NMR-Based Microbial Metabolomics and the Temperature-Dependent Coral Pathogen *Vibrio coralliilyticus*

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Abstract

Coral bleaching occurs when the symbioses between coral animals and their zooxanthellae is disrupted, either as part of a natural cycle or as the result of unusual events. The bacterium *Vibrio coralliilyticus* has been linked to coral disease globally (for example in the Mediterranean, Caribbean, Red Sea and Indian Ocean), and, like many other *Vibrio* species, exhibits a temperature-dependent pathogenicity. The temperature-dependence of *V. coralliilyticus* in regard to its metabolome was investigated. Nuclear magnetic resonance (NMR) spectra were obtained of methanol-water extracts of intracellular metabolites (the endo-metabolome) from multiple samples of the bacteria cultured into late stationary phase at both 27 °C (when this organism exhibits virulence) and 24 °C (an avirulent form). The spectra were subjected to principal components analysis (PCA), and significant temperature-based separations in PC1, PC2, and PC3 dimensions were observed. Betaine, succinate, and glutamate were identified as metabolites that cause the greatest temperature-based separations in the PC scores plots. With increasing

temperature, betaine was shown to be down regulated while succinate and glutamate were up regulated.

Brief

NMR was used to examine the metabolome of the temperature-dependent bacterial coral pathogen *Vibrio coralliilyticus* that causes coral lysing at elevated temperatures.

Introduction

Vibrio coralliilyticus has been identified as a temperature-dependent coral pathogen and linked to coral bleaching and lysing.[1-3] Bleaching of coral results from a disruption of symbiosis with its zooxanthellae[4] and is reversible with repopulation by zooxanthellae.[5, 6] Corals experience patterns of bleaching events due to seasonal fluctuations in seawater temperature.[5] Coral bleaching usually occurs during the warmer months of the year; however, corals have the ability to adapt to these increases in sea water temperature via thermally tolerant symbiotic partners.[6] Still, a growing concern is that the corals are not able to adapt to accelerated rates of temperature change, and this can prevent recovery of the bleached corals to their healthy state.[6] At temperatures of 25 °C and above, the Gram-negative bacterium *V. coralliilyticus* was found in high concentrations in the bleached coral *Pocillopora damicornis* (collected from the Red Sea and the Indian Ocean).[1] When inoculated into healthy laboratory-cultured corals at temperatures above 24.5 °C, *V. coralliilyticus* caused bleaching, and at 27 °C and higher, caused lysing. At temperatures of 24 °C and below, inoculation of the bacteria into the corals caused neither bleaching nor lysis. A relationship between elevated temperature and virulence of *V. coralliilyticus* has been hypothesized[1, 7] and is the object of active research around the world.[2, 3, 8, 9] Our objective is to characterize the effect of temperature on metabolic changes in *V.*

coralliilyticus in order to contribute to our understanding of this organism's temperature-dependent pathogenicity. Nuclear magnetic resonance (NMR)-based metabolomics was employed to address this question.

The goal of this work was to study the endogenous low molecular weight metabolites extracted from *V. coralliilyticus* cells which is an environmental stressor to corals at elevated temperatures[1, 7, 10, 11] using NMR-based metabolomics. The use of the metabolomics approach to address environmental issues holds great promise for environmental risk assessment[12] and discovery related to systems biology and the biology of numerous organisms.[11, 13] NMR-based metabolomics is successful in these areas because of a combined use of an unbiased, quantitative analytical method and multivariate statistical tools.[14] Proton (^{1}H) NMR is an extremely useful analytical technique in that hundreds of metabolites can be detected in a relatively short experimental time, and it is commonly used for metabolite identification and quantization within complex biological samples.[10, 11] This non-destructive and cost-effective method provides spectra that offer a snapshot of the metabolic state of the organism at the time of sampling, and because of the capacity for high-throughput, the metabolome can be sampled at various times during stressful events.[14] Although NMR is a relatively non-sensitive technique, its spectra are still affected by biological variability introduced during organism culture. Efforts to reduce or control the variability in metabolomics experiments are of utmost importance because of the complexity of a typical ^{1}H NMR spectrum. Peaks can be obscured because of biological or analytical variability thus making interpretation of the spectra difficult.[14] Principal components analysis (PCA) is an unsupervised pattern recognition approach that is often used in metabolomics to detect correlated patterns in the data which could then be further interpreted by identifying

specific resonances (or metabolites) causing the patterns. PCA often shows separations of groups of data caused by up/down regulation of groups of metabolites due to a stressor.[15]

