Comprehensive phenotypic profiling of mutant microbial strains with Odin

Introduction

Countless labs around the world utilize mutational analyses to probe gene function, transcriptional and translational regulation, phenotypic output, and other characteristics of their microorganisms. The advent of CRISPR-Cas9 genome editing technology and the decreasing costs of nucleic acid sequencing have paved the way for many new microbial model systems. New microbes with unknown phenotypes bring with them opportunities to utilize high-throughput phenotypic screening techniques. For example, newly identified microbes often have one or more mutations in metabolic genes whose orthologues in *E. coli* are involved in glycolysis or another major pathway. Researchers may knock out or otherwise mutate candidate genes to confirm the function or elucidate the unknown role. It is desirable in these cases to screen mutant strains for as many phenotypes as possible. Unfortunately, while other -omics methods have made significant strides in increasing throughput, phenotypic screens have lagged behind.

Phenotype MicroArrays™ (PM) from Biolog, used in conjunction with the Odin™ instrument (Figure 1) enables phenotypic screening of organisms against a large library of substrates and conditions. Odin can incubate and read up to 50 plates at a time. Data collection is achieved in two ways. Odin can read each plates' Optical Density (OD) to directly measure cell proliferation. Alternatively, one can also measure the redox dye color change to determine the metabolic activity in each well. Both measurements can be made in parallel, to collect two orthogonal data points from the same experiment, which can illuminate phenotypic differences in metabolism vs. cell growth.

PM technology allows for the differentiation of phenotypes among strains by measuring either growth or metabolic activity as a response to the various pre-selected compounds. There are 20 PM arrays used for most microbial strain phenotyping.

Figure 1. Odin is the all-in-one platform for cellular characterization. Up to 50 plates can be incubated and read in one experiment. Odin is ideal for monitoring growth curves, measuring cell respiration kinetics, and identifying unknown microbes.

PM 1-8 contain metabolic substrates including 190 carbon sources, 95 nitrogen sources, 285 di- and tri-peptides along with a variety of sulfur- and phosphate- containing compounds and other nutritional supplements. Each of these substrates were selected to probe different points along specific metabolic pathways, which will give a comprehensive overview of an organism's metabolic profile. PM 9-20 are designed to test an organism's susceptibility to various osmotic and ionic effects, pH, and a wide array of chemical inhibitors.

Phenotype MicroArrays enable researchers to simultaneously screen their organism(s) for thousands of different phenotypic traits using a single starting culture. The user prepares a cell suspension in a minimal inoculating fluid devoid of most nutrients, and aliquots that cell suspension into each of the PM plate wells. The user can supplement the inoculating fluid with a redox dye to measure respiration, or they can run without a dye to focus on growth and turbidity changes. Once plates are inoculated, they are transferred to the Odin instrument for incubation, data acquisition, and analysis.

Maltose metabolism in *E. coli* is controlled by a small set of genes activated by MalT including MalF which is a subunit of the MalFGK2 membrane transporter. This application note shows how the Odin platform can be used to compare wild type (MG1655FB) and mutant (*malF:Tn10*) strains of *E. coli*. The main goal of the experiment was to validate that deletion of MalF is sufficient to ablate the transport of maltose into the cell and therefore render the mutant unable to grow using maltose as the sole carbon source. Additionally, we will show a gain of function in tetracycline-family drug resistance resulting from the insertion of a tetracycline resistance marker via Tn10 transposon.

Methods

Phenotype Microarray Experiments

PM profiles were generated using standard Biolog procedures for *E. coli* and other gram-negative bacteria. *E. coli* strains, MG1655FB and its *malF:Tn10* mutant derivative, were grown on BUG+B (Biolog Universal Growth medium plus Blood) at 36 °C for 24 hours prior to inoculation into PM plates. A cell suspension was made in 16 mL minimal Biolog Inoculating Fluid (IF-0a) to a transmittance (T) of 42% T as measured by a Biolog turbidimeter. 112 mL of this suspension was then transferred to a sterile container with 75 mL fresh IF-0a and 900 µL Biolog Dye Mix A resulting in a transmittance of 85% T for a total of 90 mL. 22mL of this 85% T suspension was then added to PM 1 and 2 at 100 µL per well. 600 µL of the remaining 85% T suspension was then added to a new sterile container with 120 mL IF-10a and 1.2 mL additional Dye Mix A, and the resulting suspension was then used to inoculate plates PM 9-20 at 100 µL per well. To the remaining 68 mL of the 85% T suspension was added 680 µL of 2 M sodium succinate/200 µM ferric citrate solution. The resulting suspension was then used to inoculate plates PM 3-8 at 100 µL per well. Following inoculation of all plates, PM 1-20 were placed into the Odin instrument to incubate for 24 hours at 36 °C. The Odin instrument was set to read plates every 20 minutes for the duration of the experiment to record the amount of dye reduction in each well in a kinetic fashion.

Measurement of the metabolic activity in each well was accomplished by taking absorbance reads at 590 nm for 24 hours (72 measurements) and calculating AUC (area under curve) from the kinetic curves for each well of the PM 1-20 plates. A fold change value was calculated for each well of the PM 1-20 plates as the ratio AUC_ E. *coli malF:Tn10* / AUC_ *E. coli* MG1655FB to determine biologically significant phenotypes. Graphs were produced for each well by overlaying kinetic OD readings of *E.coli* WT and mutant in each well of the PM 1-20 plates and colored with cyan, yellow and green to represent decrease, no change, and increase of OD values in *E. coli malF:Tn10* mutant relative to MG1655FB.

