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Author
Hazen, Terry

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ESPP Environmental Stress Pathway Project

Anaerobic Phenotype Microarray Method for Knockout Mutant Comparison

Dominique Joyner, Aindrila Mukhopadhyay, Romy Chakraborty, Sharon Borglin, Janet Jacobsen & Terry Hazen

DOE GENOMICS:GTL ACCELERATING DISCOVERY FOR ENERGY AND ENVIRONMENT
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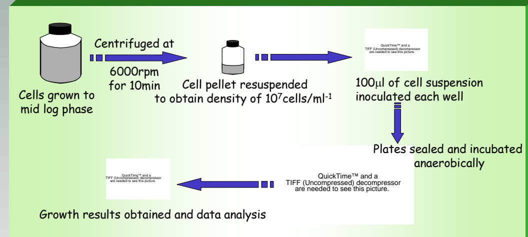
Abstract

Phenotype Microarray (PM) has been developed for the high throughput and rapid assessment of phenotypic responses of microbes to approximately 2,000 metabolites and chemicals under aerobic conditions. Previously in our lab, a method was developed for PM under anaerobic conditions. In the present work we describe a method of inoculum standardization of anaerobes to ensure repeatability of results between replicate runs. Our tests were conducted with the sulfate reducing bacterium *Desulfovibrio vulgaris* strain *Hildenborough* in a defined lactate sulfate medium. For optimization of results, several factors were tested that included growth phase of inoculum having the greatest capability for growth after inoculation, optimal centrifugation times at 6000 g for highest retention of cell pellet, optimal inoculum concentration of resuspended cells as determined by AODC which was compared to OD at 600nm and %T. Our results show that standardization was achieved as demonstrated by repeatability of growth data between biological replicates of *D. vulgaris* in the PM. The application of the anaerobic PM was tested in 2 different studies with a wild type DvH and a single crossover sensor histidine kinase mutant strain of *D. vulgaris* with a potentially interesting phenotype under salt stress. The differential expression patterns of wt *D. vulgaris* and the mutant strain of *D. vulgaris* were compared.Osmotic sensitivity to NaCl and KCl was increased in the mutant strain with inhibition of growth above 3% as compared to 6% and 5% with the wt. No protection of the mutant was conferred by the addition of osmoprotectant. In another test, the mutant strain was used for the novel application of PM technology to investigate phenotypic expression of an organism under stressed conditions. In this study, anaerobic PM of the mutant strain under osmotic stress was generated with 250mM NaCl vs 250mM KCl and compared with the expression pattern of the organism under non stressed conditions. The mutant strain amended with 250mM KCl had greater resistance to osmotic stress up to 10% NaCl and greater resistance to200mM sodium benzoate and 100mM sodium nitrite.

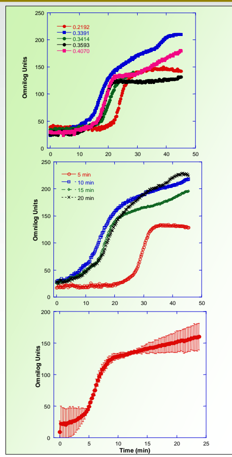
Phenotype Microarray (PM)

The Omnilog system generates a high-throughput and rapid phenotype microarray (PM) of a bacterium of interest. It is possible to investigate phenotypic expression on a wide variety of substrates. Approximately 2,000 assays are run simultaneously to include catabolic and biosynthetic metabolites, ions for osmotic effects, pH, toxic metals and a variety of inhibitory and stimulatory chemicals.

Our group has adapted the system for anoxic incubation of SRBs -specifically *Desulfovibrio* and *Desulfomicrobium* species. Inoculum standardization has been developed to ensure defined inoculum for maximum reproducibility.



Inoculum Standardization



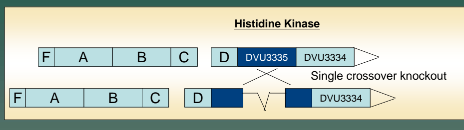
Optimal growth of a 100ul volume of SRB in defined lactate-sulfate medium containing iron is 10^8 cells/ml correlating to mid log phase growth of the SRB with a 10% inoculum. Stationary phase and early log phase cells do not generate high enough final yield to be detected by CCD camera of the Omnilog system

Cells are centrifuged to remove excess medium prior to resuspension in appropriate PM medium. Optimal centrifugation time and speed were established to generate a bacterial cell pellet that could be easily resuspended and homogenized and not result in cell death

Standardized inoculum successfully yields consistent growth patterns of strain DvH in the PM plates with multiple biological replicates.

Plot represents average of 5 biological replicates with std deviation. Of each biological replicate n=96

Mutant Generation (DvAM88) and Expected Phenotype



The Kdp two-component system in bacteria consists of an inducible high affinity transporter of K+ ions encoded by genes in the *kdpABC* operon where *kdp* is induced by low concentrations of K+ and repressed by high concentrations of K+

Single cross over method was used to knock out DVU3335 the histidine kinase present upstream of the response regulator of the *kdp* operon.

The resulting knockout DvAM88 is expected to have a deactivated *kdp* system.

