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High-throughput phenotypic screening for characterizing WHO-listed fungal pathogens and monitoring phenotypic drift in bacteria

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Introduction

Genotypic microbial analysis is often used as the standard baseline for defining new and emerging strains, however understanding the phenotypic profile of a given microbial strain is key to contextualizing the results of any study. Here, we generated baseline phenotypic profiles for eleven fungal pathogens recognized by the WHO to be of special concern in the global health community: *Aspergillus fumigatus, Talaromyces marneffei, Scedosporium prolificans, Candida spp. (albicans, auris, glabrata, parapsilosis, tropicalis,* and *krusei)*, and *Cryptococcus spp. (neoformans* and *gattii)*. Each fungal strain was grown under nearly 2,000 different conditions using Biolog's Phenotype MicroArray (PM) plates that assay for carbon, nitrogen, sulfur, and phosphate utilization; pH and osmotic stress resistance; and drug tolerance.

To investigate the occurrence of phenotypic drift in laboratory and production strains, we generated the same PM profiles for faster-growing bacteria: *Escherichia coli*, *Streptococcus thermophilus*, and *Lactobacillus casei*. We then simulated years of passages while monitoring for changes in each organism's phenotypic profile. Once phenotypic drift was captured, affected generations were then sequenced to ascertain the occurrence of mutations within each strain.

Overall, this study demonstrated that high-throughput phenotypic screening can be a useful tool for both characterizing strains of interest and monitoring lab and production strains for phenotypic instability resulting from genetic changes after repeated passaging.

Methods

Phenotype MicroArrays (PM) from Biolog, used in conjunction with the Odin™ family of instruments enables phenotypic screening with a large library of substrates and conditions against which to test your organisms.

- Side-by-side strain comparisons
- Absorbance measurements at 490 nm or 590 nm, and 740 nm
- Dye-based measurements for metabolism
- Traditional OD measurements for biomass/growth
- Reads every 2-20 minutes for kinetic determinations
- Incubates up to 50 plates at a time
- Automated software analysis and streamlined data export

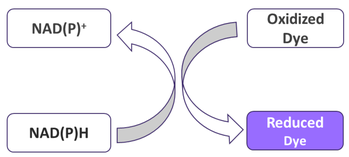

Figure 1. Tetrazolium-base redox dye is irreversibly reduced by NAD(P)H, producing a purple color which can be measured at 590 nm.

WHO-listed fungal pathogen characterization: PM profiles were generated using standard Biolog procedures for fungi. Briefly, filamentous strains were grown for 7-10 days on 2% malt extract agar at 26°C until sufficient spore/conidial development. Yeast strains were grown for 48 hours on Biolog Universal Yeast agar media at 26 °C prior to screening. Once sufficient growth was achieved, strains were transferred to FF or YT inoculating fluid and inoculated onto YT Microplates or PM1-10 and 21-25 and incubated in Odin L for 72 (YT) or 96 hours (FF) at 26°C and read every 20 minutes at 590 and 740nm.

1: Forsberg K, Woodworth K, Walters M, Berkow EL, Jackson B, Chiller T, Vallabhaneni S. Candida auris: The recent emergence of a multidrug-resistant fungal pathogen. Med Mycol. 2019 Jan 1;57(1):1-12. doi: 10.1093/mmy/myy054. Erratum in: Med Mycol. 2019 Jun 1;57(4):e7. PMID: 30085270.

