EVOLUGATE "PRESS KIT"

Gainesville, January 2010

TABLE OF CONTENTS:

ABOUT EVOLUGATE

"NOTHING IN BIOLOGY MAKES SENSE, EXCEPT IN THE LIGHT OF EVOLUTION"

Theodosius Dobzhansky (1900-1975)

SUMMARY PRESENTATION

Founded in 2005, EVOLUGATE, LLC is a privately owned biotechnology and engineering company, operating in the nascent field of experimental evolution and genomic engineering of micro-organisms for industrial and academic purposes.

EVOLUGATE has developed a technology that offers the possibility to improve existing bio-processes as well as to create new ones through the utilization of a reliable and proprietary continuous culture machine, known as the EvolugatorTM, which improves microorganisms by natural evolution and selection.

EVOLUGATE's current operations focus on the experimental adaptation of micro-organisms, including bacteria, archaea, and microbial eukaryotes such as yeast, fungi and algae. In addition, EVOLUGATE plans to develop its technology to enable the growth of plant, animal, human and stem cells in a continuous mode.

EVOLUGATE intends to become a key leading technology provider in the evolution of micro-organisms used in the biofuel, biodegradation, bioinsecticide industries, as well as in other green chemistry markets - the "missing link" between scientific exploration and industrial efficiency.

The company is located in Gainesville, Florida.

EVOLUGATE'S "CONTINUOUS CULTURE TECHNOLOGY"

Maintaining populations of microbial cells under controlled conditions of growth and environment for an indefinite duration is a prerequisite for experimentally evolving natural isolates of wild-type species or recombinant strains.

Continuous culture vessels called chemostats have been used since the 1950's to select for cells that have a higher proliferation rate under given conditions (Monod J., 1950, Ann. Inst. Pasteur. 19:390-410 ; Novick A., Szilard L., 1950, Science 112:715-716). These work by partial dilution - as a culture grows and becomes saturated, a small proportion of the grown culture is replaced with fresh medium, allowing the culture to continually grow at close to its maximum population size.

The potential power of these devices was recognized, but they have never met that potential because they invariably select mutants that evade dilution through attachment to vessel surfaces, resulting in persistent sub-populations of uncontrollable size and growth rate (wall growth). This wall growth selection prevents selection of the targeted characteristics of the researched micro-organism.

The Evolugator TM eliminates wall growth problems and this leads to many applications in the microbial field. Evolugate holds the exclusive and worldwide license under the patent rights claiming the continuous culture apparatus (Evolugator TM) and its use.

APPLICATIONS

The use of EVOLUGATE's technology is particularly appropriate in conditions in which a selective pressure can be applied on the growth of the microorganism. Within these conditions, the Evolugator TM will enable the selection of the best possible variant. Examples include:

- Increasing the efficiency of degradation of a toxic product that can be used as a nutrient source.

- Adapting a strain to a particular environment e.g. temperature, atmosphere, pressure or acidity.

- Improving strains that have been genetically manipulated in a manner that has negatively affected their growth rate, so that they recover a wild-type growth rate.

- Evolving strains that are more resistant to a toxic bio-product.

Many laboratories that work in diverse fields such as experimental evolution, biodegradation, metabolic engineering, and enzyme evolution will find that continuous culture, with the use of the Evolugator, will both complement their existing tools and allow them to explore new avenues.

MARKETS

BIO-PESTICIDES, ENTOVIA Division

Directed adaptation of entomopathogenic fungi

As an alternative to chemical pesticides, entomopathogenic fungi such as Beauveria bassiana and Metarhizium anisopliae are currently under intensive study as promising arthropod pest biological controls agents.

Strains of these fungi have been selected for control of insects and other arthropods that act as disease vectors including mosquitoes and ticks, crops pests such as whiteflies and borers, and ecologically hazardous, invading pests such as fire ants and termites. Despite their potential several factors have hindered widespread adoption of fungi as part of biological control regimes. Effectiveness under laboratory conditions often does not translate to the high mortality needed for biological control under field conditions. Significant impediments include the relatively low resistance of fungi to abiotic stresses such as solar irradiation and heat.

