|---|---|---|---|
| HB27 | wild type | 2.5 +/- .12 | 19.9 +/- .25 |
| MM8-5/pSJ17mdhA* | J17 | 0.6 +/- .01 | 5.5 +/- .17 |
| HB27/pTEXl-mdh | J17 | 3.7 +/- .06 | 29.6 +/- 1.39 |
| MM8-5 | None | 0.0 | 0.5 +/- .01 |
| MM8-5 /pTEXI-mdh | J17 | 4.2 +/- .13 | 32.6 +/- .72 |
| MM8-5 /pTEX1-D50-3 | D50-3 | 0.3 | 2.7 +/- .07 |
| MM8-5/pTEX1-P2-300 | P2-300 | 3.0 +/- .07 | 23.2 +/- .99 |

*MDH activity values recorded are averages of three replicate samples from three separate experiments for a total of nine data points. Standard deviation is less than 5%. MDH activity is reported as units of MDH per mg protein in cell lysates. One unit of MDH activity is defined as the amount of enzyme needed to convert one micromole of NADH to NAD in one minute. * = integrative vector.*

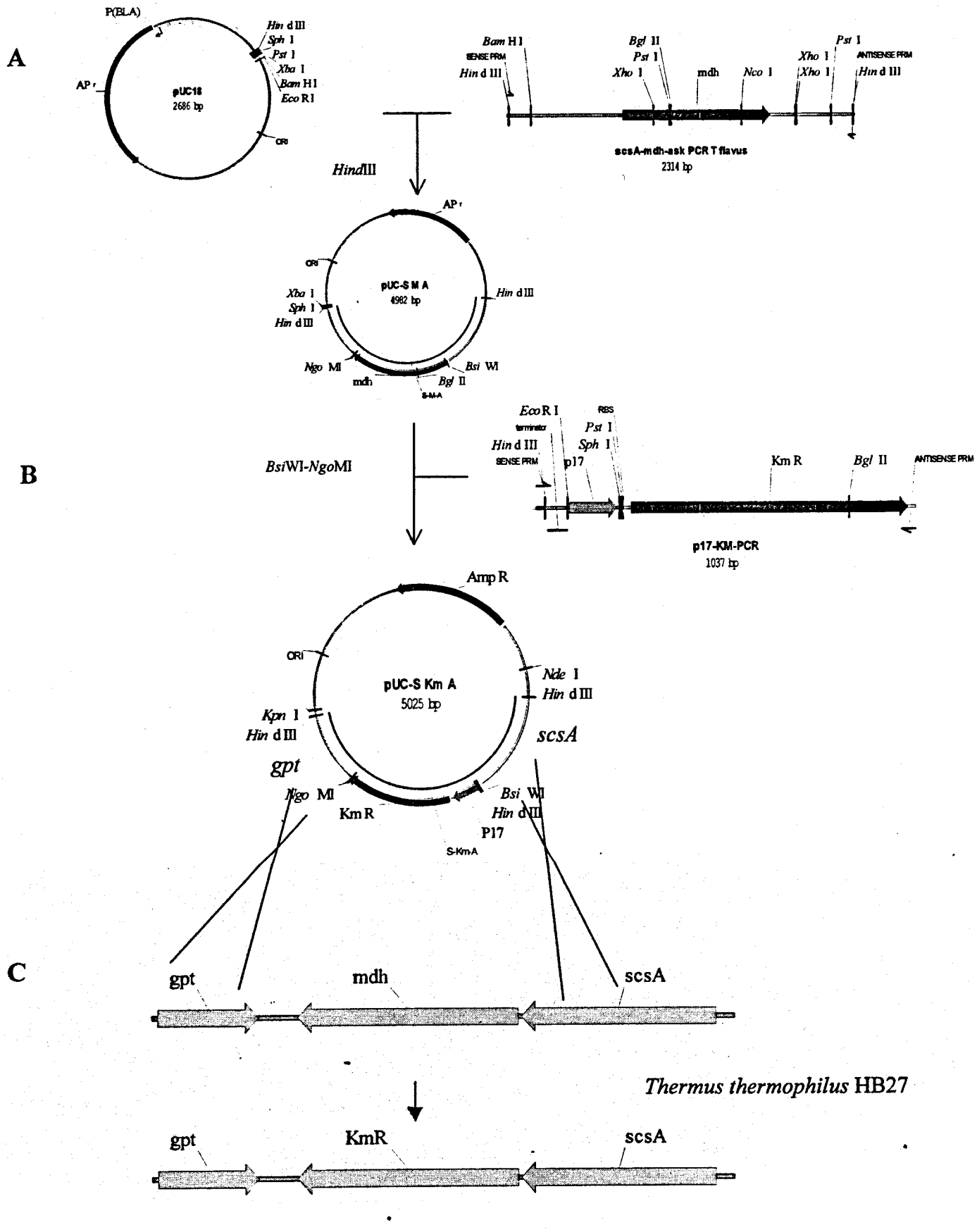

A
P(BLA)
Hin d III
Sph I
Pst I
Xba I
Bam H1
Eco R1
AP r
pUC18
2686 bp
ORI
Bam H1
SENSE PRM
Hin d III
Bgl II
Pst I
Xho I
mdh
Nco I
Xho I
Pst I
Xho I
ANTISENSE PRM
Hin d III
scsA-mdh-ask PCR T flavus
2314 bp
HindIII
AP r
ORI
pUC-S M A
4982 bp
Xba I
Sph I
Hin d III
Hin d III
Ngo MI
mdh
Bsi WI
Bgl II
S-M-A
B
BsiWI-NgoMI
Eco R I
terminator
RBS
Pst I
Hin d III
SENSE PRM
p17
Sph I
Km R
Bgl II
ANTISENSE PRM
p17-KM-PCR
1037 bp
Amp R
ORI
pUC-S Km A
5025 bp
Nde I
Hin d III
Kpn I
Hin d III
scsA
gpt
Ngo MI
Km R
Bsi WI
Hin d III
P17
S-Km-A
C
gpt
mdh
scsA
Thermus thermophilus HB27
gpt
KmR
scsA

Figure 1. Construction of *Thermus thermophilus* $_{\Delta}$*mdh* $Km^R$ MM8-5.

