Distinguishing Growth and Metabolism in Microbial Phenotyping on Odin

Introduction

The need to characterize multiple strains of the same species of microorganism has rapidly increased over the last several years. Not only are strain-level genomes now becoming available for researchers to utilize, but also studies of proteomic and regulatory variation among various clinical and environmental isolates of both eukaryotic and prokaryotic microorganisms have yielded fruitful data. Phenotypic variability among strains of the same species is a field of active study, and there are few available methods for screening many phenotypes simultaneously. Conventional methods for performing phenotypic screens are limited in their utility, as they can only handle a small number of strains or a limited number of conditions at one time.

Studies across various microorganisms have targeted intra-species variation among clinical isolate strains. For example, a recent study by Zlatkov and colleagues (2022) found that two *Escherichia coli* strains RS218 and IHE3034 take on different metabolic profiles under the same aerobic conditions, where RS218 undergoes oxidative metabolism and IHE3034 engages in anaerobic respiration. Many studies have also pointed out that there can be significant differences between pathogenic isolates both metabolically and in antibiotic resistance profiles. The issue of strain variation is further complicated when genetic factors are also considered. Two strains may exhibit preferential utilization of one or more carbon sources; however, those strains may have thousands of mutations between them making it difficult to pinpoint a causative mutation or pathway.

It is important to consider both metabolic activity and cell proliferation as there are cases in which organisms can undergo a metabolic shift without changing growth rate. For example, a 2016 study by Ene and colleagues showed that *Candida albicans* white and opaque cell phases have different metabolic profiles although both cell phases demonstrate similar doubling times. The inverse is also true: growth can be significantly impacted in response to a specific substrate or stressor, while NADH production may stay consistent overall. Microbes often have preferred nutritional sources needed for cell wall biosynthesis like N-acetylglucosamine and L-alanine which are required for peptidoglycan synthesis in *E. coli*.

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.

Phenotype MicroArrays™ (PM) from Biolog, used in conjunction with the Odin™ instrument (Figure 1) enables phenotypic screening and a large library of substrates and conditions against which to test your organisms. Odin can incubate and read up to 50 plates at a time with read intervals every 20 minutes over the course of 24-72 hours (or longer). Data collection is achieved in two ways. Odin can read the Optical Density (OD) in each well to directly measure cell proliferation. Alternatively, one can add a redox-sensitive reporter dye, and measure the color change in each well via NADH production to determine the metabolic activity. Both types of measurements can be collected at the same time, 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 panels used for most microbial strain phenotyping. 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 substrate was selected to probe different points along specific metabolic pathways, and provides 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 up to 4,800 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. This provides a simple and efficient workflow to test thousands of conditions using a single culture. Once plates are inoculated, they are transferred to Odin for incubation, data acquisition, and analysis.

This application note shows how the Odin platform was used to compare non-pathogenic (CGSC6300) and pathogenic (O157) strains of *E. coli* and elucidate phenotypic differences which can point to divergent metabolic function as well as chemosensitivity. Phenotypic variations were identified by comparing both strains' metabolic and proliferation profiles generated with or without dye respectively.

Methods

Phenotype Microarray Experiments

PM profiles were generated using standard Biolog procedures for *E. coli* and other gram-negative bacteria. *E. coli* strains, CGSC6300 and O157 (*E. coli* Genetic Stock Center and USDA, respectively), were grown on BUG+B (Biolog Universal Growth medium plus Blood) at 36 °C for 24 hours prior to inoculation into PM plates. Two cell suspensions for each strain were made in 16 mL minimal Biolog Inoculating Fluid (IF-0a) to a transmittance (T) of 42% T as measured by a Biolog turbidimeter. 15 mL of each suspension was then transferred to a sterile container with 75 mL fresh IF-0a resulting in a transmittance of 85% T for a total of 90 mL for measuring cell growth. To measure metabolic activity via dye reduction, 900 µL Biolog Dye Mix A was also added to one of each culture. 22 mL of these 85% T suspensions were then added to PM 1 and 2 at 100 µL per well. 600 µL of the remaining 85% T suspensions were then added to new sterile containers with 120 mL IF-10a and 1.2 mL additional Dye Mix A if needed, and the resulting suspensions were then used to inoculate PM 9-20 at 100 µL per well. To the remaining 68 mL of the 85% T suspensions was added 680 µL 2 M sodium succinate/200 µM ferric citrate solution. The resulting suspensions were then used to inoculate PM 3-8 at 100 µL per well. The same procedure, minus the dye addition, was followed to generate PM 1-20 plates for measuring growth. Following inoculation of all plates, PM 1-20 were placed into Odin to incubate at 36 °C. Odin was set to read plates every 20 minutes to record the amount of dye reduction and growth in each well in a kinetic fashion. OD measurements were taken at 590 nm with and without dye over 24 hours to measure cell growth and metabolic activity respectively.