Using the Evolugator TM, entomopathogenic fungi was adapted for growth from 25°C to 37°C. This technology could be used to adapt fungal strains to virtually any environmental condition including affecting host range.

BIOFUELS

Several genetically modified micro-organisms have been developed to produce biofuel such as ethanol, butanol, methanol and other chemicals from biomass.

Properties of those biofuel producing micro-organisms such as yield, temperature resistance, pH sensitivity, ethanol, butanol, methanol or other chemicals tolerance could be improved by selection in the Evolugator TM to reduce the final product cost.

EVOLUGATE contracted with BioTork LLC the exclusive license of its proprietary continuous culture technology for every biofuel application.

BIOREMEDIATION

Micro-organisms have already been used for many bioremediation processes to degrade pollutants produced by human activity, but the development of industrial waste that is more and more complex and toxic is making bioremediation and the use of biotechnology in environmental management more and more attractive. Indeed :

- The world population is growing and the industrialization of an increasing number of developing countries leads to increasing chemical waste;

- Traditional methods and treatments are often costly and harmful;

- There is a high public demand for "greener" alternatives, using non-GMOs as the only acceptable environmental friendly approach.

Using the tools of improvement by natural selection mastered by EVOLUGATE can elevate the field of waste biodegradation, from an environmental "by-market" to a viable and legitimate industry associated in many ways to modern life. Examples of how EVOLUGATE can contribute to this industry:

<u>Soil bio-remediation.</u> With its ability to evolve both aerobic and anaerobic natural strains, EVOLUGATE could greatly improve every bacterial in situ remediation process, avoiding heavy techniques like air sparging injection. This technology addresses both accidental pollution treatment and the rehabilitation of post-industry sites.

<u>Chemical hazardous materials</u>. The pressure of regulation in the past few decades has led the chemical industry to store massive quantities of hazardous materials, awaiting new treatment techniques. With its capacity to adapt bacteria for tolerance to higher concentrations of hazardous materials, EVOLUGATE could greatly improve ex situ techniques for the elimination or concentration of simple chemicals, heavy metals derivatives or radioactive substances. Ex-situ treatments are the only field of bioremediation that can use genetically modified organisms (GMOs), which can then be improved using the Evolugator TM technology.

OTHER MARKETS

For millennia, since the discovery of fermentation, people have used the power of microbial metabolism in their daily lives. These uses were generally limited to metabolic reactions that were common in nature and played a large role in the life cycle of the host organism – for instance, one can readily find fruit that is naturally fermenting on the vine, and the fermenting yeast using the grapes as a valuable source of energy and carbon for its reproduction. These abilities have been optimized for efficiency by natural selection.

More recently, advances in gene discovery (linking genetic identity to specific chemical conversions) and genetic engineering (moving genes in and out of microorganisms, or altering genes in specific ways) have made microorganisms able to perform many more chemical reactions, all of which resulting in numerous products, ranging from food additives to antibiotics to enzymes used in detergents, are now produced in or with microorganisms.

The number of products produced in bacteria (Bioconversions) is also growing because of the drive to replace polluting chemical synthesis processes by "greener" alternatives and of the increasing need to use renewable resources.

***Green Chemistry** — utilization of bacteria to produce industrially important chemicals — could potentially replace traditional chemistry, a 100 billion dollar industry, if the utilized organisms could be evolved to meet efficiency and yield demands necessary for effective replacements of chemical processes. If biofuels such as ethanol and methanol become economically viable, the potential market could reach 2.5 trillion dollars. The smaller market of production of industrial enzymes is a $1.5 billion industry, and already relies entirely on microorganisms - even small gains in efficiency and yield of the utilized micro-organisms could generate considerable revenue.*

Specialty chemicals

In a similar fashion GMO strains producing specialty chemicals can be improved. These molecules can have direct utilization like lactic acid in biodegradable polymers or could be precursors to high value pharmaceuticals.