Experimental Section

Sample Preparation: cell growth, metabolism quenching, and cell collection. *V. coralliilyticus* (ATCC BAA-450) cells were inoculated onto two glycerol artificial seawater (GASW)[16] agar plates. GASW contained 20.8 g NaCl; 0.56 g KCl; 2.34 g $MgSO_4$; 4.0 g $MgCl_2 \cdot 6H_2O$; 0.009 g K_2HPO_4; 0.0008 g $FeSO_4 \cdot 7H_2O$; 2.0 g yeast extract; 2.0 g glycerol; and 1.0 L deionized water. One plate was grown at 24 °C and the other at 27 °C for 24 h in the dark. Six colonies from each plate were used to inoculate 25mL of fresh GASW media and grown at their respective temperatures for 24 h in the dark. Then, a 4% transfer was performed of the culture into fresh GASW media (96 mL GASW media + 4 mL *V. coralliilyticus* culture) for each temperature regime, and grown in the dark for an additional 14 h. The culture was then centrifuged at 8000 g for 15 min, and the supernatant decanted. The cells were re-suspended in fresh GASW media to an OD_{610} between 2.3 and 2.5. Using this re-suspended culture, a 1% transfer was performed where 0.50 mL of the re-suspended *V. coralliilyticus* culture was added to 49.50 mL GASW media. Six samples were grown at each temperature until the cell cultures reached the late stationary phase (24 h). To harvest the cells, metabolic quenching was performed through rapid temperature reduction by adding approximately 15mL of liquid nitrogen to each 50mL final growth flask. The quenched cell growths were centrifuged at 12,000 g for 8 min at 4 °C, and the pelleted cells were washed with 10 mL 3% (w/v) NaCl (30 g NaCl in 1000 mL deionized H_2O). The salty cell solution was centrifuged again at 12,000 g for 8 min at 4 °C and the cell pellets transferred to microcentrifuge tubes. Next, the cell pellets were

washed with 1 mL 3% (w/v) NaCl and centrifuged at 16,100 g for 1 min in a microcentrifuge. The final cell pellets were flash frozen in liquid nitrogen and stored at -40 °C for 3 h. Finally, the cells were lyophilized overnight and stored at -40 °C until extraction.

Sample Preparation: endometabolite extraction. Metabolites were extracted[17-19] from the lyophilized *V. coralliilyticus* cells with hot 2:1 (v/v) MeOH:H_2O at a constant cell mass/solvent ratio of 24 mg of lyophilized cells and 1.2 mL of MeOH:H_2O. The solutions were vortex-mixed, and then centrifuged at 10,500 g for 5 min at 8 °C to remove cell debris. The supernatants containing the small molecule metabolites were then dried using a vacuum centrifuge drier (Eppendorf® Vacufuge™ , Westbury, NY) for 2.5 h at 30 °C, then no heat for the remaining time to get gelatinous, concentrated extracts. The extracts were re-suspended in 600 μL of NMR buffer (0.2 mol/L sodium phosphate, 1 mmol/L TMSP (internal standard, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, CAS: 24493-21-8, Aldrich 269913-1G), 1 mmol/L NaN_3 (CAS: 26628-22-8, Aldrich 428456-5G; for prevention of bacterial growth) in 99.9 atom % D_2O), then vortex-mixed and centrifuged. The supernatants were transferred into 5 mm NMR tubes.

NMR Data Acquisition. One-dimensional high-resolution ^1H NMR spectra were acquired on a Bruker DMX 500 MHz spectrometer equipped with a room temperature probe and a Bruker Avance II 700 MHz spectrometer with a TXI CryoProbe™. All data were collected at a temperature of 305 K using a three-pulse sequence based on the noesy1dpr pulse sequence in the Bruker pulse sequence library. This pulse sequence provided water-suppression with good baseline characteristics. Experiments were run with 16 dummy scans and 80 acquisition scans with an acquisition time of 4.68 s and a relaxation delay of 3.0 s for a total repetition cycle of 7.68 s. The mixing time was 100 ms. The spectral width was 14.0 ppm and 65536 real data

129 points were collected. Each sample analysis took about 10 min for setup and 12 min for
130 acquisition. During the 10-min setup time, the temperature was monitored for equilibration. All
131 free induction decays (FIDs) were subjected to an exponential line-broadening of 0.3 Hz. Upon
132 Fourier Transformation, each spectrum was manually phased, baseline corrected, and referenced
133 to the internal standard TMSP at 0.0 ppm using Topspin 2.1 software (Bruker Analytik,
134 Rheinstetten, Germany).

