Report

Background

Metabolic Engineering

An emerging approach to the understanding and utilization of metabolic processes is Metabolic (or pathway) Engineering (ME). As the name implies, ME is the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly. ME typically involves the redirection of cellular activities by the rearrangement of the enzymatic, transport, and regulatory functions of the cell through the use of recombinant DNA and other techniques. Much of this effort has focused on microbial organisms, but important work is being done in cell cultures derived from plants, insects, and animals. Since the success of ME hinges on the ability to change host metabolism, its continued development will depend critically on a far more sophisticated knowledge of metabolism than currently exists.

This knowledge includes conceptual and technical approaches necessary to understand the integration and control of genetic, catalytic, and transport processes. While this knowledge will be quite valuable as fundamental research, per se, it will also provide the underpinning for many applications of immediate value.

Scope

The Metabolic Engineering Working Group is concerned with increasing the science and engineering community's level of knowledge and understanding of ME. The Working Group strives to encourage and coordinate research in ME within academia, industry, and government in order to synergize the Federal investment in ME.

Introduction

In November 1995, Science Advisor John H. Gibbons of the Office of Science and Technology Policy (OSTP) released the report, "Biotechnology for the 21st Century: New Horizons." This report was a product of the Biotechnology Research Subcommittee (BRS) under OSTP, and identifies priorities for federal investment and specific research opportunities in biotechnology. These priorities include agriculture, the environment, manufacturing and bioprocessing, and marine biotechnology and aquaculture. The BRS formed several working groups to facilitate progress on some of these key priorities. The Metabolic Engineering Working Group (MEWG) was created to foster research in Metabolic Engineering, an endeavor that can contribute to all of the key priorities in the aforementioned report. The Working Group is composed of Federal scientists and engineers who participate as part of the activities of OSTP, and represent all of the major agencies involved in Metabolic Engineering research.

In its on-going efforts to promote and enhance the use of Metabolic Engineering (ME), the Working Group sponsored its second annual Interagency Grantee's Conference. This Conferenc was held June 28, 2001 at the National Science Foundation, in Arlington, VA.

The purpose of the Conference was to showcase the Grantees from the first and second the Interagency Announcements of Opportunities in Metabolic Engineering (NSF 98-49 and NSF 99-85), and review their progress on their Metabolic Engineering Research Grants.

Abstracts of Expert Presentations

Glycolytic Flux in Escherichia coli: A Gene Array Perspective Comparing Glucose & Xylose

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The simplicity of the fermentation process (anaerobic with pH, temperature, and agitation control) in ethanologenic Escherichia coli KO11 and LY01 makes this an attractive system to investigate the utility of gene arrays for biotechnology applications. Using this system, gene expression, glycolytic flux and growth rate have been compared in glucose-grown and xylose-grown cells. Although the initial metabolic steps differ, ethanol yields from both sugars were essentially identical on a weight basis and little carbon was diverted to biosynthesis. A total of 27 genes changed by more than 2-fold in both strains. These included induction of xylose-specific operons (xylE, xylFGHR, and xylAB) regulated by XylR and the cyclic AMP-CRP system, and repression of Mlc-regulated genes encoding glucose uptake (ptsHIcrr, ptsG) and mannose uptake (manXYZ) during growth on xylose. However, expression of genes encoding central carbon metabolism and biosynthesis differed by less than 2-fold. Simple statistical methods were used to investigate these more subtle changes. The reproducibility (coefficient of variation of 12%) of expression measurements (mRNA as cDNA) was found to be similar to that typically observed for in vitro measurements of enzyme activities. Using a student t-test, many smaller but significant sugar-dependent changes were identified ($p<0.05$ in both strains). A total of 276 genes were more highly expressed during growth on xylose; 307 genes were more highly expressed with glucose. Slower growth (lower ATP yield) on xylose was accompanied by decreased expression of 62 genes encoding the biosynthesis of small molecules (amino acids, nucleotides, cofactors, and lipids), transcription, and translation; 5 genes were expressed at a higher level. In xylose-grown cells, 90 genes associated with the transport, catabolism and regulation of pathways for alternative carbon sources were expressed at higher levels than in glucose-grown cells, consistent with a relaxation of control by the cyclic AMP-CRP regulatory system. Changes in expression ratios for genes encoding the Embden-Meyerhof-Parnas (EMP) pathway were in excellent agreement with calculated changes in flux for individual metabolites. Flux through all but one step was predicted to be higher during glucose fermentation, pyruvate kinase. Expression levels (glucose/xylose) were higher in glucose-grown cells for all EMP genes except the isoenzymes encoding pyruvate kinase (pykA and pykF). Expression of both isoenzymes was generally higher during xylose fermentation but statistically higher in both strains only for pykF encoding the fructose-6-phosphate activated isoenzyme, a key metabolite connecting pentose metabolism to the EMP pathway. The coordinated changes in expression of genes encoding the EMP pathway suggest the presence a common regulatory system, and that flux control within the EMP pathway may be broadly distributed. In contrast, expression levels for genes encoding the Pentose-Phosphate pathway were statistically similar regardless of sugar.

Maximizing Ethanol Production by Engineered Pentose-Fermenting Zymomonas mobilis

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Zymomonas mobilis has been metabolically engineered to broaden their substrate utilization range to include D-xylose and L-arabinose at the National Renewable Energy Laboratory in Golden, CO. Both chromosomally-integrated and plasmid-bearing Z. mobilis strains that are capable of fermenting the pentose D-xylose have been created by incorporating 4 genes: 2 genes xylA and xylB encoding xylose utilization metabolic enzymes, xylose isomerase and xylulokinase and 2 genes talB andtktA encoding pentose phosphate pathway enzymes, transaldolase and transketolase. While the proof-of principle that the metabolically engineered Z. mobilis strains are able to ferment bother glucose and xylose to ethanol has been previously established, our current research undertakes detailed quantitiative investigations on the enhanced metabolic network to maximize the ethanol production from glucose and xylose by these strains.

Two different xylose-fermenting Z. mobilis strains were grown on glucose-xylose mixtures in computer-controlled fermentors to analyze the extracellular metabolite concentrations as well as the activities of several intracellular enzymes from the xylose and glucose consumption pathways. Dynamic profiles of these enzymes show dramatic increases in the activities of the two xylose utilizing enzymes immediately after the depletion of the preferred sugar, glucose. We are now addressing the regulatory mechanisms underlying these reproducible increases. First, the issues of regulation at the protein synthesis level versus the enzyme activity level is being resolved through quantification of the key intracellular protein concentrations through proteomic analysis using 2 D gel electrophoresis techniques. In parallel, we are characterizing the intracellular concentrations of the key metabolites along the network, namely the phosphorylated carbohydrates through NMR spectroscopy. Subsequently, the issues of transporter limitations as well as the gene expression regulation and dosage effects will be addressed in the next year.