Results

For this experiment, *E. coli* WT MG1655 and a *malF:Tn10* mutant derivative strain were challenged against nearly 2,000 different conditions including metabolic substrates (C, N, S, and P), pH and osmolyte stress, and varying concentrations of a wide array of inhibitors. This was done by inoculating each strain into a set of PM 1-20 microplates along with our tetrazolium-based dye which undergoes an irreversible color change after reduction in response to NADH production. We identified significant phenotypic differences between the mutant and WT strains as outlined in the methods. We were able to identify significant differences with respect to carbon source utilization and antibiotic resistance indicative of the engineered mutation.

MalF is an integral membrane protein in *E. coli*, acting with MalG to make up part of the maltose transport system required for use of maltose or other dextrin and starch-derived sugars as an exogenous carbon source. The *malF:Tn10* mutant contains a chlortetracycline resistance marker inserted at the MalF locus via the Tn10 transposable element rendering MalF non-functional. We would expect to see a loss of function in maltose metabolism in the mutant relative to WT, in addition to a gain of function in chlortetracycline resistance.

Indeed, this is the case as was shown using Odin with PM plates 1 and 11 (Figure 2). PM 1 contains many carbon sources including maltose and maltotriose which are both processed in the same metabolic pathway after import into the cell. In line with the expected impact of the MalF deletion, the mutant was unable to metabolize either of these substrates. PM 11 contains an array of inhibitory compounds in sets of four increasing concentrations. As expected, the mutant showed significantly increased resistance to chlortetracycline at concentrations ranging from 10-50 µM (PM 11 wells A6-A8). The *malF:Tn10* strain also demonstrated a similar gain of resistance to demeclocycline at concentrations of 2-21 µM (PM 11 wells D6-D8), consistent with the acquisition of tetracycline-compound resistance (Figure 2).

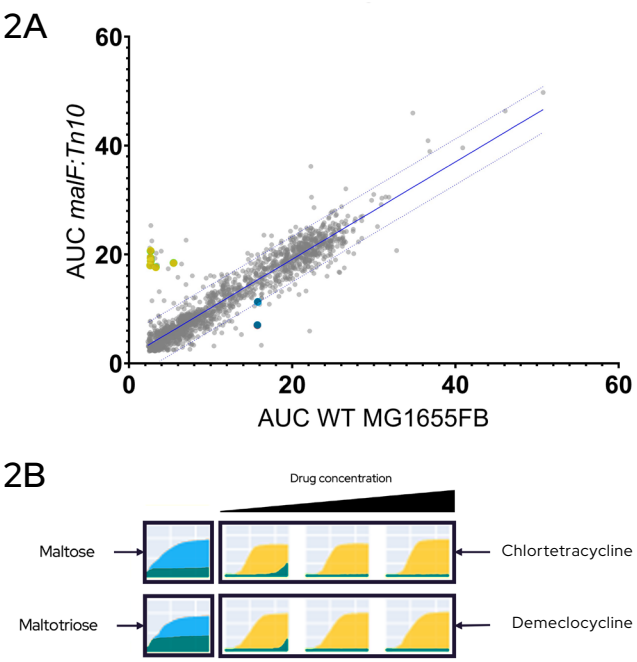

Figure 2: Gain and loss of function phenotypes *malF:Tn10* vs MG1655FB

2A: WT vs *malFTn:10* AUC values for PM 1-20 scatter plot. Green points indicate chlortetracycline and demeclocycline. Red points indicate maltose and maltotriose. Blue line indicates linear regression best fit (R^2 = 0.8772) and blue dashed lines indicate 90% prediction band. 2

B: X-axis: time (0-24 hours); Y-axis: OD_{590}. blue = loss of function relative to wild type, yellow = gain of function relative to wildtype, green = overlap. The *malF:Tn10* mutant strain (represented in green) shows a loss of function phenotype in its ability to use maltose and maltotriose as sole carbon sources, consistent with the insertion of the Tn10 transposable element at the MalF locus. The mutant also shows the expected gain of chlortetracycline resistance phenotype relative to WT (represented in blue). The mutant gained similar resistance to demeclocycline as well.

Conclusion

Odin used in conjunction with our Phenotype MicroArrays allow for the high-throughput phenotypic characterization of microbes. Researchers can assess the growth rate of a wide variety of species in response to nearly 2,000 different metabolic substrates, conditions, and industrial/pharmacologically relevant compounds. The use of a tetrazolium-based redox reporter dye provides an orthogonal measurement to growth in the form of a metabolic profile in response to each of these substrates. Analysis of these two data sets allows for the characterization of subtle phenotypes which may indicate a shift in metabolic state without a change in cell division or vice versa. Here we used metabolic analyses with PM plates in Odin to show that the *E. coli malF:Tn10* mutant is deficient for maltose and maltotriose metabolism, and resistant to tetracycline compounds as expected based on the genetic construct used to generate the knockout strain. The mutant also proved resistant to demeclocycline, which further confirmed the tetracycline-family drug resistance phenotype. This effectively demonstrates how Odin can be used to phenotypically validate mutant strain construction and to generate a phenotypic profile of a mutant organism.