SCIENTIFIC BOARD

***Steven Benner**, PhD, scientific consultant, is currently the founder and President of the Foundation For Applied Molecular Evolution, www.ffame.org. Dr. Benner received his PhD in Chemistry from Harvard University after graduating in Molecular Biophysics and Biochemistry at Yale University. In 1991, he helped found evolutionary bioinformatics, launched one of the first web-based bioinformatics servers with Gaston Gonnet, generated the first naturally organized protein sequence databases, and helped develop the MasterCatalog that generated ca. $4 million in sales.*

Dr Benner established paleomolecular biology, where researchers resurrect ancestral proteins from extinct organisms for study in the laboratory. He invented dynamic combinatorial chemistry, combining ideas from molecular evolution, enzymology, analytical chemistry, and organic chemistry to generate a strategy to discover small molecule therapeutic leads. Dr. Benner initiated synthetic biology as a field: the Benner group was the first to synthesize a gene for an enzyme, and used organic synthesis to prepare the first artificial genetic systems, with outcomes actually applied in the therapy of HIV and hepatitis B & C. These systems also support the first artificial chemical system capable of Darwinian evolution.

Along his career, Dr. Benner was distinguished with many awards among which : the National Science Foundation Graduate Fellow, the Anniversary Prize from the Federation of European Biochemical Societies in 1993, the Nolan Summer Award in 1998, the B. R. Baker Award in 2001, and the Sigma Xi Senior Faculty Award 2005.

***Jim Spain**, PhD, scientific consultant, is currently a Professor of Environmental Engineering at the Georgia Institute of Technology. Dr. Spain received his PhD in microbiology from The University of Texas and then studied the biodegradation of pesticides in the marine environment for five years as a post doctoral fellow and research scientist at the U.S. Environmental Protection Agency Marine Environmental Research Laboratory. Prior to joining Georgia Tech Dr. Spain directed the Environmental Biotechnology research program at the Air Force Research Laboratory in Panama City, Florida where he studied the biodegradation of synthetic organic compounds in the environment.*

Dr. Spain works at the interface between basic microbiology research and practical applications to solve environmental problems. His research interests in environmental biotechnology include : discovery and construction of bacteria for degradation of organic pollutants ; evolution and adaptation of microbial communities ; distribution, persistence, and biodegradation of chemical pollutants in soil and water ; photobiological hydrogen production by cyanobacteria ; and discovery of biocatalysts for green chemistry synthesis of novel materials. Dr. Spain is a former editor for Applied and Environmental Microbiology and has published widely on the biodegradation and biosynthesis of *organic compounds. He consults regularly with bioremediation companies and has discovered a number of novel microorganisms able to biodegrade pollutants previously thought to be recalcitrant.*

Valérie de Crécy*, PhD, scientific consultant, was trained as a bacterial geneticist at the Pasteur Institute (Paris) and the National Institutes of Health (Bethesda). She has worked in industrial settings (at Aventis and a consultant for a French Biotech company) and in academic settings at The Scripps Research Institute and recently at the Dpt of Microbiology and Cell Science at the University of Florida. Her work has covered many aspects of microbial metabolism (primary, secondary and regulation) and lead to around forty publications. In recent years she has focused on combining comparative genomics with experimental validation to identify novel genes and on using experimental evolution protocols to adapt bacteria to new metabolic constraints.*

Thomas Lyons*, PhD, Principal Research Scientist and Scientific Board Member, is a former faculty member in the Department of Chemistry at the University of Florida. Dr. Lyons is also a current fellow at the Foundation for Applied Molecular Evolution (FfAME). He received his PhD in chemistry from the University of California, Los Angeles where he studied the relationship between the structure of a protein and its function, with an emphasis on how changes in the structure/function relationship can lead to disease states. He then did a postdoctoral fellowship at the University of Missouri, Columbia where he studied the chemistry and genetics of nutrient uptake in microorganisms and how environmental changes alter global patterns of gene transcription. As a faculty member at the University of Florida, Dr. Lyons specialized in studying a new and largely uncharacterized family of receptors called PAQRs that can be found in nearly all organisms. He continues this research at FfAME. Dr. Lyons' research falls into two main categories. The first involves study the role of these receptors in fungal physiology, with particular emphasis on figuring out how these receptors can be manipulated to control fungal growth. The second category involves the use of a yeast-based functional assay system to study the pharmacology of human PAQR receptors, several of which are tightly linked to pathological states such as type II diabetes. Dr. Lyons' expertise spans the disciplines of biochemistry, pharmacology, microbiology and genetics.*

