Metabolomics of *Acinetobacter* Cultivations in Kleenol 30

Cultures of *A. radioresistens* 50v1 and *A. johnsonii* 2P08AA were prepared as described and harvested during early stationary phase (OD ~0.6-0.7, 7-8 h) by centrifugation (3500 rpm) at 4 ˚C for 15 min (Beckman Coulter Allegra 21R). After centrifugation, the cell pellets and supernatant fractions were separated and respectively treated. The supernatants were saved as 500 µL aliquots, dried using a DNA 110 Savant DNA SpeedVac, and stored at -80 ˚C. The pelleted cells were washed twice with 0.2x M9 and stored at -80 ˚C.

Samples were characterized by the West Coast Metabolomics Center using gas chromatography and time-of-flight mass spectrometry (GC-TOF/MS). In brief, dried cells and culture broth were separately resuspended in 2 mL of pre-chilled (-20 ˚C) and degassed extraction solvent (acetonitrile:isopropanol:water, 3:3:2), vortexed for 30 s, shaken for 5 min at 4 ˚C, clarified by centrifugation (~12000 g), and the resulting supernatant evaporated to dryness. Samples were derivatized by resuspension in 10 µL of 40 mg/mL methoxyamine hydrochloride in pyridine (30 ˚C, 1.5 h) followed by addition of 41 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (80 ˚C, 30 min); fatty acid methyl esters (C8-C30) were additionally added to serve as retention index markers (Barupal et al., 2019).

Samples were transferred to crimp top vials and separated on an Agilent 6890 Gas Chromatograph equipped with a Gerstel automatic liner exchange system (ALEX), multipurpose sample (MPS2) dual rail, Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany), and built-in gas purifier (Airgas, Radnor PA). Chromatographic separation was afforded using a 30 m (0.25 mm i.d.) Rtx-5Sil MS column (0.25 µm 95% dimethyl 5% diphenyl polysiloxane film) with an additional 10 m integrated guard column (Restek, Bellefonte PA). Carrier gas was 99.9999% pure Helium with a constant flow of 1 mL/min.

Sample volumes of 0.5 µL were injected with a 10 µL s^{-1} injection speed on a spitless injector with a purge time of 25 s. Temperature profile included 1 min at 50 ˚C for the oven temperature, an increase of 20 °C min^{-1} to 330 ˚C across 14 min, with a final hold for 5 min at

330 ˚C. A temperature of 280 °C was used for the transfer line between the gas chromatograph and Leco Pegasus IV time of flight mass spectrometer (single mass analyzer). The measured mass range was 85-500 Da, scan rate was at 17 spectra s^{-1}, electron energy was 70 eV, ion source temperature was 250°C, and detector voltage was 1850 V. Spectra were acquired using the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI) and processed using the BinBase database system (Fiehn et al., 2005; Fiehn et al., 2010; Trigg et al., 2019), which quantifies (signal-to-noise ratio of 5:1) and matches mass peaks to the Fiehn mass spectral library using retention index (RI) and reference mass spectral information, which are internally compiled by the West Coast Metabolomic Center.

Statistical Analyses

Comparisons of the cultivation kinetic parameters were conducted using unpaired Student's t-tests and one- and two-way ANOVA analyses (Microsoft Excel), where statistical relevance was accepted at $p < 0.05$, and normal distribution of the data was supported by Shapiro-Wilk Tests (Past 4.10; Hammer et al. (2001)). Metabolomic data were compared using univariate, multivariate, and visual approaches. Discrete changes in the metabolomes (*e.g.*, trends for a single metabolite) were identified using unpaired Student's *t*-tests (Microsoft Excel) with corrections for multiple testing using a false discovery rate (FDR) of FDR ≤ 0.20 (Benjamini and Hochberg, 1995). For the 700 metabolites in the total study (whole cell and extracellular metabolomes from 2 bacterial strains treated with and without Kleenol 30), normal distributions (Shapiro-Wilk Tests) were indicated for ~92% (646) of the metabolites; however, metabolites exhibiting a $p > 0.03$ (Shapiro-Wilk Tests) *and* a $p < 0.05$ (Student's *t*-tests) were carried forward to account for potential underestimations of normality given the sample size (n = 3).

Broad structural and metabolic trends were obtained by visualizing the changes in the metabolomes ($p < 0.05$) using MetaMapp[1] and Cytoscape 3.9.1[2] (Barupal et al., 2012),

[1] http://metamapp.fiehnlab.ucdavis.edu/ocpu/library/MetaMapp2020/www/
[2] https://cytoscape.org/

respectively; these combined tools constitute a statistical organizational approach that yields visual maps of metabolites that are arranged by known structural patterns and metabolic pathways. Confirmation of broad changes in the metabolomes were obtained using ChemRich[3] (Barupal and Fiehn, 2017), a statistical enrichment tool that compares groups of metabolites based on structural and biochemical classes. For this study, the standard and user-defined classifications in ChemRich included amino acids, monosaccharides, sugar acids, organic acids, fatty acids, lipid-related metabolites, nucleotide-related metabolites, and compatible solutes. Lastly, multivariate tests (canonical correspondence analyses) were conducted to correlate the metabolomic trends with the cultivation parameters and cultivation conditions (Past 4.10; Hammer et al. (2001)).

[3] https://chemrich.fiehnlab.ucdavis.edu/