Measurement of the metabolic activity was accomplished by generating AUC (area under curve) values of kinetic OD readings for each well of the PM 1-20 plates containing dye. Similar kinetic OD readings were generated for PM 1-20 plates without dye to quantify cell growth. Fold change values were calculated for each well of the PM 1-20 plates as AUC_ *E. coli* O157/AUC_ *E. coli* CGSC6300 to determine biologically significant differences. Graphs were produced for each well by overlaying kinetic OD readings of O157 and CGSC6300 in each well of the PM 1-20 plates where yellow represents the reference, or CGSC6300 strain, cyan represents the test, or O157 strain, and green represents the overlap between the two (Figure 2).

Figure 2: Schematic of Phenotype MicroArray data. Growth or metabolism were measured over time. Area denoted in yellow in the comparison graph represents the loss of function for the experimental sample relative to the reference sample. Conversely, a blue area in the comparison graph would represent the gain of function for the experimental sample. Area in green represents the overlay of the two data sets, for comparison.

Results

Escherichia coli WT, CGSC6300, and pathogenic strain O157, 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 Biolog's PM 1-20 microplates along with our tetrazolium-based dye which undergoes an irreversible color change after reduction in response to NADH production. Plates were incubated in the Odin instrument at 36 °C for 24 hours and automatically read every 20 minutes. We identified significant phenotypic differences between the pathogenic and WT strains as outlined in the methods. We were able to identify significant differences both in metabolic substrate preferences and stress tolerance between the two strains. Differences were identified within each strain by comparing the difference between their metabolic and growth profiles. Differences were identified between strains by comparing their metabolic or growth phenotypes.

Microplates PM 11-20 contain serial dilutions of a variety of inhibitory compounds, including common antibiotics as well as other inhibitors to growth and metabolism. CGSC6300 and O157:H7 showed similar resistance profiles for most inhibitors tested whether growth (no dye) or metabolism (with dye) were considered (Figure 3A & 3B). Resistance to the DNA topoisomerase drug, enoxacin, varied between strains only when growth was measured. The pathogenic strain O157:H7 showed hyper-susceptibility to the drug in terms of growth; cell density failed to significantly increase over a baseline even after 24 hours incubation at any concentration whereas the lab strain CGSC6300 was resistant. This contrasts with the metabolic profile which shows a similar increase in metabolic rate in both strains at 6 µM and 19 µM enoxacin. These results may indicate that O157:H7 is able to persist in the presence of low concentrations of drug. Under this hypothesis, persister cells are still able to metabolize the available nutrients but are unable to divide due to the DNA replication inhibition exerted by enoxacin.

Alternative carbon source utilization is a hallmark of many non-pathogenic and pathogenic strains and species alike. Here we challenged the non-pathogenic CGSC6300 and pathogenic O157:H7 *E. coli* strains with almost 200 carbon sources and observed both unique and conserved metabolic abilities for the two strains. Figure 4 includes the kinetics curves for PM 1 using the tetrazolium-based redox dye readout, indicating metabolic activity. The pathogenic O157:H7 strain was found to have loss of function phenotypes for the metabolism of several carbon sources relative to CGSC6300 including D-saccharic acid, D-serine, D-sorbitol, D-galactonic acid-γ-lactone, α-ketoglutaric acid, and a gain of function relative to CGSC6300 for metabolism of D-sucrose. There are also several carbon sources metabolized by both strains, but at differing rates (uridine and L-asparagine) where CGSC6300 can rapidly metabolize these substrates, and O157:H7 utilizes the substrates at a lower rate.