A. The region surrounding the *mdh* gene from *Thermus flavus* was amplified from chromosomal DNA using the following PCR primers: forward

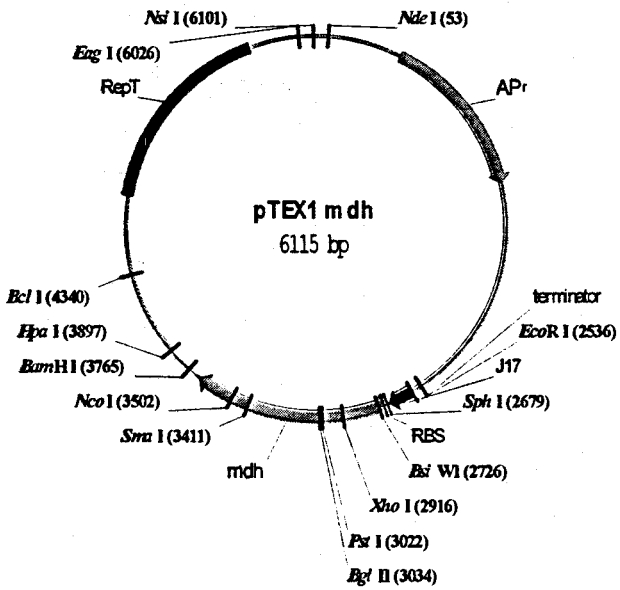
5′-ACAACAAAGCTTCGGGCAAAGGGGGAACGGAGGTCCT-3′ and reverse

5′-ACAACAAAGCTTGAGCCTTTTGACCTCGTCCTGGGG-3′. These primers were designed to amplify the sequence of the malate dehydrogenase and the regions immediately flanking the malate dehydrogenase gene from *T. flavus* according to published DNA sequences [Nishiyama, 1991 #880; Nishiyama, 1995 #556; Nishiyama, 1986 #1093] Restriction sites were added into the PCR primers to give 5′ and 3′ *Hind*III sites. The 2.3 Kb PCR product containing the *mdh* gene and flanking regions was cloned into the *Hind*III site of pUC18. The resulting plasmid is 4982 bp and was designated pUC S-M-A. B. Plasmid pUC S-M-A was digested with *Bsi*WI and *NgoMI* removing the all but the first 20 bp of the *mdh* gene sequence. A kanamycin resistance cassette was amplified using plasmid pTEX J17 as the template and using the following PCR primers: forward.

5′-ACAACACGTACGGATTACGCCAAGCTTCATGGCCTAA-3′ and reverse

5′-ACAACAGCCGGCTCGTTCAAAATGGTATGCGTTTTG-3′. Restriction sites were added into the PCR primers to give a 5′ *Bsi*WI and 3′ *NgoMI* site. The cassette contains a strong constitutive *Thermus* promoter (J17) upstream of the themostable kanamycin nucleotidyltransferase cassette ($Km^R$) . To prevent transcription read through from the native mdh promoter a transcriptional termination sequence was cloned upstream of the J17 promoter. The kanamycin resistance cassette was digested with *Bsi*WI and *Ngo*MI and ligated into the *Bsi*WI-*Ngo*MI digested pUC S-M-A. The *mdh* gene was replaced with a kanamycin resistance cassette and the resulting plasmid was designated pUC- S Km A. C. After transformation of plasmid pUC- S Km A into *T. thermophilus* HB27 a double crossover homologous recombination event replaces *mdh* with the $Km^R$ determinant. The $_{\Delta}$*mdh* $Km^R$ *T. thermophilus* HB27 strain subsequently isolated was designated MM8-5.

A


Nsi I (6101)
Nde I (53)
Eag I (6026)
RepT
APr
pTEX1 m dh
6115 bp
Bcl I (4340)
terminator
Hpa I (3897)
EcoR I (2536)
BamH I (3765)
J17
Nco I (3502)
Sph I (2679)
Sma I (3411)
RBS
mdh
Bsi WI (2726)
Xho I (2916)
Pst I (3022)
Bgl II (3034)

B

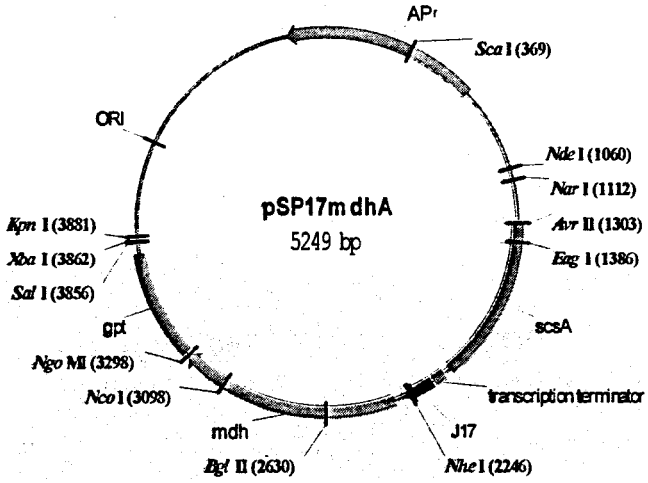

APr
Sca I (369)
ORI
Nde I (1060)
Nar I (1112)
pSP17m dhA
5249 bp
Kpn I (3881)
Avr II (1303)
Xba I (3862)
Eag I (1386)
Sal I (3856)
scsA
gpt
Ngo MI (3298)
transcription terminator
Nco I (3098)
mdh
J17
Bgl II (2630)
Nhe I (2246)