135 **Metabolite Identification.** Metabolites present in the samples were identified using comparisons
136 between processed 1D ^{1}H NMR spectra and libraries of standard spectra, one developed in-house
137 and SBASE-1-1-2 from AMIX (version 3.8.3, Bruker Biospin GmbH). Some metabolites were
138 identified using selective TOCSY experiments.[20] A ^{13}C (zgpg30) and a HSQC spectrum
139 (hsqcedetgpsisp2.2 Bruker pulse sequence) of a representative *V. coralliilyticus* sample provided
140 carbon chemical shifts and H-C correlations that could then be compared to appropriate libraries
141 (such as the MMC Database[21] and KEGG[22]) of spectra.

142 **Data Analysis.** Spectra were bucketed (from 9.5 to 0.5 ppm) using AMIX software. A bucket
143 size of 0.005 ppm was used. Water (4.82 – 4.63 ppm) and a peak at 3.3 ppm which could be an
144 inadvertent methanol contaminant or the methyl groups of betaine (3.29 – 3.24 ppm) were
145 excluded from every bucket table. All data was mean-centered before PC analysis, and no other
146 scaling was found necessary.

147 **Results and Discussion**

148 NMR-based metabolomics has been utilized to understand the effects of temperature on
149 the metabolome of *V. coralliilyticus*. We have taken care to reduce the analytical variability of
150 our data via multiple trials, repeated extractions and repeated data processing by multiple
151 individuals. Data from two trials are reported here. One trial was completed in 2007 and resulted

in two successful batches, and another trial was completed in 2008 resulting in two successful batches. Each batch consists of five or six bacterial cultures at each of the two temperatures. The extraction procedure beginning with the lyophilized cell material from the two 2008 batches was performed twice, at two different times, but with the identical extraction procedure, to test for repeatability of the extraction procedure and NMR data collection. The same procedure (except for one known exception discussed below) was followed for growth, quenching, and extraction executed in each trial involving two different groups of people in 2007 and 2008.

Pattern Recognition and Statistical Analysis. PCA was used to analyze the *V. coralliilyticus* spectra to observe patterns in the scores data, and loadings were used to identify specific resonances (or metabolites) causing the patterns. The analysis proceeded with comparisons within the batches, based on temperature (intra-batch) and then comparisons between batches (inter-batch). The intra- and inter-batch PCA results for the 2007 and 2008 data are visualized with scores and 2D loadings plots shown in Figures 1 and 2. The samples from both 2008 batches were re-extracted and are represented with "R" appended to the name.

Intra-batch comparisons. Student's t-tests for significant differences in intra-batch temperature comparisons showed that there is significant separation in PC1 and often in PC2 or PC3 dimensions (see Table 1). Loadings plots show that the intra-batch separations of B01_2007, and B03_2008 were largely due to variability in betaine, succinate, and glutamate peak intensities (Figure 2). Intra-batch separations in B02_2007 and B04_2008 are caused by mostly the same metabolites except that lactate intensities contribute more significantly to the loadings (~1.30 ppm). Also, the overall separation is smaller in B04_2008 compared to the separations in B01_2007, B02_2007 or B03_2008.

The cell growth procedure described in the Experimental Section was followed for both years except that in the 2007 procedure, the 27 °C cell cultures were grown in the presence of incidental light through the glass in the top of the shaker. This could have introduced some intra-batch biological variability within the 2007 data; therefore, the procedure was changed in 2008 so that the cell cultures at both temperatures were grown consistently in the absence of light. In addition to the possible introduction of biological variability in the 2007 data, some inconsistency was possible when conducting inter-batch comparisons with the 2008 data. This could be important if there were interactions of media or metabolites with light; however, comparisons of PC loadings plots (Figure 2) for the 27 °C spectra from each year indicate that the variations in spectra are due to the same metabolites.