Metabolic Engineering of Solvent Tolerance in Anaerobic Bacteria

E. Terry Papoutsakis
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Understanding solvent (and other toxic chemical) tolerance of microorganisms is crucial for the production of chemicals, bioremediation, and whole-cell biocatalysis. It is also very important basic knowledge. Past efforts to produce tolerant strains have relied on selection under applied pressure and chemical mutagenesis, with some good results, but not consistently so. We desire to examine if Metabolic Engineering (ME) and genomic approaches can be used to construct more tolerant strains for bioprocessing. The accepted dogma is that toxicity is due to the chaotropic effects of solvents on the cell membrane. Impaired membrane fluidity and function inhibit cell metabolism, and result in cell death. We have found that in C. acetobutylicum, several well-defined genetic modifications not related to membrane function impart solvent tolerance (by 40-70%) without strain selection. This suggests that we need to re-examine the accepted dogma. The objective of this research is to identify genes that contribute to solvent tolerance and to use genetic modifications (involving these genes) to generate solvent tolerant strains. In view of the large number of possible genes that may be involved in determining solvent tolerance, we use DNA microarrays based on the genome sequence of C. acetobutylicum. DNA microarrays were designed and constructed in our laboratory in order to examine the large-scale transcriptional program of the cells in response to various levels of butanol and other solvent challenges. Many genes belonging to several classes (molecular pumps, chaperonins (HSPs), primary metabolism, ATPases, sporulation, transcriptional regulators, carbohydrate metabolism) were identified as changing gene expression under solvent stress. Several of these genes will be explored in ME studies.

Metabolic Engineering of Methylobacterium extorquens AM1

Steven Van Dien
University of Washington

A stoichiometric model of central metabolism was developed based on new information regarding metabolism in this bacterium to evaluate the steady-state growth capabilities of the serine cycle facultative methylotroph Methylobacterium extorquens AM1 during growth on methanol, succinate, and pyruvate. The model incorporates 20 reversible and 47 irreversible reactions, 65 intracellular metabolites, and experimentally-determined biomass composition. The flux space for this underdetermined system of equations was defined by finding the elementary modes, and constraints based on experimental observations were applied to determine which of these elementary modes give a reasonable description of the flux distribution for each growth substrate. The predicted biomass yield, on a carbon atom basis, is 49.8%, which agrees well with the range of published experimental yield measurements (37-50%). The model predicts the cell to be limited by reduced pyridine nucleotide availability during methylotrophic growth, but energy-limited when growing on multicarbon substrates.

Mutation and phenotypic analysis was used to test model predictions regarding key enzymes for growth on C3 and C4 compounds. Three enzymes involved in C3-C4 interconversion pathways were predicted to be mutually redundant: malic enzyme, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate synthase. Insertion mutations in the genes from the genome sequence that are predicted to encode these enzymes were made, and these mutants were capable of growing on all substrates tested, confirming the model predictions. Likewise, citrate synthase and succinate dehydrogenase were predicted by the simulations to be essential for all growth substrates. In keeping with these predictions, null mutants could not be obtained in these genes. In addition, a random approach using transposon mutagenesis was used to generate mutants with impaired growth on succinate or pyruvate. A mutant in a gene predicted to encode a subunit of the NADH-quinone oxidoreductase was obtained, and was unable to grow on succinate or pyruvate but grew normally on methanol. Since this function is necessary for the entry of NADH into the electron transport chain, this finding supports the model prediction that NADH must be oxidized to ultimately yield ATP during multicarbon growth, but not with methanol as the carbon source. A transposon mutant in a putative a-ketoglutarate dehydrogenase gene was also unable to grow on succinate or pyruvate. However, the model does not predict this enzyme activity to be required for growth on any substrate. In situations such as this in which the phenotype does not agree with predictions, the model has helped to identify errors in the current understanding of Methylobacterium extorquens AM1 central metabolism.

Engineering Plant One-Carbon (1-C) Metabolism

David Rhodes
Purdue University

Primary and secondary metabolism intersect in the one-carbon (C1) area, with primary metabolism supplying most of the C1 units and competing with secondary metabolism for their use. This competition is potentially severe because secondary products such as lignin, alkaloids and glycine betaine require massive amounts of C1 units. Many current metabolic engineering projects aim to change levels of these products, or entail reducing the supply of C1 units. It is therefore essential to understand how C1 metabolism is regulated at the metabolic and gene levels so as to successfully engineer C1 supply to match demand. Our project aims to acquire this understanding. Specific objectives are: (1) to clone complete suites of C1 genes from maize and tobacco, and to incorporate them into DNA arrays; (2) to use sense and antisense approaches as well as mutants to engineer alterations in C1 unit supply and demand; and (3) to quantify the impacts of these alterations on gene expression (using DNA arrays), and on metabolic fluxes (by combining radio- and stable isotope labeling, MS, NMR and computer modeling).

Four findings from Year 1 are summarized. All were unexpected and have implications for engineering C1 metabolism: (1) Unlike other eukaryotes, plants have methylenetetrahydrofolate reductases that use NADH rather than NADPH as reductant, and are not allosterically inhibited by AdoMet. (2) DNA arrays show that formate dehydrogenase and a cluster of enzymes for methyl group synthesis and transfer are more highly expressed in roots than leaves. (3) Metabolic flux analysis and modeling of tobacco engineered to convert choline to glycine betaine suggests a crucial role for a chloroplast choline transporter. (4) Plants have an unsuspected source of formate ö the irreversible hydrolysis 10-formyltetrahydrofolate, via an enzyme previously known only in prokaryotes. The first and last of these findings depended on genomics-based approaches, and illustrate the value of bioinformatics in metabolic engineering.