Methods Continued

Phenotypic drift monitoring: *E. coli* ATCC® 11775™ *(E.c.), S. thermophilus* ATCC® 19258™ *(S.t.),* and *L. casei* ATCC® 393™ *(L.c.)* were grown at 36°C on Biolog Universal Growth +5% sheep's blood, M17, and Lactobacilli MRS media (ATCC® Medium 416), respectively, with *(S.t.* & *L.c.*) or without (*E.c.*) 5% CO_2. Passage 0 (P0) strains were streaked for single colonies and one colony was sub-cultured onto a fresh plate every 24 hr (*E.c.* and *S.t.*) or 48 hr (*L.c.*) for up to 40 passages. PM profiles were generated using standard Biolog procedures with dye to measure metabolic activity (Figure 1). P0 strains were inoculated on plates PM 1-20 in triplicate, incubated, and read every 20 minutes in Odin L for 24 hrs at 36°C to establish a baseline. P15 *L.c.* and P20 *E.c./S.t.* were then tested on PM1-20 and compared against respective P0 strains to identify altered phenotypes. A subset of PM plates with measurable phenotypic drift were then replicated to ensure reproducibility. *E.c.* and *S.t.* P40 strains were again tested on the respective PM subset in triplicate to monitor for additional drift and/or stability of P20 phenotypes. Area Under the Curve (AUC) values were compared across triplicates using Sidak's multiple comparison test.

Phenotypic drift sequencing: We used the Illumina® DNA Prep Kit to prepare genomic DNA from P20 *E.c.* and P40 *E.c.*, then performed whole-genome sequencing on Illumina® NextSeq®. Reads (150 bp) underwent filtration with fastp 0.23.2, and sequence quality was assessed using FastQC v0.11.9. Reference-based assemblies were conducted using the ATCC® 11775™ reference sequence (P0 *E.c.*). Subsequently, reads from P20 *E.c.* and P40 *E.c.* (19 and 20 M, respectively) were mapped to the P0 *E.c.* reference sequence using bwa version 0.7.17-r1188. Variants were called using lofreq, and a consensus sequence was generated using bcftools 1.17. Variants with frequencies ≥95% were identified as the variant nucleotide.

Results: WHO-Listed Fungal Pathogens

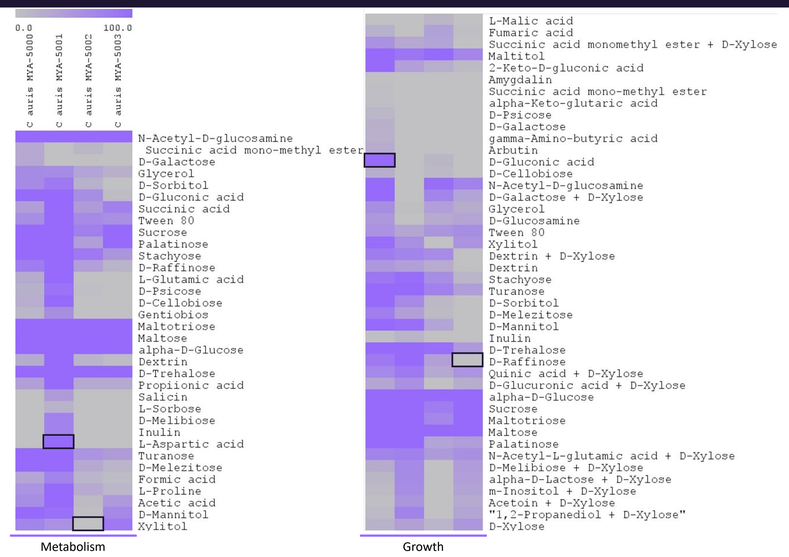

Figure 2. Differentiation of major *Candida auris* clades. Growth and metabolism were monitored for 72 hours. Four *C. auris* strains from Clades A, B, C, and D (MYA-5000, 5001, 5002, and 5003 respectively) for 72 hours on a YT Microplate. Color indicates Area Under the Curve for each substrate representing total growth/metabolic output.

- *Candida auris* is a well-known multi-drug resistant fungi which is cause for concern among medical researchers and clinicians globally[1].
- We confirmed that *C. auris* was resistant to over 67% of the fungal inhibitor compounds in our PM library and susceptible triclosan and thallium(I) acetate, and high concentrations of miltefosine and lithium chloride (not shown).
- We profiled four *C. auris* strains representative of the 4 major clades using YT Microplates which test carbon source utilization, and our platform is able to differentiate the 4 clades using their unique metabolic and growth characteristics (Figure 2).