Inter-batch comparisons. In the PC scores plots (Figure 1), the groupings of batch-to-batch (or year-to-year, not shown) data lead to the conclusion that there is some variation between the batches which we have not been able to control. Indications point to fundamental biological or culture-condition variability or end-point variation in the growth process in the *V. coralliilyticus* data. The NMR spectra and statistical data generated from B03_2008 and B04_2008 and the re-extracted B03R_2008 and B04R_2008 have been generated in a repeatable fashion following the extraction protocol. Figure 1 (middle and bottom) shows the PC1 vs. PC2 scores plot of all of the 2008 spectra and their re-extracted counterparts. The respective groups of data are not significantly different (except for B03/R_2008_24 in PC2 with a Student's t-test p-value of 0.034, while all other groups have p-values greater than 0.05) indicating that the re-extraction and NMR collection procedures were performed with minimal introduction of variability.

Metabolite Profiling and Identification. A representative ^{1}H NMR metabolite profile is shown in Figure 3 with peak annotations of some of the polar metabolites based on our identification protocol. The metabolites betaine, succinate, glutamate, and lactate are responsible for the separations in principal component space in intra-batch comparisons with the 2D loadings being heavily weighted with betaine and succinate in PC1 and PC2. The betaine peak at 3.27 ppm does not contribute to the loadings because it is in an excluded region (3.29 – 3.24 ppm).

Betaine, the most commonly identified osmolyte in plants, mammals, and bacteria,[23, 24] has peaks at 3.27 ppm and 3.90 ppm, which are the two most intense peaks in the ^{1}H NMR spectra of *V. coralliilyticus*. Osmoprotectants aid the cells in reestablishing turgor pressure in the event of increased osmolality.[23, 25] Most organisms are unable to synthesize betaine *de novo* and rely on transport mechanisms such as ProP and ProU which are in *E. coli* cells.[26-30] These two transport systems are capable of transporting proline, betaine, carnitine, ectoine, and choline into the cell.[23, 26-30] *Vibrio* species such as *Vibrio parahaemolyticus* have been reported to utilize these transport mechanisms for importing betaine and ectoine.[31] Even though some *Vibrio* species have synthesis systems for these osmolytes,[31, 32] uptake from the environment is the preferred method.[32] Another osmoprotectant that causes separations in the 2D loadings plot is glutamate with ^{1}H NMR peaks (multiplets) around 2.35 ppm and 3.77 ppm. Among the many metabolic pathways in which glutamate is involved, in *E. coli*[33] and *Vibrio costicola*[34] cells, it is synthesized to balance the K^{+} uptake from the media. The media used for *V. coralliilyticus* cell growth contains K^{+}, and ^{1}H NMR spectra of the fresh media indicate the presence of betaine (data not shown) suggesting that betaine is preferably transported into the *V. coralliilyticus* cells from the media, and synthesis of glutamate is in response to the accumulation of K^{+} from the media, among other possibilities. Betaine, succinate, and glutamate peak intensities are

219 dependent on the temperature at which the *V. coralliilyticus* cells were grown. Betaine peaks
220 decrease in intensity at the higher temperature (27 °C) while the opposite is true for glutamate
221 and succinate; therefore, betaine is down regulated and glutamate and succinate are up regulated
222 with increasing temperature. According to the KEGG compound database, glutamate is present
223 in 18 metabolic pathways, succinate is in 13, and betaine is in two. Glutamate is involved in
224 various pathways including the urea cycle and various metabolisms of nitrogen and amino
225 groups. Succinate is most notably involved in the citric acid cycle while betaine is used in the
226 ABC transport mechanism. All three metabolites aid in the production or metabolism of various
227 amino acids.[22]

228 In conclusion, intra-batch temperature-dependent separation in PCA was consistently
229 observed. The separations in the PCA scores plots indicate metabolomic differences between the
230 virulent (27 °C) and threshold-of-virulency (24 °C) forms of *V. coralliilyticus* based on betaine,
231 succinate, glutamate, and other traces. However, the inter-batch reproducibility showed
232 substantial variability. Re-extraction of the 2008 batches, instrumental reanalysis, and multiple
233 exclusions in the PCA analysis were attempted to identify systematic errors which may have
234 contributed to the inter-batch variability. Factors such as instrument variation, baseline or
235 referencing errors, extraction errors or incidental chemical contamination, were not implicated in
236 the variability. Therefore, we believe there was significant biological variability during the
237 growth of *V. coralliilyticus*. The biological variability could be explained by collecting samples
238 along the growth curve of *V. coralliilyticus* which will introduce the dimension of time, or
239 observing the metabolome of individual colonies of *V. coralliilyticus* to identify any phenotypic
240 variations. Other experiments that would further explain the temperature-dependent changes in
241 the metabolome would be the induction of a temperature change during cell growth,[35] or