**Figure 2.** A. **Expression vector pTEX1-mdh contains the *T. flavus* malate dehydrogenase downstream of the strong constitutive *Thermus* promoter J17. The *mdh* gene from *T. flavus* was amplified from chromosomal DNA using the following PCR primers: forward *mdh***

**5′-ACACAGAATTCGCATGCTCAAGAAGGCCCTGGGCTAA-3′ and reverse *mdh***

5′-ACACACGGATCCTGCGCCAGCATGGGGTGGTATAAA-3′. Restriction sites were added into the PCR primers to give 5′ *Eco*RI and 3′ *Bam*HI. Plasmid pTEX1 was digested with *Eco*RI and *Bam*HI and a *Eco*RI-*Bam*HI digested *mdh* PCR product was ligated into the vector to create pTEX1-*mdh*. **B.** Integrative vector pSJ17mdhA has the J17 promoter-*mdh* gene cassette flanked by the *scsA* and *gpt* chromosomal regions.

# APPENDIX C:

## Inducible and Constitutive Expression Using New Plasmid and Integrative Expression Vectors for Thermus

Inducible and Constitutive Expression Using New Plasmid and Integrative Expression Vectors for *Thermus* Sp.

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**Running Title: Expression Vectors for *Thermus* Sp.**

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Phone: 847-768-0686, Fax: 847-768-0669

SUMMARY

This report describes the construction and use of improved promoter probe and expression vectors for use in *Thermus* species. Two plasmid promoter probe vectors and an integrative promoter probe vector were constructed. All three of the promoter probe vectors contain unique restriction sites located upstream of a promoterless thermostable kanamycin nucleotidyltransferase cassette ($Km^R$) that were used for the isolation of promoter fragments. Fragments of *Thermus thermophilus* HB27 chromosomal DNA were cloned into these promoter probe vectors and the constructs were transformed into *T. thermophilus* HB27. Several candidates with strong promoters cloned upstream of the $Km^R$ gene were isolated and characterized. Three expression vectors were constructed based on one of these promoters, J17, that functions in both *Thermus* and *Escherichia coli*. Plasmid based pTEX-PL and pTEX-RBS are expression vectors with the *Thermus* J17 promoter upstream of a multiple cloning site. An integrative vector pTG-J17 has the J17 promoter upstream of a MCS flanked by portions of the *leuB* gene. An inducible expression vector was constructed using the heat shock inducible promoter (70°C to 85°C) from the *dnaK* gene of *T. flavus* with a multiple cloning site downstream. To demonstrate the utility of these expression vectors, the malate dehydrogenase gene (*mdh*) from *Thermus flavus* was cloned and expressed in both *E. coli* and *T. thermophilus* HB27.

Keywords: *Thermus*, *thermophilus*, *flavus*, promoter, expression, vector, inducible, plasmid, integrative.

# INTRODUCTION

*Thermus* species are gram negative thermophilic microorganisms that grow at temperatures between 50°C and 82°C [Brock, 1969 #1356; Oshima, 1974 #1337]. While thermostable enzymes comprise a significant and increasing percentage of biotechnology products thermophiles are not currently used as production hosts in biomanufacturing processes. Rather, genes that encode industrially important thermostable enzymes are cloned and expressed in mesophilic microorganisms for commercial production. This is because a multitude of genetic tools is available to allow the expression of genes of interest in a few mesophilic microorganisms such as *E. coli* and *Bacillus subtilis*, while genetic expression studies in thermophiles are less well developed. Due to the lack of convenient and effective genetic tools for thermophiles, genetic and biochemical studies are frequently performed by cloning genes from thermophiles and expressing them in mesophiles.

Shuttle vectors with both mesophilic and thermophilic replication functions have been constructed and various thermophilic selection markers (Km $^{R}$, *trpB, leuB* and β-*gal*) have been used [Raven, 1995 #1357]. Expression of cloned genes on plasmid [Koyama, 1990 #935]; [Lasa, 1992 #772]; [Mather, 1992 #832; Lee, 1994 #628; Wayne, 1997 #299; Maseda, 1995 #558] and integrative [Weber, 1995 #213] [Tamakoshi, 1997 #313] vectors in *Thermus* has also been achieved. However, most of the cloning and expression vectors used for genetic studies in *Thermus* species do not possess a combination of features that make them convenient to use for a variety of applications. While plasmid and integrative vectors have been used for the expression of cloned genes in *T. thermophilus* these existing vectors do not contain convenient multiple cloning sites, terminator sequences to prevent transcriptional read-through from vector DNA, ability to replicate in *E. coli* and *T. thermophilus,* and selectable antibiotic resistance genes that function in both *E. coli* and *T. thermophilus*. A key limitation in gene expression studies in *Thermus* thus far has been the lack of inducible promoters. The vast majority of expression vectors used in *E. coli* and for genetic studies generally employ inducible promoters. Furthermore, there is no consensus in the literature concerning the relative merits of plasmid versus integrative vectors for use in *T. thermophilus*. The plasmid and integrative vectors described here using identical reporter genes and promoters can subsequently be used to address this issue.