Significant findings from Year 2 include: (1) Confirmation of the crucial role for a chloroplast choline transporter in conversion of choline to glycine betaine by metabolic flux analysis and modeling of transgenic tobacco expressing choline monooxygenase and betaine aldehyde dehydrogenase in the chloroplast, or choline oxidase and betaine aldehyde dehydrogenase in the cystosol. (2) Analysis of 14C-formaldehyde and 14C-serine metabolism in leaves of near-isogenic maize lines differing for alternative alleles of a single locus conferring glycine betaine accumulation (Bet1/Bet1) or lack thereof (bet1/bet1), show markedly different fluxes of radiolabel into choline moieties under salinity stress. Despite these large differences in flow of C1 units into choline moieties, no significant differences between near-isogenic maize lines were found in the mRNA transcript abundances of any of the C1 enzymes, with the single exception of phosphoethanolamine-N-methyltransferase, which shows a modest 2-fold down-regulation in the glycine betaine-deficient (bet1/bet1) line. The latter result suggests control of C1 flux into choline moieties primarily by post-transcriptional mechanisms.

Tobacco lines expressing antisense methylenetetrahydrofolate reductase, antisense S-formylglutathione hydrolase, and formate dehydrogenase have been derived and are currently being characterized for C1 gene expression and metabolic fluxes. A dynamic kinetic model of the intersecting transmethylation, methionine salvage and Smethylmethionine cycles has been developed, and is being used to explore the effects of altering one or more enzyme levels on metabolism of U-13C5-methionine.

Carbohydrate Engineering for Generating Sialylated Glycoproteins in Insect Cells

Michael J. Betenbaugh
Johns Hopkins University

Insect cells are used to generate of a variety of biotechnology products. Many of the most valuable biotechnology products are glycoproteins that include oligosaccharides attached to the protein at particular amino acids. These oligosaccharides can be extremely important to the therapeutic activity of biopharmaceuticals in humans. Unfortunately, processing in insect cells yields glycoproteins with different oligosaccharides from those generated by human and other mammalian hosts. While mammalian cells produce complex oligosaccharides often terminating in the sugar, sialic acid, insect cells typically generate simplistic oligosaccharides terminating in mannose or N-acetylglucosamine. Since these covalently-attached carbohydrates can significantly affect a protein's structure, stability, biological activity, and in vivo circulatory half-life, the objective of this project is to manipulate carbohydrate-processing pathways in insect cells to generate complex sialylated glycoproteins. The sialylation reaction involves the addition of a donor substrate, cytidine monophosphate-sialic acid (CMP-SA) onto a specific acceptor carbohydrate via an enzymatic reaction in the Golgi apparatus. Evaluation of the nucleotide-sugars in Sf-9 and High Five insect cells grown in serum-free medium revealed negligible levels of CMP-SA to suggest a limitation in the donor substrate levels. Consequently, the genes responsible for generating CMP-SA must be engineered into insect cells using metabolic engineering strategies. Unfortunately, the mammalian genes were unknown so bioinformatics approaches were implemented to identify putative human genes based on known bacterial sequences. When the enzymes encoded by these genes are expressed with baculovirus vectors, sialic acids and the donor substrate (CMP-SA) can be generated in insect cells at levels exceeding those typically observed in mammalian cell lines. Furthermore, the enzymes have broad substrate specificities which may allow for the generation of glycoproteins with different sialic acid termini. In addition to producing the donor substrate, CMP-SA, the correct acceptor carbohydrate acceptors must be generated in insect cells. Collaborating scientists are generating correct carbohydrate acceptors by expressing favorable glycosyltransferase enzymes such as galactose transferase and by evaluating methods to inhibit unfavorable cleavage reactions. The completion of the sialylation reaction will be obtained by expressing the catalyzing sialyltransferase enzyme in the presence of these correct acceptor and donor substrates. Engineering the sialylation reaction into insect cells may increase the value of insect cell-derived products as vaccines, therapeutics, and diagnostics. Humanizing insect cells and other recombinant DNA hosts will make expression systems more versatile and may ultimately lower biotechnology production costs. In the future a particular host may be chosen based on its efficiency of production rather than its capacity to generate particular oligosaccharide profiles.

Modeling Metabolic Pathways: A Bioinformatics Approach

Imran Shah
University of Colorado

The overall goal of this project is to develop novel bioinformatics tools to aid metabolic engineering (ME). The final final product this project is a predictive computational system for metabolic pathway elucidation utilizing high-throughput biomolecular data (mostly genomic sequence and expression), background biological knowledge and novel inference techniques. To achieve this goal we are developing bioinformatics software to address the following challenges: (i) biochemical data representation and integration from public domain sources, which is necessary to effectively compute with biomolecular information; (ii) the accurate assignment of biocatalytic function to protein sequences using machine learning methods, which is necessary to place putative proteins in a biochemical context, and (iii) the elucidation of pathways by heuristic search, which is necessary to automatically relate sets of putative enzymes in a broader metabolic context. When implemented the system will be made available to the ME community through interactive web-accessible software. We are approaching the problem in a general manner so that the system will be useful in annotating whole microbial genomes, in finding alternative routes in a partially complete pathways, or even elucidating pathways that have not been observed before.

In Silico Analysis of the Escherichia Coli Metabolic Genotype and the Construction of

Selected Isogenic Strains
Bernhard O. Palsson
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Small genome sequencing and annotation are leading to the definition of metabolic genotypes in an increasing number of organisms. We show how in silico metabolic genotypes are formulated based on genomic, biochemical, and strain-specific data. Such metabolic genotypes have been formulated for E. coli, H. influenzae, and H. pylori. The in silico models are based on the philosophy of using applicable physico-chemical (such as stoichiometric structure) and capacity (maximum fluxes) constraints on the integrated functioning of the metabolic networks. Given these constraints, optimal phenotypes can be computed and compared to experimental data. They are found on the edge of the allowable solution spaces ö a space that basically represents the reaction norm of the defined genotype ö where the governing constraint on cellular functions can be identified. For E. coli, this process leads to quantitative prediction of growth and metabolic by-product secretion data in batch, fed-batch, and continuous cultures, and to the accurate prediction of the metabolic capabilities of 73 of 80 mutants examined. Furthermore, we present mathematical methods that allow for the analysis, interpretation, prediction, and engineering of the metabolic genotype-phenotype relationship, and for the interpretation of expression array data.

Key refs:
J.S. Edwards and B.O. Palsson, "The Escherichia coli MG1655 in silico metabolic genotype; Its definition, characteristics, and capabilities," Proc. Natl Acad Sci (USA), 97: 5528-5523 (2000).