collection of metabolomic data for *V. coralliiltyicus* at other temperatures. Using NMR-based metabolomics in conjunction with a robust statistical analysis tool (PCA), several metabolites which are involved in osmo-regulation and energy production pathways in *V. coralliilyticus* were shown to have changed in correlation to temperature. Therefore, this research has demonstrated temperature-dependent metabolomic changes in *V. coralliilyticus* and provides new insight into the temperature-dependent virulency of this organism.

Disclaimer. Commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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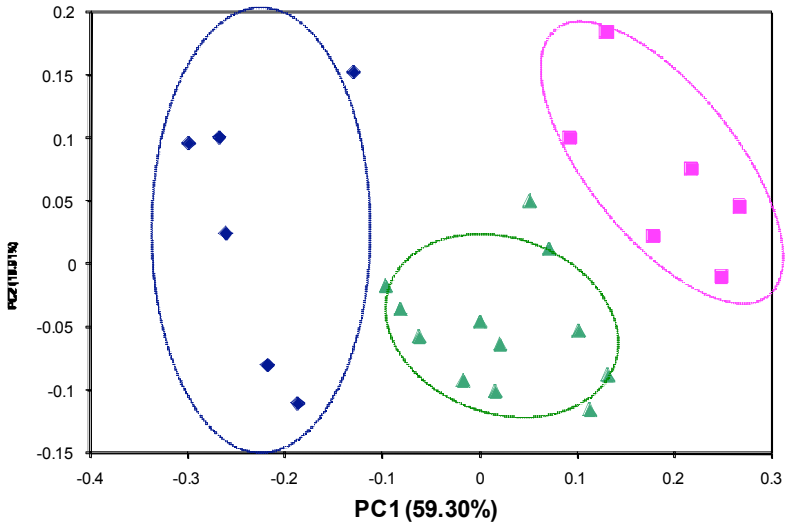

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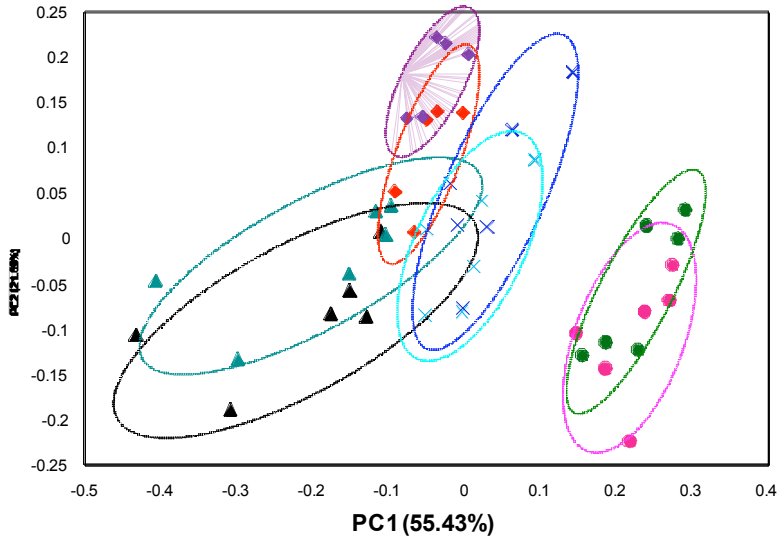