This paper describes the development of genetic tools for the isolation of promoters and the expression of genes in *Thermus* species using both constitutive and inducible promoters. These genetic tools will be applicable in a variety of biotechnological applications such as directed evolution of mesophilic genes to create thermostable derivatives or the expression of genes in thermophiles.

## MATERIALS AND METHODS

Cultures, Plasmids and Culture Procedures. The Km $^{R}$ plasmid pUC19EKF-TSP3 was a gift from Dr. Shuang-yong Xu, New England Biolabs. *Thermus thermophilus* HB27 Pro$^{-}$ was grown in TT rich medium [Lasa, 1992 #772]. DH5$_{\alpha}$ was used as the *E. coli* host. DH5$_{\alpha}$ competent cells were purchased from Life Technologies (Rockville, MD) and grown using nutrient broth or nutrient agar (Difco, Detroit, Michigan). Unless otherwise noted, kanamycin was included in liquid or agar medium at 40$_{\mu}$g/ml in experiments involving either *T. thermophilus* or *E. coli* cultures. Plasmid pBlueScript KS+ was obtained from Stratagene (La Jolla, CA) and pTG100 was described earlier [Weber, 1995 #213].

Chemicals , Biochemical Assays and Enzymes. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4 ligase was purchased from Life Technologies (Rockville, MD). Antibiotics were purchased from Sigma Chemical C. (St. Louis, MO). Malate dehydrogenase activity was determined using a spectrophotometric assay according to published procedures [Horinouchi, 1987 #1064]. Cell-free extracts were prepared for the malate dehydrogenase activity tests by growing *Thermus* cultures in TT media at 55°C overnight until mid to late log phase growth was achieved. When heat shocking was employed, cell cultures were incubated for 10 minutes at 85°C. Cell pellets were harvested by centrifugation at 10,000 x g for 10 minutes at room temperature, washed once with TES buffer (50mM Tris-HCL; 5 mM EDTA; 50 mM NaCL, pH 7.5) and resuspended in 0.1 M potassium phosphate (pH 7.0) buffer. The cell suspensions were disrupted by sonication using a Branson 350 sonifier equipped with a microtip. *E. coli* cell free extracts were heat treated at 90$^{0}$C for 10 minutes to destroy native *E.*

*coli* MDH activity. The cell free extracts were centrifuged at 10,000 x g for 15 minutes at room temperature to remove cellular debris. Malate dehydrogenase activity was assayed by following the decrease in absorbance of NADH at 340 nm at 25°C due to the conversion of oxalacetate to L-malate. One unit of malate dehydrogenase activity is defined as the amount of enzyme needed to oxidize 1 micomole of NADH per minute.

Recombinant DNA Studies. Recombinant techniques were standard unless otherwise noted (Sambrook, et al., 1989). Transformation of *Thermus* was carried out according to Koyama et al. [Koyama, 1986 #1102].

## RESULTS AND DISCUSSION

**Construction of Promoter Probe Vectors.** Two plasmid based promoter probe vectors have been constructed for use in *Thermus* ssp. pKANPROII (Fig. 1 A) and pTIGT1 (Fig. 1 B). The plasmid pUC19EKF-TSP3 [Wayne, 1997 #299] which expresses kanamycin nucleotidyltransferase via an uncharacterized *T. aquaticus* promoter and is stabily maintained in *T. thermophilus* HB27 was utilized in the construction of promoter probe and expression vectors. Both promoter probe vectors, pKANPROII and pTIGT1, are similar and are based on a fusion of the Thermus replication genes of pUC19EKF-TSP3 with pUC19 and pBlueScript KS+ respectively. Both pKANPROII and pTIGT1 contain promoterless kanamycin resistance genes with unique restriction sites located immediately upstream except that the multiple cloning site of pTIGT1 contains more unique restriction enzyme recognition sites. pKANPROII and pTIGT1 replicate in both *Thermus* and *E. coli* and allow the detection of inserted DNA fragments that encode promoters that allow the expression of the kanamycin resistance in *Thermus* and *E. coli* transformants.

An integrative promoter probe vector designated pTPRO1 (Fig. 1C) was constructed to quantify promoter activity and to examine the expression of genes present as a single integrated copy. Integrative promoter probe vectors are valuable because the promoter and gene of interest are not subject to copy number or DNA superhelicity effects. pTPRO1 contains pUC19 sequences and can replicate in E. coli. However, pTPRO1 lacks the ability to replicate in *Thermus* as a plasmid, rather it integrates by a double crossover event so that the insertion into

the chromosome is stable and permanent. pTPRO1 has a promoterless kanamycin resistance gene flanked by regions of the 3-isopropylmalate dehydrogenase gene (*leuB*) [Weber, 1995 #213] which is the region where integration into the chromosome occurs. To prevent transcription read through from the *leu*B promoter a transcriptional termination sequence from the *T. flavus* phenylalanyl tRNA synthetase operon [Keller, 1992 #774] was cloned upstream of the kanamycin nucleotidyltransferase gene. The termination sequence eliminated kanamycin expression in *E. coli* and *Thermus* for pTPR01 while transcriptional read-through was not a problem for pKANPROII or pTIGT1 either in *E. coli* or in *Thermus*. Unique restriction sites upstream of the $Km^R$ gene in pTPRO1 are available for cloning of promoter fragments.