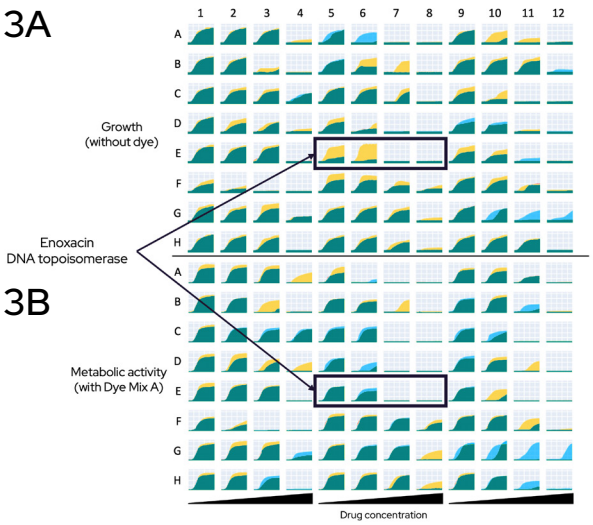

Figure 3A: PM 11 Kinetic growth curves. OD measurements indicating growth are represented on the y axis, and time (0-24 hours) is represented on the x axis. Left to right in the outlined wells is growth in the presence of ~3-fold increasing concentrations of enoxacin (6 - 175 µM) O157:H7 shows hypersensitivity to enoxacin relative to CGSC6300 which can grow at 6 and 19 µM but not at 58 and 175 µM.

Figure 3B: PM 11 Kinetic metabolic curves generated by measuring the increase in metabolic activity. OD measurements as a result of dye reduction, or metabolic activity, are represented on the y axis, and time (0-24 hours) is represented on the x axis. Both strains show similar metabolic profiles in the presence of low concentrations of enoxacin suggesting that the metabolic activity of O157:H7 is able to persist in the presence of the drug, without actively dividing.

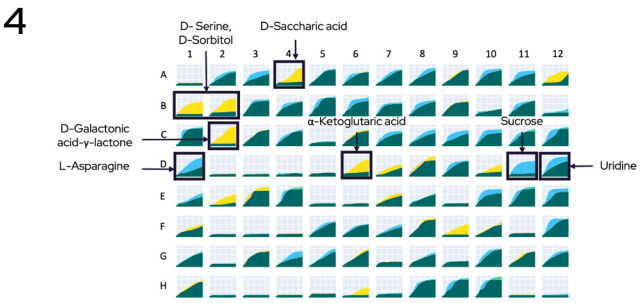

Figure 4: PM 1 Kinetic metabolic curves, demonstrating alternative carbon source utilization: OD measurements representing metabolic activity are represented on the y axis, and time (0-24 hours) is represented on the x axis. O157:H7 shows loss of function for the ability to metabolize several carbon sources including D-saccharic acid, D-serine, D-sorbitol, D-galactonic acid-γ-lactone, α-ketoglutaric acid, and shows a gain of function relative to CGSC6300 for metabolism of D-sucrose.

Conclusion

Odin, when used in conjunction with Phenotype MicroArrays allows for the high-throughput characterization of a variety of microorganisms. Researchers can assess the growth rate of an organism in response to thousands of different metabolic substrates and conditions. The measurement of growth rate allows for the identification of phenotype changes that indicate the impact of varying substrates on growth. The inclusion of a tetrazolium-based redox reporter dye provides a metabolic profile in response to each of these substrates, and allows for the identification of subtle phenotypic differences, some of which may indicate a shift in metabolic state without a change in the rate of cell division. Here we used the growth and metabolic analyses with PM and Odin to show that the *E. coli* pathogenic strain O157 was able to utilize several carbon sources more effectively than the lab strain, CGSC6300, to facilitate increased cell growth without changing total metabolic output.

References:

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