J.S. Edwards, R.U. Ibarra, and B.O. Palsson, "In silico predictions of Escherichi coli metabolic capabilities are consistent with experimental data," Nature Biotechnology, 19:125, 2001

Metabolic Engineering of Microorganisms
Jay D. Keasling
University of California

The goal of this work is to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants. The objectives of this work are: (1) to find and clone a gene that encodes an enzyme capable of degrading diethylphosphate, (2) to clone and express a pathway for complete mineralization of p-nitrophenol phosphate, (3) to clone and express a phosphotriesterase capable of hydrolyzing parathion, (4) to develop a co-culture biofilm capable of degrading parathion (as a proof-of-concept), and (5) to combine all of the genes in a single organism for complete mineralization of parathion or paraoxon.

Metabolic engineering offers the opportunity to expand the role of bioremediation. Traditional metabolic engineering involves overexpression of a desired protein and leads to a high metabolic burden on the cell. The purpose of this work is to develop strategies to help reduce this burden and make an engineered organism more environmentally effective.

Parathion (O,O-diethyl-O-p-nitrophenyl phosphorothioate), an organophosphate pesticide which has been widely used and is highly toxic, was chosen as the model compound for this project. Parathion is also structurally and functionally similar to many chemical warfare agents (including VX and soman).

Metabolic Engineering of Isoprenoid Production
Jay D. Keasling
University of California

The objectives of this work are (i) to maximize the production of the isoprenoid precursor isopentenyl diphosphate in E. coli by expressing the genes for either the mevalonate-dependent or the mevalonate-independent synthesis pathway using the metabolic engineering tools developed in this laboratory; (ii) to maximize production of the primary precursors to the terpenoids: geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate; (iii) to introduce into E. coli the genes for specific classes of terpenoids and optimize production of these ãnaturalä terpenoids; and (iv) to use laboratory evolution of terpene cyclases to produce novel terpenoids or to change the distribution of products made by terpenoid biosynthetic enzymes.

To accomplish this work, we are (i) cloning the genes encoding the enzymes in the non-mevalonate IPP biosynthetic pathway and express these genes under the control of inducible promoters on high, medium, and low-copy plasmids; (ii) cloning the genes for synthesis of DMAPP, GPP, FPP, and GGPP and express these genes under the control of inducible and constitutive promoters on high, medium, and low-copy plasmids; (iii) cloning the genes for various plant and fungal terpenes and express these genes under the control of inducible and constitutive promoters on high, medium, and low-copy plasmids; and (iv) mutating the terpene cyclases genes using mutagenic PCR and gene shuffling. For the maximization of IPP, DMAPP, and GGPP production, we will express the genes for lycopene synthesis and look for deep red colonies (containing large quantities of lycopene).

Metabolic Engineering to Study the Regulation/Plasticity of, and to Modify Diterpene Metabolism in Trichome Gland Cells

George J. Wagner
University of Kentucky

Plant trichome glands represent potential "green-factories" for the biosynthesis of useful chemicals (molecular farming). These factories require only energy from the sun, carbon dioxide from the air, water, and minerals as feedstocks. Before this potential can be realized, however, the regulation and plasticity of carbon flow in trichome glands must be better understood, and protocols for engineering glands to produce desired chemicals must be developed. The specific objectives of this project are 1) to investigate the regulation/plasticity of carbon flow in the biosynthesis of trichome-exudated diterpenes of glands, and 2) to study the feasibility of introducing heterologous genes into glands to facilitate molecular farming. Exudating plant trichome glands are specialized tissues that occur on the aerial surfaces of about 30% of higher plants. They produce exudates that serve the plant in pest/insect resistance, temperature control, etc. We isolated a gland-specific c-DNA library, which yielded a P450 gene involved in the conversion of cembratriene-ol (CBT-ol) to cembratriene-diol (CBT-diol), the major diterpene of the experimental tobacco, T.I. 1068. This plant can accumulate up to 17% of leaf dry weight as trichome exudate, and CBT-diol accounts for 60% of exudate weight. Knockdown of the P450 gene activity (using antisense and co-suppression strategies) resulted in a 20-fold increase in CBT-ol and a corresponding decrease in CBT-diol. Exudate from high CBT-ol plants was more toxic to aphids, and high CBT-ol plants had greatly reduced aphid colonization. Thus, we have metabolically engineered the last step in the biosynthesis of the major exudate diterpene and significantly altered natural-product-based aphid resistance in this plant. Knockdown strategies (antisense, co-suppression, and RNA interference) are being applied to determine the function of additional trichome-specific genes, and to determine the impact of altering their activities on exudate chemistry. Full-length genes of known function will be introduced into host plants, trichome-specifically, to determine the ability glands in these plants to accommodate heterologous diterpene biosynthetic genes. A trichome-specific promoter has been isolated that can serve in planned transformation experiments designed to metabolically engineer glands.

Aromatic Amino Acid Biosynthesis in Archaeoglobus fulgidus

H.G. Monbouquette
University of California Los Angeles

The aromatic amino acid synthesis pathway has been engineered successfully for the synthesis of natural and unnatural chiral amino acids, which are important drug intermediates, as well as other industrially important aromatics, such as indigo. Production of aromatics via engineered microbes offers both environmental and economic advantages including exclusive use of aqueous solvent and non-toxic intermediates, and lower raw material cost. Intense interest therefore has developed in the enzymes of these metabolic pathways. A. fulgidus is representative of the third, most primitive domain of life, and the aromatic amino acid synthesis pathways have not been explored in these microorganisms despite the fact that they may offer a far more robust set of biosynthetic enzymes well suited both for in vivo and in vitro synthesis applications. Recently, the entire genome of A. fulgidus was sequenced and a thorough study of open reading frames for sequences homologous to known enzymes was conducted. It is noteworthy that a number of enzymes involved in common aromatic amino acid synthesis routes were not identified on the genome. Our goal is to identify these new enzymes/pathways by a functional proteomics approach made possible by our demonstrated ability to culture A. fulgidus to the 100-liter scale, and to identify, isolate, sequence, clone and express (in E. coli) new enzymes from this microbe. This project will establish a functional proteomics approach involving coordinated use of high-throughput LC/MS-based enzyme assays, DNA microarrays, and gene cloning and expression for fast screening of enzyme activities and for identification of genes in hypothesized metabolic pathways.

