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Application of FT-IR Spectroscopy for Fingerprinting of *Zymomonas mobilis* Respiratory Mutants

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Abstract. *Z. mobilis* ATCC 29191 and its respiratory knockout mutants, *kat-, ndh-, cytB-,* and *cydB-*, were grown under anaerobic and aerobic conditions. FT-IR spectroscopy was used to study the variations of the cell macromolecular composition. Quantitative analysis showed that the concentration ratios—nucleic acids to lipids, for *Z. mobilis* parent strain, *kat-, ndh-, cytB-,* and *cydB-* strains, clearly distinguished *Z. mobilis* parent strain from its mutant derivatives and corresponded fairly well to the expected degree of biochemical similarity between the strains. Two different FT-IR-spectra hierarchical cluster analysis (HCA) methods were created to differentiate *Z. mobilis* parent strain and respiratory knockout mutant strains. HCA based on discriminative spectra ranges of carbohydrates, nucleic acids, and lipids allowed to evaluate the influence of growth environment (aeration, growth phase) on the macromolecular composition of cells and differentiate the strains. HCA based on IR spectra of inoculums, in a diagnostic region including the characteristic nucleic acid vibration modes, clearly discriminated the strains under study. Thus it was shown that FT-IR spectroscopy can distinguish various alterations of *Z. mobilis* respiratory metabolism by HCA of biomass spectra.

Keywords: FT-IR spectroscopy, *Zymomonas mobilis*, respiratory mutants, HCA, oxidative stress

1. Introduction

Discrimination between different strains of microorganisms can be based on the whole organism biochemical fingerprinting. For that purpose, Fourier-transform infrared (FT-IR) spectroscopy is one of the methods of choice, proven to be efficient for quantitative analysis of the cell macromolecular composition. FT-IR spectroscopy is a time- and chemicals-saving biophysical method that enables characterization, screening, discrimination, and identification of intact microbial cells or cell components. Main advantages of this whole-organism fingerprinting method are small sample amounts, and simple, time-saving sample preparation without chemical pretreatment, allowing to obtain real-time information about the macromolecular composition of cells.

It has been shown that FT-IR spectroscopy has a sufficient resolution power to distinguish between single-gene knockout mutants in yeast [1], *Bacillus subtilis* and its gerD and gerA mutants [2], well-defined discrimination of different phenotypes of *Staphylococcus aureus* in liquid media for diagnostic and research purposes [3]. FT-IR microspectroscopy of leaves was used to develop a rapid

method for screening of mutant plants for a broad range of cell wall phenotypes [4] and to identify different classes of *Arabidopsis* mutants [5]. Lately FT-IR spectroscopy was used to study the bacterium *Enterobacter cloacae* and several of its biofilm mutants [6].

For certain reasons, respiratory mutants of the Gram-negative bacterium *Zymomonas mobilis* might represent special interest for FT-IR analysis and strain fingerprinting. *Zymomonas mobilis* is a facultatively anaerobic, obligately fermentative bacterium with a highly active ethanol fermentation pathway. At the same time, it possesses aerobic respiratory chain, supporting high oxygen uptake rates. Due to still unknown mechanisms, respiration in this bacterium is poorly coupled to ATP synthesis [7]. Recently, we have constructed a type-II NADH dehydrogenase knockout strain (*ndh-*) [8], a knockout of the cytochrome b subunit of the bc_1 complex (*cytB-*), a knockout of the subunit II of *bd* terminal oxidase (*cydB-)*, and catalase knockout (*kat-*) strain [9]. All these mutant strains are able to grow under aerobic conditions, but have distinct alterations of respiratory metabolism to various degrees. All the mutant strains show clear indications of oxidative stress: relative to the parent type they have 3-4 times upregulated transcription of superoxide dismutase, as measured by quantitative RT-PCR [9]. Apparently, the mechanism of oxidative stress should be different for each strain, as far as the alterations of respiratory metabolism differ. Macromolecular components, analyzed by FT-IR spectroscopy, like cell membrane lipids, proteins, and nucleic acids, are the primary molecular targets of reactive oxygen species during oxidative stress [10]. Hence, our aim was to investigate the macromolecular composition of these respiratory mutants by FT-IR spectroscopy, to see if it is possible to discriminate various alterations of respiratory metabolism by hierarchical cluster analysis of biomass spectra.