**Isolation of *Thermus* Promoters.** Random chromosomal fragments from *T. thermophilus* or *T. flavus* were ligated into the promoter probe vector pKANPROII at unique restriction enzyme sites upstream from the promoterless kanamycin nucleotidyltransferase gene. The ligation mixture was used to transform *T. thermophilus* HB27. Transformants were incubated at 55°C for 4-5 days on TT plates containing 40 $\mu$g/ml kanamycin (Km). Several hundred colonies containing inserts ranging from 100 bp to 1.0 kb that enabled expression of the kanamycin resistance gene in *T. thermophilus* HB27 were isolated. One hundred $Km^R$ colonies were randomly chosen and replica plated onto TT plates containing 100 $\mu$g/ml, 500 $\mu$g/ml, and 2000 $\mu$g/ml of Km. All transformants grew well on plates containing 100 $\mu$g/ml of Km. Thirty-three transformants grew on plates containing 500 $\mu$g/ml of Km and twenty transformants were found to grow on 2000 $\mu$g/ml of Km. Plasmid DNA was isolated from all of the *T. thermophilus* HB27/pKANPROII constructs and transformed into *E. coli* $DH5_\alpha$. Approximately 20% of the colonies contained promoters that allow expression of $Km^R$ in both *E. coli* and *T. thermophilus* HB27.

A 145 bp promoter designated J17 was isolated based on its ability to produce high levels (2000 $\mu$g/ml Km) of kanamycin resistance in *Thermus* and its ability to also function in *E. coli*. Although the expression of the kanamycin nucleotidyltransferase gene under control of the J17 promoter in *Thermus* yields high levels of kanamycin resistance, the expression of kanamycin nucleotidyltransferase in *E .coli* is moderate (40 $\mu$g/ml Km). The promoter J17 also functions at high levels (2000 $\mu$g/ml Km) as a single copy when cloned upstream of the Km gene in the integrative promoter probe vector pTPRO1. Another *Thermus*-derived promoter that functions

in both *Thermus* and *E. coli* (albeit at reduced levels) was designated vv12 and was also used in the subsequent construction of expression vectors.

**Expression Vector Construction.** Vectors were constructed for the expression of heterologous and homologous genes in *Thermus*. Promoter J17 was used to construct three expression vectors pTEX1-RBS, pTEX1-PL, and pTEX3-int (Fig. 2 A and B). All of these vectors have a transcriptional terminator sequence cloned upstream of the J17 promoter to prevent transcriptional read-through from the vector. They also have a multiple cloning site downstream of the J17 promoter. The pTEX1 vectors are plasmid expression vectors that contain a kanamycin nucleotidyltransferase gene under the control of a broad host range promoter (vv12) allowing for expression of kanamycin resistance in *Thermus* and *E. coli*. Promoter vv12 replaces the uncharacterized promoter originally used to express kanamycin resistance in pUC19EKF-TSP3 and allowed the size of the resulting plasmid to be reduced by about a kilobase pair. pTEX1-RBS has a *Thermus* ribosome binding site (RBS) [Maseda, 1995 #558] cloned upstream of the MCS with a *Eco*RI site 6 bp downstream. In pTEX1-PL the RBS has been deleted. pTEX3-int has the J17 promoter cloned into the *Thermus leuB* derived integration vector pTPRO1 with unique restriction sites downstream of the promoter for cloning and expression of foreign genes.

To obtain a vector for inducible expression of cloned genes we constructed pTEX2-dnaK (Fig. 2 C) by the replacement of the constitutive J17 promoter in pTEX1-PL with the promoter from the *dnaK* gene of *T. thermophilus*. The *dnaK* promoter is activated by heat shock: specifically transferring *Thermus* cultures from $70^0$C to 85°C [Osipiuk, 1997 #266]. Like the pTEX1 expression vectors pTEX2-dnaK has a transcriptional terminator upstream of the *dnaK* promoter, a MCS downstream, and $Km^R$ under the control of a broad host range promoter (vv12).

**Assay of pTEX-1 and pTEX2-dnaK Expression Vectors.** To determine the utility of the expression vectors described here we cloned the malate dehydrogenase gene (*mdh*) (Fig 2. D), including it's ribosome binding site, into the expression vectors pTEX-1-PL and pTEX2-dnaK. Quantitative gene expression studies can be performed with *T. thermophilus* cultures using kanamycin resistance as the reporter gene; however, that requires the use of radioactive reagents (Maseda and Hoshino, 1995). Prior to this study *mdh* was not used as a reporter gene for studies of *T. thermophilus*, but just as has been reported in studies of other microorganisms (Horinouchi et al., 1987), malate dehydrogenase levels in crude protein extracts of *Thermus* cultures can be