The following was accomplished in the first year of this project: (1) the 15 A. fulgidus open reading frames (ORFs) homologous to known genes in the aromatic amino acid synthesis pathways were cloned in E. coli and were sequenced, (2) a putative gene for a novel bifunctional phosphoribosyl (PRA) anthranilate transferase/indoleglycerol phosphate (IGP) synthase was found to be two separate genes, (3) prephenate dehdrogenase activity was confirmed for the over-expressed product of a putative trifunctional chorismate mutase/prephenate dehydratase/prephenate dehydrogenase gene, (4) over-expressed shikimate dehydrogenase was purified and partially characterized, and (5) a method for determining 95% confidence intervals for DNA microarray data was developed. Of the 15 cloned ORFs, nine were over-expressed as soluble products. An effort to obtain soluble products of the remaining genes and to characterize the recombinant enzymes is continuing. A preliminary characterization of the recombinant shikimate dehydrogenase was conducted. The enzyme exhibits similar kinetics to the E. coli enzyme, albeit at a temperature optimum of ~90 °C. The prephenate dehydrogenase activity of the putative trifunctional enzyme suggests that this may indeed be a novel fusion of catalytic functions, although chorismate mutase and prephenate dehydratase activity has not been confirmed. Work is ongoing to develop LC/MS as a tool for high throughput enzyme assays and to refine the DNA microarray technique such that LC/MS and DNA microarrays may be used in complementary fashion to identify new enzymes and metabolic pathways. This approach will be used in the second year of the grant to identify the novel enzyme(s) catalyzing the first two steps in the shikimate pathway as well as the phosphorylation of shikimate.

Conference Agenda

June 28, 2001 -- Room 110

8:15 Welcoming and Introductory Remarks

MARYANNA HENKART, Chair, Biotechnology Research Working Group
FRED HEINEKEN, Chair, Metabolic Engineering Working Group -- Introduction

8:30 Glycolytic Flux in Escherichia coli: A Gene Array Perspective Comparing Glucose & Xylose by LONNIE INGRAM
8:50 Maximizing Ethanol Production by Engineered Pentose-Fermenting Zymomonas mobilis by DHINAKAR KOMPALA
9:10 Metabolic Engineering of Solvent Tolerance in Anaerobic Bacteria by TERRY PAPOUTSAKIS
9:30 Break
9:45 Welcoming Remarks by MARY CLUTTER, Chair, Subcommittee on Biotechnology
10:00 Metabolic Engineering of Methylobacterium extorquens AM1 by STEVEN VAN DIEN
10:20 Engineering Plant One-Carbon (1-C) Metabolism by DAVID RHODES
10:40 Carbohydrate Engineering for Generating Sialylated Glycoproteins in Insect Cells by MICHAEL BETENBAUGH
11:00 Break
11:15 Modeling Metabolic Pathways: A Bioinformatics Approach by IMRAN SHAH

11:35 In Silico Analysis of the Escherichia Coli Metabolic Genotype and the Construction of Selected Isogenic Strains by BERNHARD PALSSON
11:55 Lunch
1:00 Discussion: in vitro Metabolic Engineering
2:00 Break
2:15 Metabolic Engineering of Microorganisms by JAY KEASLING
2:55 Metabolic Engineering to Study the Regulation/Plasticity of, and to Modify Diterpene Metabolism in Trichome Gland Cells by GEORGE WAGNER
3:15 Aromatic Amino Acid Biosynthesis in Archaeoglobus fulgidus by HAROLD MONBOUQUETTE
3:35 Open Discussion
4:25 Closing Remarks
4:30 Adjourn

Agency Activities in Metabolic Engineering

U.S Department of Agriculture

The Agricultural Research Service (ARS) and the Forest Service (FS) conduct metabolic engineering research through the Federal laboratory system while the Cooperative State Research, Education, and Extension Service (CSREES) supports metabolic engineering research through competitive research grants and through formula-based programs in cooperation with the states.

USDA research activities encompass animal sciences, plant sciences, commodity conversion and delivery, environmental sciences (air, soil, water), human nutrition, and integration of agricultural systems.

Metabolic engineering technologies are being developed and applied across the above research areas and include the following goals:

- To modify microbial metabolism for the production of commercially useful products, chemicals, biofuels, and biomolecules from agricultural commodities and resources.
- To develop genetic and other techniques for altering metabolic pathways to understand basic processes associated with microbial based natural or newly developed biocontrol agents resulting in elimination, decreased use, or increased environmental bioremediation of both agricultural wastes and agricultural chemicals such as herbicides, insecticides, fungicides, or biocides.
- To improve efficiency of production and decrease losses due to environmental stresses, diseases, pathogens, parasites, or pests by altering host metabolism using genetic or other techniques to apply metabolic engineering at the tissue, organ, or whole organism level of animals or plants, alone or in combination with the microorganisms associated with these hosts.

Ongoing research includes:

- Metabolic engineering for the development of superior fuel ethanol producing microorganisms. Microorganisms that normally use multiple substrates are being engineered for enhanced ethanol production, and microorganisms that normally make ethanol are being engineered to use multiple substrates.
- Metabolic engineering for the development of superior solvent producing anaerobic bacteria. Specifically, the fermentative enzymes involved in butanol production are being analyzed in order to manipulate metabolic fluxes from acidogensis to solventogenesis.
- Metabolic engineering of anaerobic bacteria for improved animal performance. The specific approach is to enhance xylan degradation of feed material by introducing into the rumen a genetically modified bacterium that overproduces xylanase.
- Metabolic engineering of toxigenic fungi and host plants. Specifically, the genes involved in aflatoxin biosynthesis have been identified and a master switch gene discovered. By engineering plants to favor production of a metabolite that interferes with this master gene, aflatoxin production may be prevented in the host plant.

- Modify metabolite distribution in plants. One specific approach is to transfer the liquid wax producing capability of jojoba into a metabolic pathway for commercially viable oilseed rape and soybeans.

National Institute of Standards and Technology

NIST has internal research programs in the Biotechnology Division, and extramural collaboratively funded research and development programs through the Advanced Technology Program that are related to the scientific field known as Metabolic Engineering. Each of these programs have different foci and management structures, but share the overall goal of fostering the commercialization of recent scientific advances in areas related to biotechnology, such as biocatalysis and metabolic engineering.

Biotechnology Division (Intramural)

In the intramural programs of the Biotechnology Division (http://www.cstl.nist.gov/biotech) , which is one of five Divisions of the Chemical Sciences and Technology Laboratory, the mission is to advance the commercialization of biotechnology by developing the scientific/engineering technical base, reliable measurement techniques and data to enable U.S. industry to quickly and economically produce biochemical products with appropriate quality control. The mission is carried out in collaboration with industry, other government agencies and the scientific community. The primary research efforts that relate to Metabolic Engineering are in Bioprocess Engineering, Structural Biology, DNA Technologies, and Biomolecular Materials groups.