2. Materials and Methods

Z. mobilis ATCC 29191, the type-II NADH dehydrogenase knockout strain (*ndh-*) [8], the knockout of the cytochrome b subunit of cytochrome bc_1 complex (*cytB-*), the knockout of the subunit II of *bd* terminal oxidase (*cydB-)*, and catalase knockout (*kat-*) strain [9] were constructed, by disruption of the respective genes with the chloramphenicol-resistance determinant, using homologous recombination [8, 9]. Inoculum biomass was grown under microaerophilic conditions in liquid medium with glucose for 24 h. The batch cultures were grown under anaerobic and aerobic conditions in plastic 20 mL test-tubes and in 50 mL glass flasks, respectively, and aerobic cultures were stirred at 200 rpm. Growth medium contained 20 g/L glucose, 5 g/L yeast extract and mineral salts, pH 6 and temperature +30°C. Biomass samples were collected at exponential (7 h) and stationary (24 h) growth phases. Cells were washed twice with distilled water and centrifuged. FT-IR analysis was performed using 5–15 μL of washed cell water suspension poured out by drops on a silicon plate and dried at $T < 50$°C. Absorption spectra were recorded on an HTS-XT microplate reader (Bruker, Germany) over the range 4000–400 cm^{-1}, with a resolution of 4 cm^{-1}. Quantitative analysis of carbohydrates, nucleic acids, proteins, and lipids in biomass was carried out as in [11]. Data were processed with OPUS 6.5 software. Hierarchical cluster analysis (HCA) was used to create dendrograms from *Z. mobilis* and its knockout mutant IS absorption spectra using Ward's algorithm.

3. Results and Discussion

FT-IR spectra of *Z. mobilis* ATCC 29191 parent type and its mutant biomass grown under aerobic or anaerobic conditions were analyzed by quantitative analysis and HCA.

Comparison of recorded absorption spectra showed changes of band profiles in spectral regions 1140–1110 and 1665–1645 cm^{-1}, thus indicating differences in the macromolecular composition of parent and mutant cells. Bands at 1183 (P=O; C–O), 1095 (P=O; carbohydrates), 1108, 1515 (protein, NH_3^+ of α-amino groups, tyrosine), 1521 (NH_3^+ of side-chain amino groups), 1660 and 1657 cm^{-1} (Amide I, β-sheet) changing the shape and/or intensities were used for discrimination [12]. The shift between 1515 and 1521 cm^{-1} specifies the changes in cell protein composition depending on the growth conditions—anaerobic/aerobic, exponential/stationary phase.

Quantitative analysis of all samples was done to gain data on the carbohydrate, nucleic acid, protein, and lipid concentrations in biomass (data not shown). Variations of concentrations were not wide (2–8% depending on a component), yet well-expressed analysis of these data showed that the cell macromolecular composition depends on the growth conditions. In all strains lipid concentrations were higher under aerobic growth conditions than anaerobic growth. For example the content of lipids in *Z. mobilis* parent strain cells was 4% dry weight (DW) and 2% DW under aerobic and anaerobic growth conditions correspondingly. It is known that the increase of lipid content is one of cells responses to stress. The content of total carbohydrates was higher in mutant strain cells and was influenced by growth conditions (+/− oxygen, 7 or 24 h). For example, the carbohydrate concentration in *cydB-* mutant at exponential phase (after 7 h) under aerobic environment was 21% DW but under anaerobic conditions 17% DW. Data analysis of quantitative results showed that discrimination of parent and mutant strains can be based on nucleic acid and lipid concentrations in inoculum's cells. As the concentrations of nucleic acids and lipids in parent and mutant inoculums biomasses were in various proportions, their ratio was used for the strain differentiation. Nucleic acid to lipid concentration ratios for *Z. mobilis* parent strain, *kat-*, *ndh-, cytB-*, and *cydB-* strains were 6.95, 4.42, 5.33, 5.16, and 5.13 ($\pm$0.3) correspondingly. Notably, the values of this ratio clearly distinguished Z. *mobilis* parent strain from its mutant derivatives and corresponded fairly well to the expected degree of biochemical similarity between the strains. Thus, the catalase knockout strain differed from all the respiratory chain mutants. There was more similarity found between *cytB-* and *cydB-* strains, each with partially disrupted electron transport, than between either of them and *ndh-*, having near-zero respiration rate.