PM Characterization of DvAM 88

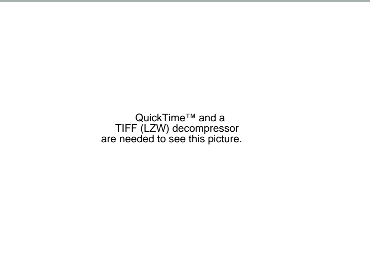
PM overview of growth characteristics of DvAM88 show little difference compared to DvH when grown on LS4D

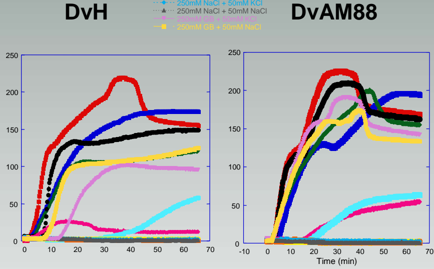
Pretreatment of DvAM88 with salts produced no real differences between DvHAM88 vs DvH on the PM array- however growth characteristics of the 2 strains inoculated into MT plates were different with pretreatment

Osmotic and ionic strength expression patterns generated from no pretreatment vs salt pretreatment were compared for DvAM88 and DvH utilizing the array of substrates provided on PM Plate #9.

K+

Salt Stress:
DvH 250mM NaCl
DvAM88 250mM NaCl
DvH 250mM KCl
DvAM88 250mM KCl



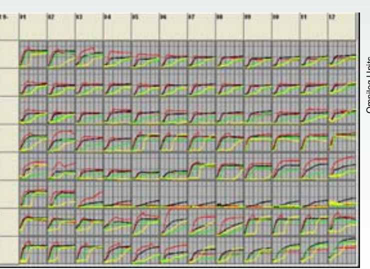


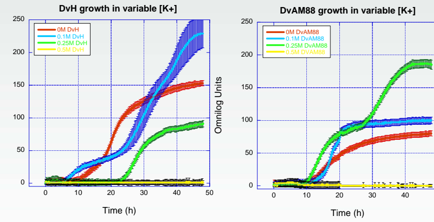
50% growth with 250mM NaCl
25% growth with 500mM NaCl
0% growth with 750mM salt

~100% growth with 250mM KCl and NaCl
25% growth with 500mM KCl and NaCl
0% growth with 750mM salt

K+ deficient

LS4D DvH
LS4D DvA M88
K+ (-) DvH
K+ (-) DvA M88



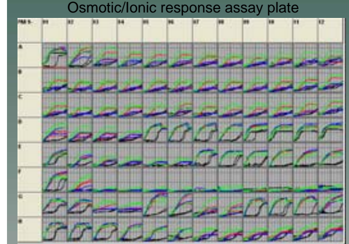


DvAM88 with 0M K+ represents growth of DvH with alternate K+ channels. The growth is not as robust as the wt in which the KDP transporter is active. DvAM88 also exhibits the best growth at 0.25M indicating that it has some resistance to K+ ions as its primary (?) K+ transport system is knocked out. For wt 250mM is the MIC generating 50% yield and an extended lag. 0.5M was inhibitory to both mutant and wt.

DVH Phenotype Expression Under Stress

We have started a preliminary screen of DvH expression patterns under a variety of stresses to include air, salt, nitrite, nitrate and heat. We have established MICs for these stressors and for most have run a high-throughput biomass production for comparative assessment of genes and proteins that are up or down-regulated upon exposure to these stresses. Our goal is to tie the phenotypic changes to microarray and proteomic data to correlate the genotype expression with the phenotype expression. Characterization of the phenotypic differences on the array of substrates as a result of stress exposure can provide insight into the mode of stress response and survival.

Osmotic/Ionic response assay plate



Green: no stress
Black: K+ starvation
Red: 250mM KCl
Blue: 250mM NaCl

- Growth of DvH under K+ starvation is inhibited on SO4 , NO3 and Urea
- Pretreatment with NaCl does not protect against further NaCl stress
- Pretreatment with KCl does not inhibit growth on NaCl

Biological Conclusions

- A mutation in the histidine kinase of the kdp system is expected to effect DvH under hypo-ionic conditions- specifically low K+. Based on the hyperosmotic results we conclude that the inactivation of the kdp transporter in DvAM88 made the strain more resistant to hyperosmotic stress
- Inactivation of the kdp transporter allows us to see the efficiency of the remaining K+ transporters wrt growth of DvH. Based on the hypoosmotic results we conclude that the other transporters are sufficient for growth of DvH and therefore the kdp transporter is not crucial for survival. Based on growth characteristics, remaining transporters must be regulated by ionic strength as only at inhibitory levels of K+ does the mutant gain enough K+ for 'normal' growth
- DvH and DvAM88 have similar growth on all substrates when grown in LS4D~ 100 mM salt. When K+ is omitted from LS4D, DvH is not capable of growth on several compounds. Interestingly, on these substrates where DvH without K+ can not grow, DvAM88 without K+ can grow- however, not as robustly as DvH with K+

PM Conclusions

- The PM array provides an overview of the growth phenotype of a strain under predicted and unpredicted conditions
- The PM was successfully adapted for the anaerobic SRB DvH and successfully used for the comparative assessment of the phenotypic expression of wt vs mutant
- We introduce the novel application of the PM for screen of phenotypic expression of a strain under a combination of stressed vs non-stressed conditions

Acknowledgement

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