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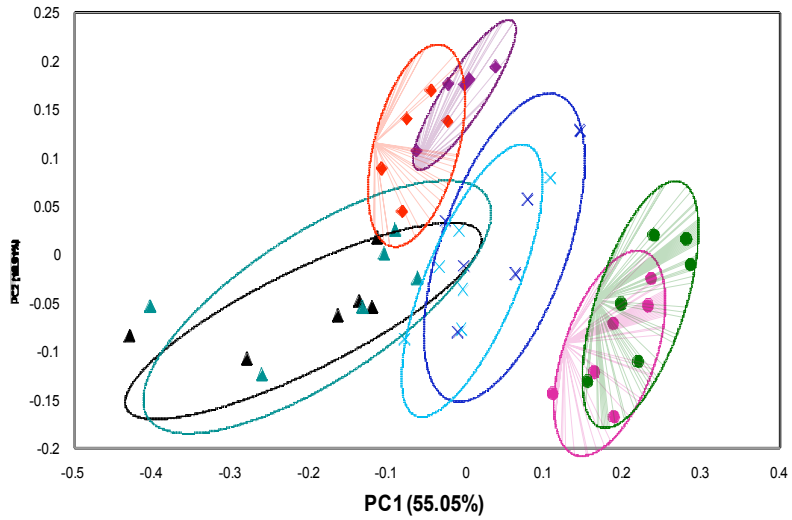

Figure 1. (top) 500 MHz PC1 vs. PC2 scores plot showing both temperatures of B01_2007 (24: dark blue diamond; 27: green triangle) and B02_2007 (24: pink square; 27: green triangle) spectra, and (middle) 500 MHz and (bottom) 700 MHz PC1 vs. PC2 scores plot showing both temperatures of B03_2008 (24: red diamond; 27: pink circle), B03R_2008 (24: plum diamond; 27: green circle), B04_2008 (24: black triangle; 27: light blue X), and B04R_2008 (24: blue-green triangle; 27: dark blue X) spectra. Ovals represent 60% Hotellings T2 confidence intervals. Separations between spectra based on temperature (intra-batch) are visibly evident by groupings and indicate fundamental differences between the virulent and non-virulent *V. coralliilyticus* samples. Separations between batches (inter-batch) are attributed to biological variability during cell culture. The 2008 re-extracted spectra (denoted with "R") overlay with the corresponding, originally extracted spectra indicating lack of variability in the endometabolite extraction procedure. Calculated p-values do not indicate significance in separations between the originally and re- extracted groups in either PC (with the exception of B03_24/R in PC2). 700 MHz data provides increased signal to noise ratios.

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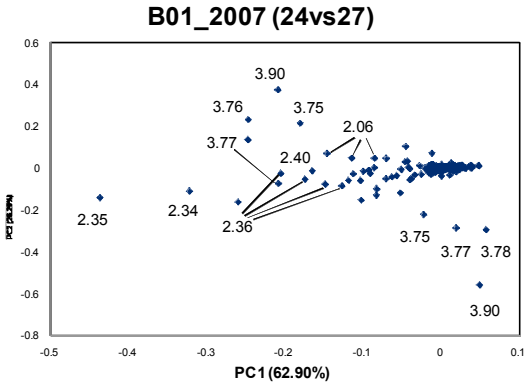

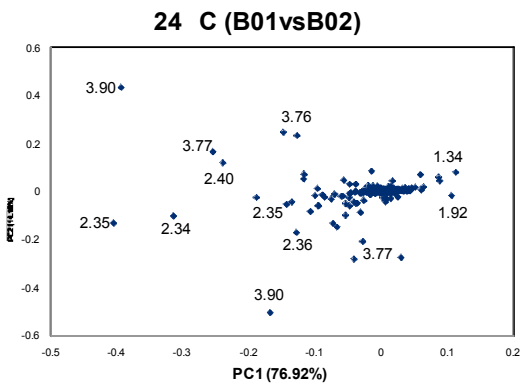

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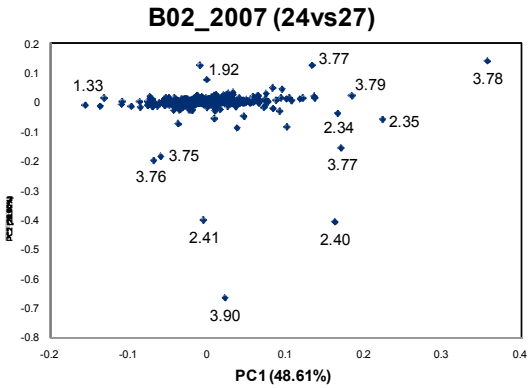