readily quantified using a convenient spectrophotometric assay. The *mdh* gene from *T. flavus* was amplified by PCR using chromosomal DNA as a template and then ligated into the multiple cloning site of pTEX1–PL and into pTEX2-dnaK to yield pTEX1-MDH and pTEX2-dnaK-MDH respectively. The two promoters used in the pTEX constructs (J17 and *dnaK*) function in *Thermus* as well as *E. coli*. Malate dehydrogenase activity was first assayed in *E. coli* because *Thermus thermophilus* HB27 MDH protein is stable at 90°C and it's activity in *E. coli* can be easily monitored in heat-treated cell extracts. The results shown in Table 1 clearly demonstrate the expression of the *mdh* gene in *E. coli* from both pTEX1-MDH and pTEX2-dnaK-MDH. In *Thermus*, there is background interference from the native malate dehydrogenase enzyme. Nonetheless, the expression of the *mdh* gene under control of the J17 promoter in pTEX1-MDH results in higher levels of expression of MDH protein than is seen with *T. thermophilus* controls. To induce the expression of *mdh*, *T. thermophilus* HB27 containing the expression vector pTEX2-dnaK-MDH was incubated for 10 minutes at 85°C prior to the preparation of cell free lysates. The results are summarized in Table 1. In the absence of heat induction there is no apparent increase of MDH activity in comparison with *T. thermophilus* HB27 controls indicating that the *dnaK* promoter is weak or inactive under these conditions. Both *T. thermophilus* HB27 and HB27/pTEX1-MDH cultures were subjected to heat shock as controls and no increase in malate dehydrogenase activity was detected (data not shown). When the pTEX2-dnaK-MDH culture is subjected to a heat shock the MDH level increases from 2.4 units/mg to 3.3 units/mg indicating an induction of transcription by the *dnaK* promoter in expression vector pTEX2-dnaK-MDH. This confirms that the *Thermus dnaK* promoter regulates transcription through heat induction as described earlier by Osipiuk *et al.* [Osipiuk, 1997 #266]. Osipiuk *et al.* noted that the *dnaK* promoter induced a 30 times more *dnaK* mRNA after heat shock whereas we see only a 37.5% increase in MDH activity. The *dnaK* promoter appears much weaker in the pTEX2-dnaK-MDH expression vector. It has been shown that promoters yield different levels of expression when cloned upstream of alternative (non-native) genes [Makrides, 1996 #18]. We are not sure if *mdh* mRNA is being produced but not optimally translated or if *mdh* mRNA levels are also much lower. Northern blot and RT-PCR studies underway in our laboratory to answer some of these questions.

Tools for the isolation and characterization of *Thermus* promoters, and tools for the expression of genes in *Thermus* have been developed. Both plasmid and integrative promoter

probe vectors were constructed and used to isolate numerous promoters from *T. thermophilus*. Approximately 20% of the *T. thermophilus* promoters obtained in this study were also capable of functioning in *E. coli*. Strong *Thermus* promoters were generally observed to be of only moderate to low strength in *E. coli*. Plasmid and integrative expression vectors were constructed using promoters capable of functioning in both *Thermus* and *E. coli*. Additionally, an expression vector with an inducible promoter was constructed using the heat shock regulated promoter of the *dnaK* gene of *T. thermophilus*. The use of these expression vectors was demonstrated by expressing the *T. thermophilus mdh* gene in both *E. coli* and in *T. thermophilus*.

The promoter probe and expression vectors reported here comprise a related set of plasmid and integrative vectors that utilize the same reporter genes and promoters and are capable of functioning in both *E. coli* and *T. thermophilus*. Moreover, these vectors contain convenient multiple cloning sites, small size, and terminator sequences to eliminate transcriptional read-through from vector promoters into cloned DNA. These vectors will prove to be useful in genetic studies and also in addressing the issue of the relative merits of plasmid versus integrative vectors for use in *Thermus* species.

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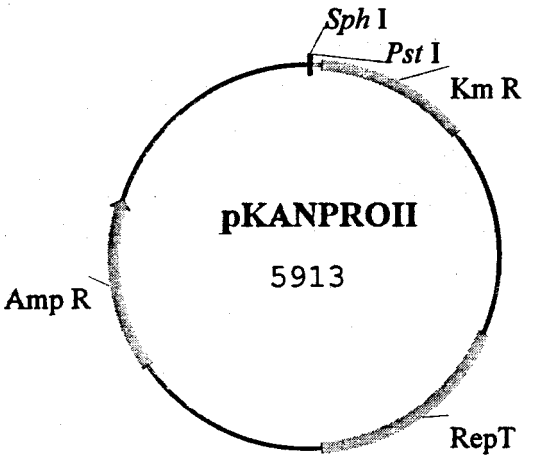
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5913
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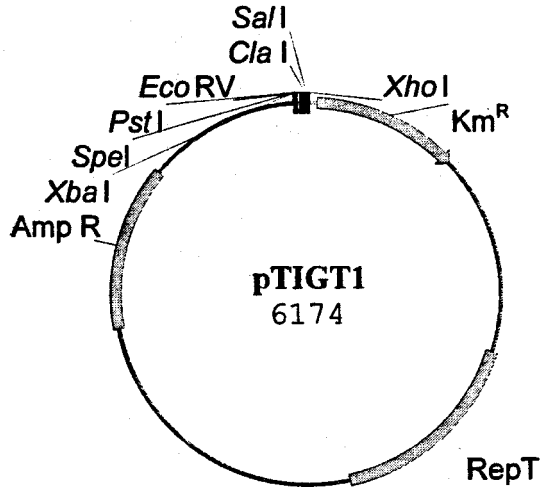
RepT

A.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Km^R
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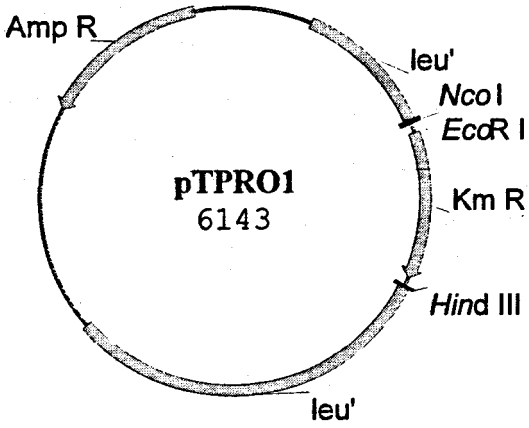
RepT