The Bioprocess Engineering (http://cstl.nist.gov/div831/bioprocess) activity includes biophysical property evaluation where thermophysical and thermochemical properties are being obtained, evaluated, codified and modeled for biochemicals, proteins and biosolutions of interest in metabolic pathway engineering. A research program in biocatalysis is underway to solve technical roadblocks in the commercial development of enzymes that build new complex molecules used in advanced drug or food product design. Other investigations include developing DNA-based reference standards for detecting and quantifying biotech crops, and fluorescence standards for interpreting DNA microarrays.

The Structural Biology activity includes x-ray and NMR measurements of atomic structures of prototypical proteins, enzymes, enzyme-substrate complexes and model DNA systems. A research program in biothermodynamics uses state-of-the-art calorimetric methods to study protein-protein and protein-substrate interactions, and computational models are developed that relate structure to function. Physical and biochemical methods are used to characterize protein behavior, including the study of membrane-embedded proteins to understand signal transduction. Computational chemistry and modeling develops methods to model the energetics and dynamics of interactions between substrates and active sites of enzymes. Modeling techniques to understand the relationship between protein sequence and structure are being developed.

The DNA Technologies activity includes development of methods and standards for DNA profiling for forensic and other uses. Research is being conducted to develop the next generation of DNA profiling based on polymerase chain reaction (PCR) technology including new methods development for rapid DNA extraction, amplification, separation, and computer imaging. DNA sequencing develops specific reference materials and technical expertise that are essential for DNA Genomic research in the public and private sector. This activity also provides quality assurance expertise to the developers of technology that proposes to use DNA recognition sites on silicon chips for the diagnosis of human genetic diseases. Research on DNA damage and repair is developing methods to characterize DNA damage on a molecular scale using GC/MS techniques. Studies of both in-vivo and in-vitro systems are underway to understand both damage (as low as one base per million) and repair mechanisms.

The Biomolecular Materials activity develops generic measurement technologies utilizing both optical and electrochemical approaches for applications in clinical diagnostics, bioprocessing, and environmental monitoring. Research on lipid membranes and membrane proteins is being performed to provide an understanding of materials and methods that will enhance the development of this important class of molecules in sensor and other applications. The light-sensitive protein, bacteriorhodopsin is being studied as a potential source for the storage and retrieval of information. Studies are underway to understand and control the mechanism of this optical transition, and to develop methods of immobilizing this protein to increase its stability.

Advanced Technology Program (Extramural)

The Advanced Technology Program within NIST provides funding to support innovative research and development, which are likely to lead to inventive new technologies and products that will have positive economic benefits for the United States. ATP has in the past, and continues to fund projects in Metabolic Engineering. These projects include the modification of enzymatic pathways in microorganisms and improved bioprocessing technologies to produce, in a cost-effective way, monomers used in the synthesis of thermoplastics, essential cofactors for human health, disease-targeted therapeutics and desulfurized crude oil. Support also has been provided to companies seeking to engineer the synthesis of isoprenoids in yeast and biopolymers in the fibers of cotton plants. The production of better goods at lower costs and the utilization of renewable biosystems are potential benefits to be derived from these projects. As documented in more than a dozen White Papers submitted to ATP, industries' future commitments for applications of metabolic engineering are expansive and cover wide areas including immobilized biocatalysis, novel bioreactors, value-added crops, better nutrition and an improved environment

Department of Defense

The Department of Defense (DoD) currently supports a broad range of research in the area of metabolic engineering through the Army Research Office (ARO) and other Army research activities, the Air Force Office of Scientific Research (AFOSR), the Office of Naval Research (ONR), and the Defense Advanced Research Projects Agency (DARPA). The specific focus of the ARO, AFOSR, ONR, and DARPA efforts will be summarized and future directions in metabolic engineering research and technology development will be addressed.

The broad needs for the DoD that can be served through research efforts in metabolic engineering are summarized below. These science and technology targets will provide enhanced and expanded capabilities for the missions of the services and provide greatly expanded capabilities for the civilian sector.

- Materials
- Processes
- Devices
- Fabrication Schemes
- Information Processing

Current interests in metabolic engineering at ARO are focused on two related topics: the characterization of biochemical pathways and enzymatic mechanisms and the genetic manipulation of protein structure and function. The goal is to develop a detailed understanding of how macromolecules have been tailored to execute their designated functions and how they interact with other macromolecules. With this information, it will be possible to engineer enzymes and metabolic pathways to exhibit a set of specific functions and properties, according to Army needs. ARO currently supports research in several areas, including: how molecular transport, subcellular compartmentalization, and reaction sequences are involved in enzymatic regulation and superstructural formation; understanding and manipulating aminoacylation of tRNAs to produce, using cellular translation machinery, new polymeric peptide materials containing non-natural amino acids; the role and regulation of "stress" proteins differentially expressed in response to environmental or external stimuli; and the design and implementation of unique enzymatic strategies for the biodegradation of environmental pollutants.

For the AFOSR, space and aerospace materials are often produced by complex sequences of reactions involving toxic solvents and expensive catalysts. Some materials are derived from structures that are difficult to synthesize with traditional chemistry.

Because of their remarkable specificity and efficiency, biocatalysts can enable the synthesis of a wide range of materials. They can catalyze de novo synthesis from renewable feedstocks, specific reactions in synthesis of monomers that are difficult to accomplish with conventional chemistry, and modification of polymers or composites at several stages of synthesis and assembly. Biocatalysts have substantial potential for deposition of thin films of organic or inorganic material including silicates. Development of biocatalytic approaches to synthesis will enable the development of materials with novel properties, reduce the cost of the material and eliminate the environmental impact of toxic chemical reagents.

AFOSR-supported work at the Air Force Research Laboratory has also led to the discovery of new catabolic pathways used by bacteria for the biodegradation of synthetic organic compounds. A variety of novel enzymes catalyze key steps in the pathways. The objective of the current work is to characterize the enzymes to determine the reaction mechanisms and then to explore the potential for use of the enzymes as biocatalysts for the synthesis of chemical feedstocks used in the production of space and aerospace polymers. Strategies are also being developed for the biological destruction of chemicals by bacterial enzymes.