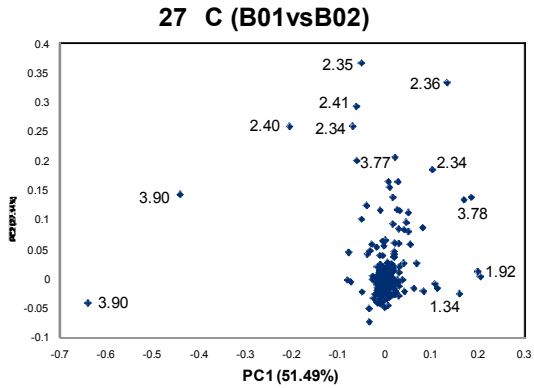

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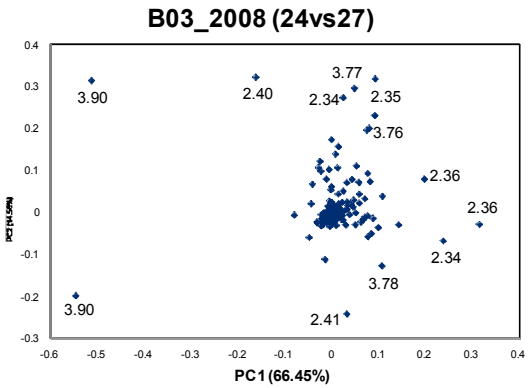

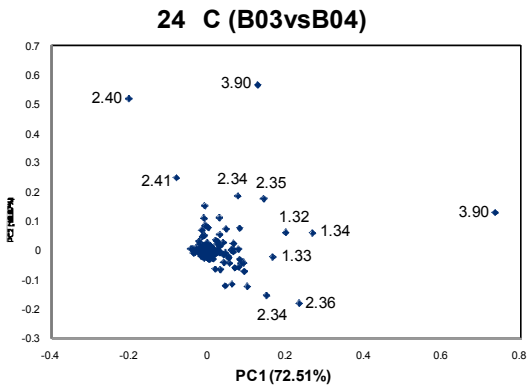

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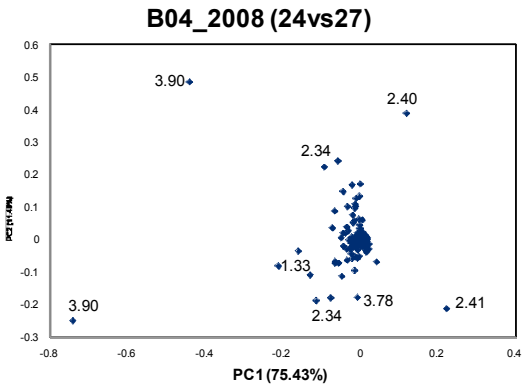

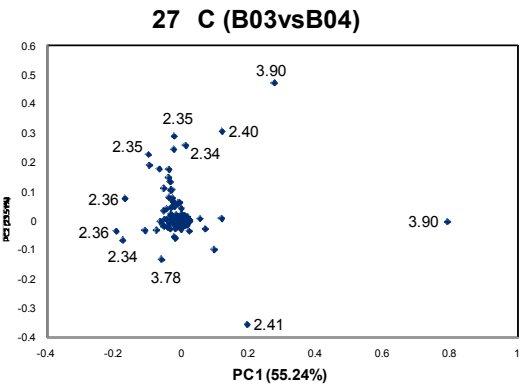

Figure 2. 2D loadings plots of 2007 and 2008 intra- (left) and inter-batch (right) comparisons. Loadings represent betaine (3.90 ppm), succinate (2.40 ppm), glutamate (2.10, 2.35, and 3.77 ppm), acetate (1.92 ppm), and lactate (1.30 ppm). These loadings in PC1 and PC2 space explain the majority of the variance between the respectively compared groups with the lowest combined explained variance being 77.51% for B02_2007 (24vs27).