B.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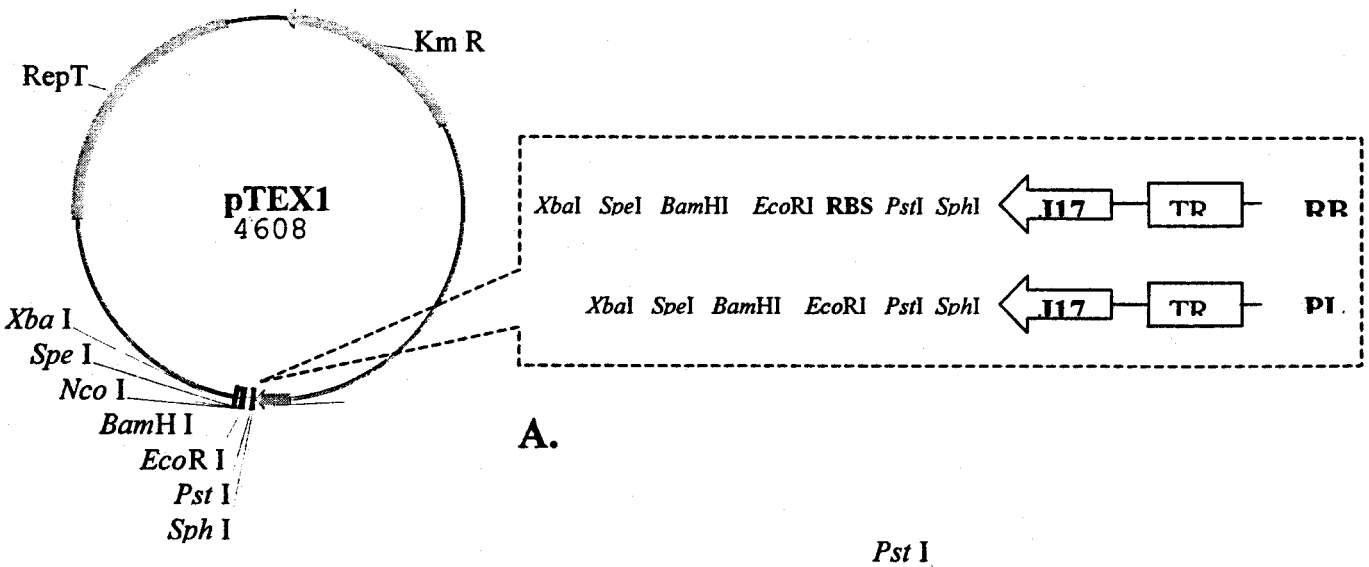
leu'
Nco I
EcoR I
pTPRO1
6143
Km R
Hind III
leu'

C.

Legend Figure 1.

Fig. 1. A 3.2 kb *Xho*I and *Sal*I fragment from plasmid pUC19-EKF-Tsp3 (7.7 kb) contains the promoterless $Km^R$ gene and a *Thermus* replication origin (RepT). (A) This 3.2Kb fragment was ligated into *Sal*I digested pUC19. Plasmids were chosen based on the orientation of the insert that yielded the unique restriction sites *Pst*I and *Sph*I upstream of the promoterless kanamycin nucleotidyltransferase gene. This construct was designated pKANPROII and is 5.9 kb. (B) The same 3.2 kb fragment was cloned into the *Xho*I site of pBluescript KS + to create pTIGT1. Constructs were also chosen based on the orientation of the insert that yielded the unique restriction sites *Xba*I, *Spe*I, *Pst*I, *Eco*RV, *Cla*I, *Sal*I and *Xho*I upstream of the promoterless kanamycin nucleotidyltransferase gene. Promoter probe vector pTIGT1 is 6.2 kb in size. (C) Integrative promoter probe vector pTPRO1 was constructed by inserting, into the *Nsi*I site of pTG100, a fragment containing a promoterless $Km^R$ gene and the *Thermus flavus* phenylalanyl tRNA synthetase operon transcription terminator sequence and unique restriction sites *Eco*RI and *Nco*I upstream of the kanamycin nucleotidyltransferase gene flanked by portions of the *leuB* gene of *T. flavus*.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J17
TR
RR
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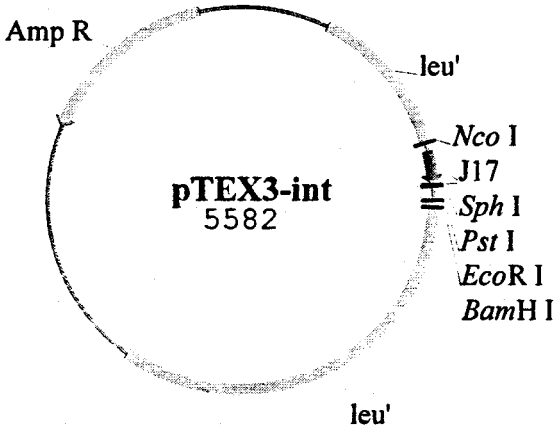
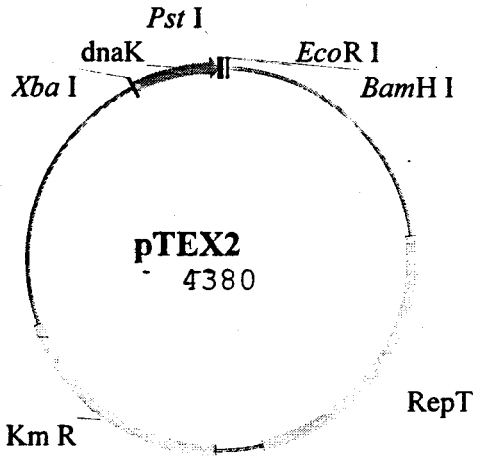
J17
TR
PI

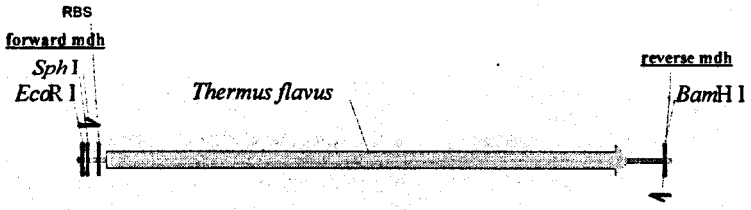
A.