Next step was to create HCA method for differentiation of *Z. mobilis* parent and knockout mutant strains. It was established that vector normalized, 2nd-derivative spectra in three spectral ranges 1185–950, 1483–1360, and 3022–2832 cm^{-1}, were functional for HCA of all samples at various growth conditions and phases. This dendrogram clearly showed two distinct clusters of aerobically and anaerobically grown strains. All inoculum samples of parent and knockout mutant strains were clearly discriminated and formed one subcluster. These results showed that the biochemical composition of cells is influenced and can be changed by choosing appropriate fermentation conditions.

Since the dendrogram of the above mentioned HCA and quantitative analysis data discriminated *Z. mobilis* parent and knockout mutant strains even using the spectra of inoculums, another HCA method was created. The second derivative inoculums spectra were analyzed in several regions: 1665–1645, 1544–1510, 1301–1086, 1220–1174, and 1120–1086 cm^{-1} to choose the diagnostic peaks or regions for HCA. As characteristic were chosen two regions: 1120–1086 and 1301–1086 cm^{-1}, and as diagnostic region was selected 1301–1086 cm^{-1} including the characteristic nucleic acid vibration modes. HCA dendrogram (Figure 1) clearly shows discrimination between *Z. mobilis* parent and knockout mutant strains, difference between *kat-* and respiratory mutants, differences between respiratory mutants and the similarity of *cydB-* and *cytB-* mutants.

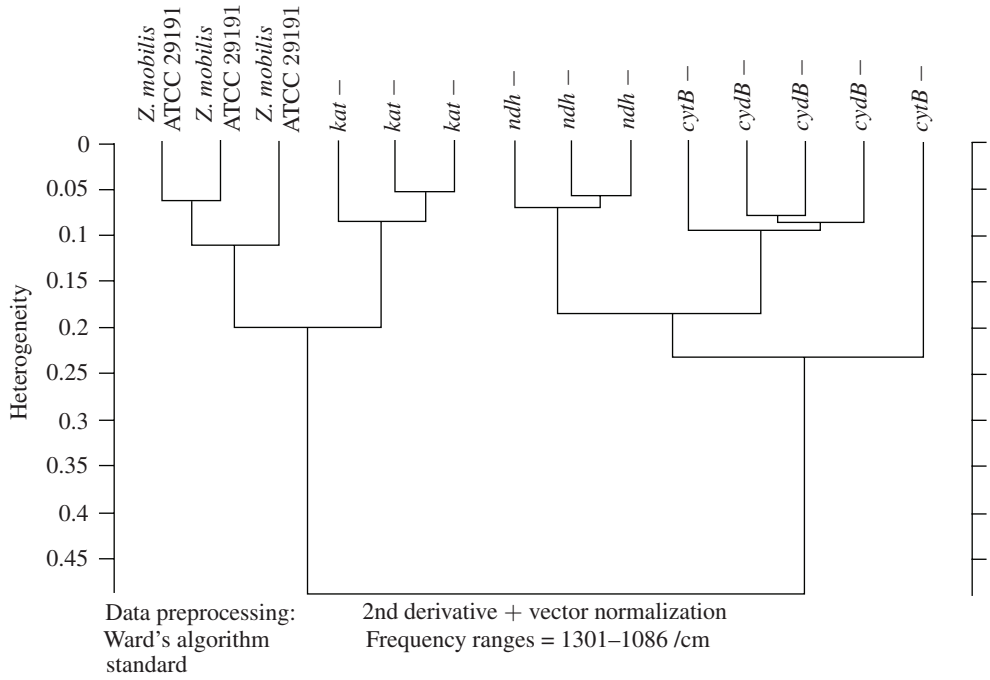

Figure 1: HCA of *Z. mobilis* ATCC 29191 and its knockout mutant inoculums spectra of 3 folds experiment (Ward's algorithm, Vector normalization, 2nd derivative, region 1301–1086 cm^{-1}).

These results are in agreement with quantitative analysis data and estimated from the mutant construction.

4. Conclusions

FT-IR quantitative analysis showed variations of the cell macromolecular composition depending on the strain peculiarities and growth conditions. Discrimination of *Z. mobilis* ATCC 29191 parent strain and its knockout mutant strains can be based on the ratio of nucleic acid to lipid concentrations. HCA showed to be effective for Z. *mobilis* ATCC 29191 parent strain and respiratory knockout mutant strain discrimination on the basis of inoculum IR-spectra and indicated the influence of growth environment (aeration, growth phase) on the macromolecular composition of cells.

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