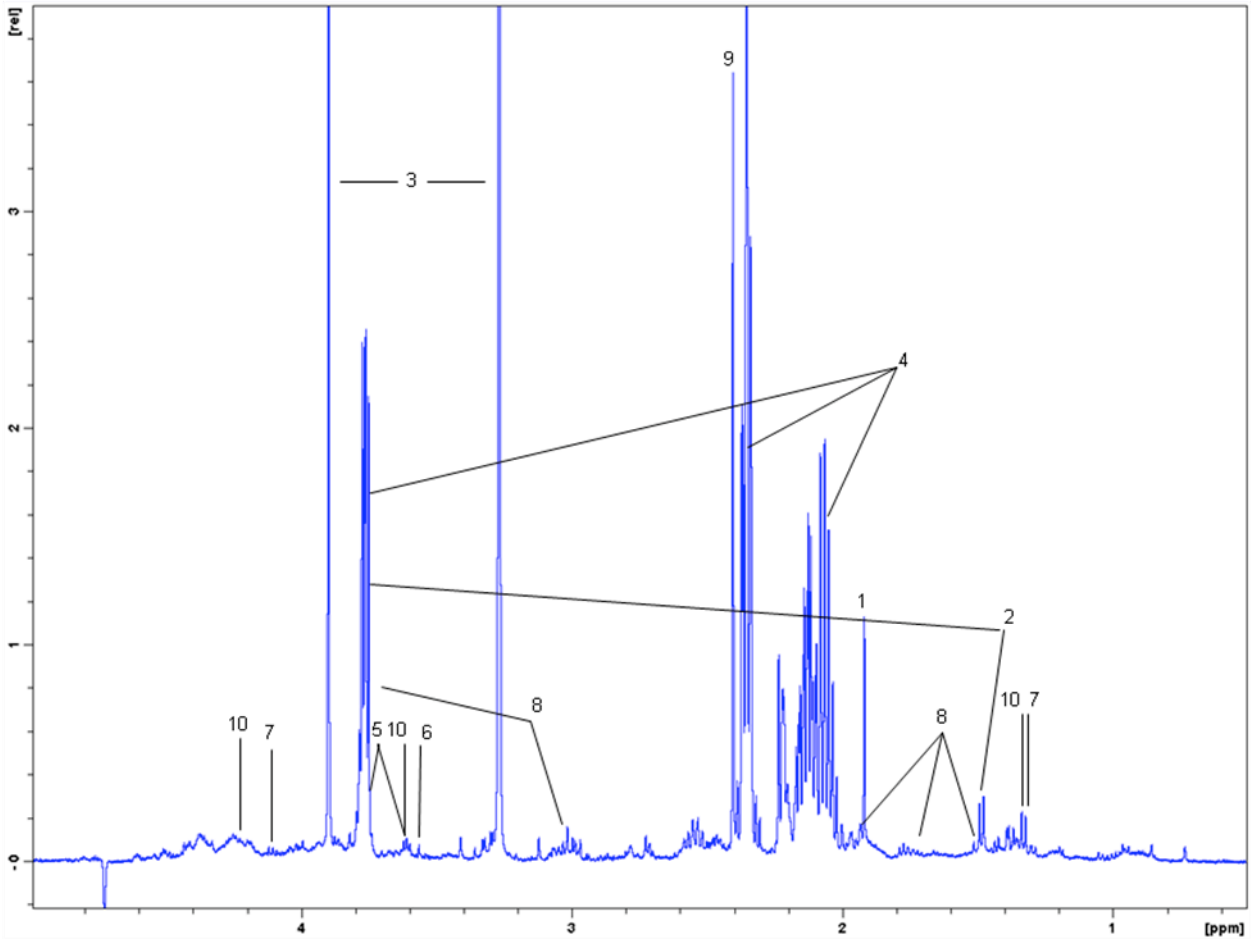

Figure 3. A representative (batch 03, 24 °C) ^{1}H NMR metabolite profile of *V. coralliilyticus* with some peak assignments. Key to spectrum: 1. acetate, 2. alanine, 3. betaine, 4. glutamate, 5. glycerol, 6. glycine, 7. lactate, 8. lysine, 9. succinate, and 10. threonine.

Table 1. p-Values* Calculated from Student's T-Test for Temperature Comparison.

Temperature Comparison of Batches (24 °C vs. 27 °C)**	PC1	PC2	PC3
B01_2007	**<0.001**	0.30	**<0.001**
B02_2007	**0.0067**	**0.0021**	0.50
B03_2008	**<0.001**	**<0.001**	0.23
B03R_2008	**<0.001**	**<0.001**	0.44
B04_2008	**0.0059**	0.075	0.55
B04R_2008	**0.0051**	0.12	0.17

* p-values are calculated using Student's two-tailed t-test assuming unequal variances, with $\alpha = 0.05$.

** Significance in separations is defined for values less than 0.05 and are shown in bold.

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