Amp R
leu'
pTEX3-int
5582
Nco I
J17
Sph I
Pst I
EcoR I
BamH I
leu'

B.


Pst I
dnaK
EcoR I
Xba I
BamH I
pTEX2
4380
RepT
Km R

C.


RBS
forward mdh
Sph I
EcoR I
Thermus flavus
reverse mdh
BamH I

D.

Legend Figure 2.

Fig 2. (A) Expression vectors pTEX1-RBS and pTEX1-PL were constructed by insertion of a transcriptional terminator sequence (TR) upstream of the $Km^R$ gene in plasmid pKANPROII-J17 (5.86 kb). Plasmid pKANPROII-J17 TR was digested with *Pst*I and *Bam*HI removing the $Km^R$ gene. A PCR fragment containing multiple unique restriction enzyme sites (*Xba*I, *Spe*I, *Bam*HI and *Eco*RI) and the CAT gene from Tn9, flanked by two *BamH*I restriction sites was digested with *Pst*I and *Bgl*II and ligated into the pKANPROII-J17 TR *Pst*I-*Bam*HI digest. Transformants were selected in *E. coli* for $CM^R$ and examined for $Km^S$. The resulting construct pKII TR MCS CAT was digested with *Bam*HI and ligated back to itself to remove the CAT gene. Transformants were selected in *E. coli* for $Amp^R$ and colonies were gridded to detect $Cm^S$ clones. The resulting construct pKII TR MCS was digested with *Sca*I-*Dra*I and a kanamycin gene cassette, consisting of the vv12 promoter upstream of the $Km^R$ gene, was blunt end ligated into the vector. The resulting pTEX1-RBS construct was $Km^R$ and $Amp^S$. Plasmid pTEX1-RBS has a *Thermus* RBS cloned upstream of the MCS with an *Eco*RI site 6 bp downstream. In the pTEX1-PL the RBS has been deleted. (B) pTEX3-int was constructed by replacing the promoterless $Km^R$ gene from pTPROI with the J17 promoter and several unique restriction sites downstream for cloning of genes. (C) Inducible expression vector pTEX2-dnaK consists of: The *dnaK* promoter was amplified from *T. thermophilus* chromosomal DNA using the following PCR primers: forward *dnaK*

5′-ACAACAGAATTCTCTAGAGGGTGTCCCCGGCGCGCACCA-3′ and reverse *dnaK*, 5′-ACAACACTGCAGGTACGTCTTCACCTCGCCTCCC-3′. Restriction sites were added into the PCR primers to give 5′ *Eco*RI, *Xba*I and 3′ *Pst*I sites. Plasmid pTIGT1 was digested with *Xba*I and *Pst*I and a *Xba*I-*Pst*I digested *dnaK* promoter PCR product was ligated into the vector.

The resulting construct pTIGT1-dnaK was digested with *ScaI-DraI* and a kanamycin gene cassette, consisting of the vv12 promoter upstream of the $Km^R$ gene, was blunt end ligated into the vector. The resulting pTEX2-dnaK construct is $Km^R$ and $Amp^S$.

(D) The *mdh* gene from *T. flavus* was amplified, including it's ribosome binding site, from chromosomal DNA using the following PCR primers: forward *mdh* 5'-ACACAGAATTCGCATGCTCAAGAAGGCCCTGGGCTAA-3' and reverse *mdh* 5'-ACACACGGATCCTGCGCCAGCATGGGGTGGTATAAA-3'. Restriction sites were added into the PCR primers to give 5' *Eco*RI and 3' *Bam*HI sites. The *mdh* gene PCR product was cloned into pTEX1-PL and into pTEX2-dnaK. Plasmid pTEX1-PL was digested with *Eco*RI and *Bam*HI and a *Eco*RI-*Bam*HI digested *mdh* PCR product was ligated into the vector to create pTEX1-MDH. Plasmid pTEX2-dnaK was digested with *Eco*RI and *Bam*HI and a *Eco*RI-*Bam*HI digested *mdh* PCR product was ligated into the vector to create pTEX2-dnaK-MDH.

Table 1. Malate dehydrogenase activity of *Thermus* expression vector constructs.

| Culture and Condition | MDH activity (Units MDH/mg) |
|---|---|
| *E. coli* DH5$_\alpha$ | 0 |
| DH5$_\alpha$/pTEX1-MDH | $3.8^{+/-}$ 0.13 |
| DH5α/pTEX2-dnaK-MDH | $2.4^{+/-}$ 0.11 |
| *Thermus thermophilus* HB27 | $2.4^{+/-}$ 0.26 |
| HB27/pTEX1-MDH | $3.4^{+/-}$ 0.09 |
| HB27/pTEX2-dnaK-MDH | $2.4^{+/-}$ 0.18 |
| HB27/pTEX2-dnaK-MDH [HS 85°C] | $3.3^{+/-}$0.20 |

Values recorded are average of three replicate samples. Standard deviation is indicated.

HS 85°C = The culture was heat shocked by exposure to 85°C for 10 minutes prior to the preparation of cell free lysates.