Nemat Keyhani*, PhD Member of the Advisory Board, scientific consultant, is currently an Associate Professor in the Department of Microbiology and Cell Science at the University of Florida. He received his Ph.D. in Biochemistry and Molecular Biology from the Department of Biology at Johns Hopkins University under Dr. Saul Roseman. Prior to joining the University of Florida, his post-doctoral work involved studies on microbial pathogenesis and biodegradation of chitin by marine bacteria. Since joining the University of Florida, his research has focused on molecular, genetic and physiological studies of the entomopathogenic fungi, Metarhizium anisopliae and Beauveria bassiana and their ability to target diverse member of the Arthropoda. He has established the first extensive transcriptome analysis of B. bassiana, is a member of the M. anisopliae genome sequencing effort, and is the lead principal investigator and founder of the B. bassiana Genome Sequencing Consortium.*

PUBLICATIONS

On Sept 26, 2007 an article has been published on line for publication in Applied Microbiology & Biotechnology prior to the printed publication

Development of a novel continuous culture device for experimental evolution of bacterial populations.

de Crécy E, Metzgar D, Allen C, Pénicaud M, Lyons B, Hansen CJ, de Crécy-Lagard V

Abstract Appl Microbiol Biotechnol. 2007 Nov ;77(2):489-96. Epub 2007 Sep 26.

The availability of a robust and reliable continuous culture apparatus that eliminates wall growth problems would lead to many applications in the microbial field, including allowing genetically engineered strains to recover high fitness, improving biodegradation strains, and predicting likely antibiotic resistance mechanisms. We describe the design and implementation of a novel automated continuous culture machine that can be used both in time-dependent mode (similar to a chemostat) and turbidostat modes, in which wall growth is circumvented through the use of a long, variably divisible tube of growth medium. This tube can be restricted with clamps to create a mobile growth chamber region in which static portions of the tube and the associated medium are replaced together at equal rates. To functionally test the device as a tool for re-adaptation of engineered strains, we evolved a strain carrying a highly deleterious deletion of Elongation Factor P, a gene involved in translation. In 200 generations over 2 weeks of dilution cycles, the evolved strain improved in generation time by a factor of three, with no contaminations and easy manipulation.

On August 26, 2009 the following article has been published in the open access journal BMC Biotechnology:

Directed evolution of a filamentous fungus for thermotolerance

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Abstract

Background: Filamentous fungi are the most widely used eukaryotic biocatalysts in industrial and chemical applications. Consequently, there is tremendous interest in methodology that can use the power of genetics to develop strains with improved performance. For example, Metarhizium anisopliae is a broad host range entomopathogenic fungus currently under intensive investigation as a biologically based alternative to chemical pesticides. However, it use is limited by the relatively low tolerance of this species to abiotic stresses such as heat, with most strains displaying little to no growth between 35-37°C. In this study, we used a newly developed automated continuous culture method called the Evolugator™, which takes advantage of a natural selection-adaptation strategy, to select for thermotolerant variants of M. anisopliae strain 2575 displaying robust growth at 37°C.

Results: Over a 4 month time course, 22 cycles of growth and dilution were used to select 2 thermotolerant variants of M. anisopliae. Both variants displayed robust growth at 36.5°C, whereas only one was able to grow at 37°C. Insect bioassays using Melanoplus sanguinipes(grasshoppers) were also performed to determine if thermotolerant variants of M. anisopliae retained entomopathogenicity. Assays confirmed that thermotolerant variants were, indeed, entomopathogenic, albeit with complex alterations in virulence parameters such as lethal dose responses (LD50) and median survival times (ST50).

Conclusion: We report the experimental evolution of a filamentous fungus via the novel application of a powerful new continuous culture device. This is the first example of using continuous culture to select for complex phenotypes such as thermotolerance. Temperature adapted variants of the insect-pathogenic, filamentous fungus M. anisopliae were isolated and demonstrated to show vigorous growth at a temperature that is inhibitory for the parent strain.

Insect virulence assays confirmed that pathogenicity can be retained during the selection process.

In principle, this technology can be used to adapt filamentous fungi to virtually any environmental condition including abiotic stress and growth substrate utilization.

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