Results: Phenotypic Drift

Table 1 : Total accumulated phenotypes. Most altered phenotypes are changes in sensitivity to antimicrobial compounds while *E. coli* was the only one to show a shift in metabolic substrate preferences.

Species	When Phenotype appeared	# Significant changes	
		Metabolic	Sensitivities
E. coli	P20 and increased in P40	1	2
	P20 and Preserved in P40	4	20
	P40	18	27
S. thermophilus	P20 and Preserved in P40	0	14
	P40	0	12
L. casei	P15	0	8

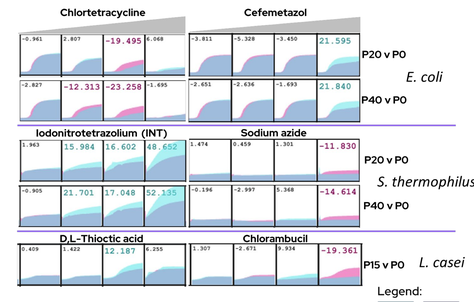

Figure 3 : Kinetic metabolic curves, demonstrating phenotypic drift. OD590 measurements representing metabolic activity are shown on the y-axis, and time (0-24 hours) is shown on the x-axis.

Well	Substrate	Diff. AUC	p-val
A8	L-arginine	-19.468	<0.0001
A9	L-asparagine	-11.567	<0.0001
A11	L-cysteine	-20.029	<0.0001
B2	Glycine	-12.056	<0.0001
B5	L-leucine	-11.465	<0.0001
B6	L-lysine	-16.383	<0.0001
B7	L-methionine	-31.412	<0.0001
B8	L-phenylalanine	-19.034	<0.0001
B9	L-proline	-16.757	<0.0001
B10	L-serine	-24.323	<0.0001
B11	L-threonine	-24.004	<0.0001

Table 2 : Nitrogen utilization defects in *E. coli* P40. *E. coli* showed a gradual shift away from being able to utilize more than half of amino acids as the sole source of exogenous nitrogen.

- We simulated years of passaging without selective pressure in *E.c, S.t.,* and *L.c.*and screened passage 0, 15/20, and 40 on PM1-20 plates.
- *E.c.* showed the most significant phenotypic drift with a total of 78 altered profiles followed by *S.t.* with 26, and *L.c.* with 20 (Table 1).
- Sensitivity phenotypes included both increased and decreased resistance for all three species (Figure 3). *E.c.* gained resistance to cefmetazole and lost resistance to chlortetracycline, *S.t.* lost resistance to sodium azide and increased its responsiveness to INT, and *L.c.* gained resistance to D,L-thioctic acid and lost resistance to chlorambucil (Figure 3).
- *E.c.* showed significantly ($p<0.0001$) reduced ability to metabolize several amino acids (Table2) indicating a loss of fitness in nitrogen metabolism relative to P0.
- Using whole genome sequencing, we identified a total of 10 genetic variants in P20 *E.c.*, comprising 7 SNPs and 3 deletions, when the reads were aligned to the P0 *E.c.* reference sequence.
- P40 *E.c.* exhibited 4 additional SNPs and 1 insertion. Among these variants, there were four affected coding sequences: Actin cross-linking toxin VgrG1, Galactoside O-acetyltransferase, and two hypothetical proteins.

Conclusions

- Large-scale phenotypic profiles for emerging and important pathogens using our platform can allow quick identification beyond the species level.
- Using Odin to monitor the phenotypic stability of production and research strains over time can complement genome sequencing to identify mutations.
- It is necessary to monitor for phenotypic drift in strains after as few as 15 passages, as seen with *L. casei. E. coli* and *S. thermophilus* showing significant drift in metabolic and sensitivity phenotypes which worsened over time.
- After 20 passages, we detected 10 genetic variations in E. coli and 27 phenotypic changes. This increased to an additional 55 phenotypic changes and 14 genetic variations affecting 4 genes by passage 40.
- For bioproduction and experiments, use authenticated strains and minimize passaging to ensure reliable and reproducible results.