One of the metabolic engineering foci at ONR, currently, is the microbial synthesis of energetic materials (EM) and EM precursors for the purposes of cost and environmental impact. Practically all such materials are non-natural products and their biosynthesis therefore requires the re-engineering of existing pathways and/or the assembly of new or hybrid pathways in one or more host organisms. An example of a simple EM precursor now under study is 1,2,4-butanetriol, which as its energetic trinitrate is used as a plasticizer in propellant and explosives formulations. More advanced EM targets, such as RDX, HMX and Cl20, involve high density fused ring cores with multiple nitramino (C-N(NO2)) substituents. While these are very difficult targets, they suggest worthwhile research goals such as the biosynthesis of highly electron withdrawing substituents on carbon (as in C-nitramino) or the assembly of strained heterocyclic rings. Clearly, a theoretical/experimental approach to the prediction of the true scope of enzyme reaction specificity, with energetic boundaries, would be particularly valuable in the design of pathways for EM biosynthesis. Other non-polymeric targets, besides EM, would include novel photonic/electronic/optical materials. Persons interested in metabolic engineering opportunities at ONR are strongly advised to communicate with Dr. Harold J. Bright (703-696-4054, brighth@onr.navy.mil) before submitting a proposal.

DARPA's metabolic engineering programs are driven by an interest in protecting human assets against biological threats and using biology to enhance both human and system performance. The general concept of this thrust is to understand how nature controls the metabolic rate of cells and organisms (e.g., extremophiles, hibernation) and apply this understanding to problems of interest to DoD. Examples of current investments in metabolic engineering include efforts to develop technologies for engineering cells, tissues and organisms to survive in the battlefield environment so they can be used as sensors. DARPA is also developing technologies that permit the long-term storage of cells including human blood. More complete descriptions of current DARPA programs and solicitations in these areas can be viewed at http://www.darpa.mil/dso.

U.S. Department of Energy

The Department of Energy is supporting over $25 million in metabolic engineering research, largely through the Offices of Science (SC), Energy Efficiency and Renewable Energy (EE), and Environmental Management (EM). The research falls in two main categories: 1) basic research, which involves the advancement of metabolic engineering fundamental knowledge and capabilities, and 2) applied research, which employs metabolic engineering techniques in development of target products. The basic research efforts of the Department reside within SC, whereas most of the applied research in this area is conducted within EE. In general, these research efforts are conducted by universities, national laboratories, and industry.

The Department's goals related to metabolic engineering research are to:

- To expand the level of knowledge and understanding of metabolic pathways and metabolic regulatory mechanisms related to the development of novel bio-based systems for the production, conservation, and conversion of energy.
- Apply metabolic engineering techniques to enhance and develop plants and microorganisms for use in the production of chemicals and fuels or for environmental remediation of waste sites.

Metabolic engineering research within SC is supported predominantly through the Office of Basic Energy Sciences (BES) and Biological and Environmental Research (OBER). Most of BES's metabolic engineering research resides within the Energy Biosciences program which has the mission to generate the fundamental knowledge required for the development of novel bio-based systems for the production, conservation, and conversion of energy. A significant part of the program has been and continues to be aimed at the development of metabolic engineering capabilities related to plants and fermentative microbes. These activities include defining metabolic pathways, characterization of the catalytic properties of enzymes, determining metabolic regulation mechanisms, development of gene transfer capabilities, kinetic analysis of the flow through a pathway, and in a few instances the actual metabolic engineering of specific pathways. The program focuses on the development of basic scientific knowledge as opposed to the development of specific processes.

The metabolic engineering research within OBER resides in three divisions: Health Effects and Life Sciences Research, Medical Applications and Biophysical Research, and Environmental Sciences. Most of the research is conducted in association with the human genome, microbial genome, structural biology, and environmental remediation programs. OBER's research in this area is directed toward enhancing fundamental knowledge of metabolic pathways and addresses the development of tools and capabilities to elucidate the kinetics and mechanisms of microbial metabolic pathways; to create useful pathways for biotransformation of metals for biodegradation of toxic organics; and to understand complex relationships between genes, the proteins they encode, and the biological functions of these proteins in the whole organism.

In complement with its core research efforts, SC is conducting joint research with EM in support of their environmental restoration efforts and with EE in support of their fuels and chemicals production efforts. These newly formed partnerships demonstrate the spirit of collaboration and coordination within the Department, which combines science with technology to fulfill DOE's research missions.

Metabolic engineering research within EE is supported through the offices of Transportation Technologies (OTT), Industrial Technologies (OIT), and Utility Technologies (OUT). As applied R&D efforts, the focus is on specific research and market issues within the purview of the respective office. For example, research in OTT focuses on ethanol production using bacteria and yeast that feed on sugars derived from non-agricultural feedstocks. In OIT, the focus is on the development of bioprocesses and new chemical synthesis routes using whole organisms or enzymes in the production of chemicals and materials. Finally, the research in OUT focuses on the use of photosynthetic microorganisms, such as cyanobacterium or alga blue or green algae in the production of hydrogen. In each of these program efforts, the R&D activities address metabolic engineering to increase the production of the product(s) desired by either enhancing existing pathways, constructing new pathways, or designing alternative pathways.

Environmental Management (EM) has a modest biotechnology research effort in support of its mission in waste management related to the clean-up and restoration of the U.S. national laboratory sites. The focus of this research involves bioremediation, including intrinsic, chemical bioaugmentation, and phytological approaches to clean-up chlorinated compounds, heavy metals, and other hazardous organics. Metabolic engineering approaches are being used to improve the effectiveness and efficiency of their environmental clean-up efforts by enhancing, augmenting, or creating new metabolic pathways within target organisms or plants. More recently, EM has teamed with SC to pursue basic research needs in various areas of national laboratory clean-up issues and waste management.

Environmental Protection Agency

Developing Metabolic Engineering Strategies

The mission of the Environmental Protection Agency is to protect human health and the environment from adverse effects of anthropogenic activity. Included in this mission are various elements for which metabolic engineering can play a useful role.

One prominent concern is the introduction of chemicals to the environment which may have detrimental effects on humans and other biota. As mandated by statute and implemented by rule, the Agency routinely conducts evaluation of chemicals intended for use, currently in use, or determined to exist at significant levels in the environment. From these evaluations, the Agency may decide to implement management strategies designed to limit the potential for adverse effects.

The application of novel technologies such as the use of biotechnology as a substitute to conventional manufacturing and processing of raw materials into final products is consistent with the mission of the Agency. EPA implements this by supporting development of technologies which 1) use chemical substitutes that are less toxic; 2) produce more efficient activity resulting in decreased requirement for the chemical or; 3) develop engineering procedures which produce little or no toxic end products. Finally, consistent with the pollution prevention ethic is the reevaluation of chemical stewardship from one of "cradle to grave" to a more multigenerational philosophy in which a chemical may be utilized successively in different forms prior to final disposal. Metabolic engineering has a role to play by enabling the development of biological mechanisms for production or use that meet one or more of these criteria.

While it is generally accepted that chemical-based technologies have evolved to provide a higher standard of living for the general population, it is also recognized that the use of some chemicals, either through the chemical characteristics or the handling, synthesis or disposal, have produced negative effects on human health and/or the environment. Advances in technology allow scientists to better predict the potential for adverse effects from exposure to chemicals as well as mechanisms to diminish the negative effects of chemical production such as production of toxic byproducts and disposal of the chemical. The approach, which strives to identify synthetic pathways that are less polluting than existing pathways and that encourages the development of nontoxic chemical products, is referred to as "Green Chemistry". The use of metabolic engineering to evaluate the potential for increased risk from chemicals, by allowing the study of responsible metabolic pathways and by permitting modification of such pathways to reduce risk, is another way in which metabolic engineering firs within the EPA mission.

Finally, basic research, which utilizes methods of metabolic engineering, can provide longer range approaches to assist EPA in its overall mission of protecting human health and the environment. The EPA supports extramural metabolic engineering research through the Technology for a Sustainable Environment (TSE) program, which awards grants in the area of pollution prevention. Since 1995, the TSE program has funded metabolic engineering research related to methanol conversion, solvent tolerance, biopolymer production and pesticide production-all focused on the elimination of pollution at the source

National Institutes of Health

National Institute of General Medical Sciences

The National Institute of General Medical Sciences (NIGMS) supports metabolic engineering research, usually in the form of grants to investigators in universities (R01s) or in small businesses (SBIRs). These grants support basic research in two general areas: (1) the development of microbial or plant-based metabolic routes to useful quantities of ãsmallä molecules such as polyketides; (2) the development of a much better understanding of the control architecture that integrates the genetic and catalytic processes in normal and aberrant cells. During fiscal 2002, the NIGMS is providing $13.6 million (47 grants) for the support of research directly involving metabolic engineering. Examples of funded projects include (1) a study of the pikromycin biosynthetic pathway, and (2) an in silico studies of E. coli growth.

National Science Foundation

The **Directorate of Engineering** supports several investigators in the area of metabolic engineering. One common feature of these research projects involves purposeful changes in organism behavior for increased product yields and levels for both wild type and recombinant systems. In addition, the improved biodegradation of toxic compounds is also being approached through metabolic engineering. Biological processes of this type have significant industrial potential, but in many cases still require the necessary biochemical engineering to translate them into a scalable process. In order to obtain the highest yields of metabolite products, restructuring of the central pathways for carbon catabolism and dispersal of incoming carbon into synthetic pathways will be necessary. Because of the tight integration among these pathways and the energy-producing pathways, restructuring of this central core of metabolism will require a systems approach, which considers the interactions of the pathways concerned with the other metabolic subsystems in the cell. The system is complicated by regulation at both genetic and enzyme levels of all of these interacting metabolic subsystems. Therefore, an important aspect of the engineering research is the development of the mathematical systems, and control theory needed for a quantitative analysis and understanding of the metabolic changes which are initiated by the manipulation of the enzymatic, transport and regulatory functions of the cell. Examples of metabolic engineering research supported in this Directorate include: (1) the use of linear optimization theory for the network analysis of intermediary metabolism, (2) the development of methods to select the internal fluxes for experimental measurement based on their sensitivity to experimental error, (3) the development of a method to determine flux control coefficients using transient metabolite concentrations, and (4) a study of network rigidity to help overcome the cell control mechanisms that resist flux alterations at branch points in metabolic pathways.

The **Directorate for Biological Sciences** (BIO) supports a broad range of research activities directed at increasing the knowledge base required for metabolic engineering. Examples of several BIO activities with implications for Metabolic Engineering include the following: (1) the ãArabidopsis Genome Research Initiative:ä a multinational research cooperation to sequence the entire genome of the model plant, Arabidopsis thaliana, in order to establish baseline genomic data for plants, and to develop microarrays and other technology that can be used for further applications; (2) the ãPlant Genome Research Programä which supports research on plant genome structure and function. Research supported by this program is characterized by a systems approach to plant genome research that builds upon recent advances in genomics, bioinformatics, and plant biology. This program has already funded over 70 groups of investigators, often consortia of several universities and industries, to carry out sequencing and functional genomics projects. Supported efforts range from sequencing agriculturally important genomes (maize, soybean, tomato), to technology development, to focused applications (stress tolerance, pathogen responses, cotton fibers). (3) The "Microbial Observatories Initiative includes the study of novel microorganisms in soils, marine sediments, and aquatic environments. The tremendous diversity of currently undescribed microorganisms offers potential metabolic engineering spin-offs such as new pathways for biodegradation of environmental toxins and novel pharmaceuticals. (4) The BIO Directorate is in the second year of the ã2010 Projectä that supports research to determine the function of all genes in Arabidopsis thaliana by the year 2010. In the first year, 26 awards were made in support of creative and innovative research designed to determine the function of networks of genes and to develop new tools for functional genomic approaches.

The **Directorate for Geosciences** supports research related to ME in marine ecological systems. Examples of research areas include: (1) determination of the physico-chemical requirements for the maintenance, growth, and regulation of marine microbes; (2) identification, isolation, and determination of the function of enzymes responsible for useful degradation processes; (3) exploration of marine viruses and how they can be used in genetic engineering; (4) development of molecular assays for harmful species of marine microbes; (5) determination of cellular and biochemical control of trace metal limitation; (6) characterization of enzymes and genes associated with nitrogen fixation in cyanobacteria; and (7) identification and characterization of marine microbes and consortia that degrade, detoxify, or metabolize marine pollutants.

The **Directorate for Mathematical and Physical Sciences** supports a number of projects involving metabolic engineering. Of particular interest is the use of new enzymes to facilitate catalytic processes such as the desymmetrization of achiral molecules and the development of new bacterial strains that will be useful for the conversion of petrochemical and other industrial byproducts into useful or benign derivatives. Theoretical work continues to explore the basis of information encoding which is the foundation of molecular genetics and its associated properties of self-replication and the nonrandom organization of genetic material into specific shapes. Bridges to the experimental realm provide ever more elegant examples of synthetic structures that mimic genetic principles. These experiments are expanding our understanding of the underlying chemistry of genetic and biochemical processes and provide the basis for such functional examples of chemical systems patterned after living systems as enzyme mimics. Additionally, the increasing understanding of the specific ways that drug molecules interact with gene-derived entities is the basis for a new era of chemotherapy.