Integrated Proteomics and Metabolomics reveal altered metabolic regulation of *Xanthobacter autotrophicus* under electrochemical water-splitting conditions

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ABSTRACT

Biological-inorganic hybrid systems are a growing class of technologies that combine microorganisms with materials for many purposes including chemical synthesis, environmental remediation, and energy generation. Recently, hybrid systems have been developed toward the sustainable generation of value-added chemicals from the plentiful and potentially renewable resources of electricity, water, and air. These hybrid systems typically consider microorganisms as catalysts that essentially perform only the reaction of interest, however other metabolic activity may influence that reaction and thus the output of the entire system. This possibility renders the investigation of biological responses to the hybrid environment critical to future system development and optimization. The present study investigates this phenomenon in a recently reported hybrid system that uses electrochemical water-splitting to provide reducing equivalents to the nitrogen-fixing bacteria *Xanthobacter autotrophicus* for efficient reduction of N_2 to biomass that may be used as fertilizer. Using integrated proteomic and metabolomic methods, we find a pattern of differentiated metabolic regulation under electrochemical water-splitting (hybrid) conditions. We further report an increased expression of proteins of interest, namely those responsible for nitrogen fixation and assimilation, which indicate increased rates of nitrogen fixation and support previous observations of faster biomass accumulation in the hybrid system compared to typical planktonic growth conditions. This work complicates the inert catalyst view of biological-inorganic hybrids while demonstrating the power of multi-omics analysis as a tool for deeper understanding of those systems.

INTRODUCTION

The integration of microorganisms with inorganic materials has recently attracted attention for demonstrating the synergy of biological and abiotic processes for a variety of chemical transformations. [1-7] Transformations of particular interest include environmental remediation[5, 8], the growth of biomass as biofertilizer, [9] and the production of valuable chemical products including ammonia[7, 10, 11] and biofuels[12, 13]. Many examples of these biological-inorganic hybrid systems exhibit sustainable methods of generating value-added chemicals from the plentiful and potentially renewable resources of electricity, water, and air. [9, 10, 14-16] The hybrids offer a promising future toward chemical synthesis, especially if we can accurately identify the interplay between the biological and inorganic aspects that allows for their impressive synergy.

While microorganisms in these systems may be intended as specific catalysts for the reaction of interest, evidence suggests that they adjust relevant metabolic activity in response to the growth conditions of the unique hybrid environment. [6, 17] Investigation of those potential biological adaptations thus presents an opportunity to uncover fundamental characteristics at the interface of biological and inorganic components in hybrid systems. Within the last few years, research in our group has utilized an integrated proteomics- and metabolomics-based approach uncovered altered metabolic pathways in hybrid systems that combine different microorganisms with abiotic components including *Sporomusa ovata* (*S. ovata*) with electrochemical water splitting[17] and *Xanthobacter autotrophicus* (*X.* autotrophicus) with semiconducting quantum dots. [6] Additional studies have used one or more omics tools to study the metabolism of many microorganisms. [18] This metabolic information has implications for the design and optimization of chemical transformations accomplished by hybrid systems, especially for reactions that utilize major biological pathways where a metabolic shift is liable to affect the balance of key reactions. The present study investigates such metabolic adaptation in a hybrid system that combines the bacteria *X. autotrophicus*[19-23] with electrochemical water-splitting. Our group has demonstrated the synergy that *X. autotrophicus* displays when interfaced with inorganic materials such as electrodes conducting electrochemical water-splitting, [9, 16] semiconducting quantum dots, [6] and perfluorocarbon nano-emulsions. [24] The simultaneous ability of *X. autotrophicus* to fix both N_2 and CO_2 renders it attractive for the development of sustainable technologies including the interface with electrochemical water-splitting which has previously been utilized to produce biofertilizer from the bacteria. [9] However, a lack of fundamental knowledge regarding potential changes to expected metabolic activity presents a barrier that must be overcome to further develop such technology.

To better understand any potential interactions between the bacteria and electrochemical conditions, we hypothesized that the application of integrated proteomic and metabolomic methods would allow us to determine whether differential metabolic regulation occurs under electrochemical water-splitting conditions at organism-wide and pathway-specific scales. Here, we report an altered metabolism of *X. autotrophicus* under electrochemical water-splitting that favors an increased capacity for nitrogen fixation and carbon dioxide fixation with a high intracellular nitrogen content. Our findings support the addition of integrated multi-omics analysis to other characterization methods at the interface of materials and microbiology and will support the design of hybrid systems for synergistic chemical transformation.

RESULTS AND DISCUSSION

Cultures of *X. autotrophicus* (ATCC 35674, DSM 432) were grown in a minimal media lacking nitrogen and organic carbon **(Supplementary Table 1)** and supplied with a gas mixture of 60:20:18:3 N_2:H_2:CO_2:O_2 gas mixture for autotrophic and diazotrophic growth following a previously-reported protocol. [6] Samples were inoculated to produce 100mL of liquid culture under either electrochemical water-splitting (hybrid) conditions or H_2-fed conditions (for more information, vide infra and see the section Hybrid and H_2-fed condition setup in SI). The hybrid conditions consist of a single-cell chamber with previously-developed cobalt-phosphate (CoPi) and cobalt phosphorus (Co-P) electrodes for simultaneous oxygen evolution reaction (OER) and hydrogen evolution reaction (HER), respectively (Fig. 1). [25-27] These catalysts are biocompatible[25] and their combined reactions generate sufficient reducing equivalents, mostly in the form of H_2, that are able to serve as the primary source of reducing equivalents during growth of *X. autotrophicus*. [9] In addition to the H2 produced by the electrochemical water-splitting, the cultures were purged with a gas mixture in a ratio of 78:20:2 N2:CO_2:O_2 for 10-minutes each day and sealed in the gas-tight bottle the remainder of the time during the experiment. The H_2-fed conditions consisted of 100mL of culture in an Erlenmeyer flask contained in a sealed gas-tight jar with a gas mixture of 60:20:18:3 N_2:H_2:CO_2:O_2 replenished daily. Cultures were grown under each condition for four days with t=0 occurring at the initial inoculation before samples were collected for metabolomic and proteomic analysis.

The metabolomic and proteomic analyses were conducted following previously reported protocols[6] on all cultures (n = 3 for both groups, see Metabolomics separation and analysis and proteomics analysis in SI) revealed distinct alterations to bacterial metabolism at this timepoint. Among 2,570 identified proteins across all samples, 499 were upregulated in the hybrid condition (fold change > 1.5, $p < 0.05$) and 380 were downregulated (fold change < 0.66, $p < 0.05$) while of the identified 87 metabolites 20 were upregulated in the hybrid condition and 4 were downregulated (Fig. 2b, 3b). Principal component analysis revealed that both proteins (Fig. 2a) and metabolites (Fig. 3a) were separated into groups by experimental condition, indicating a significant shift in the overall metabolism. Differences in individual protein expression and metabolite abundance were further investigated referencing the Kyoto Encyclopedia of Genes and Genomes[28] to compare the regulation of specific biological pathways.

Alterations to the expression of multiple types of proteins and metabolites reflect an increase in nitrogenase expression and key ammonia-assimmilating proteins that indicate high levels of intracellular nitrogen under hybrid conditions. Biological nitrogen fixation of N_2 into NH_3 was considered to be of particular importance as it is responsible for the assimilation of 100% of extracellular nitrogen into biomolecules in the absence of other nitrogen sources. [29] Due to its simultaneous necessity and large demand of both ATP and reducing equivalents, [30] nitrogen fixation is tightly regulated in *X. autotrophicus*. [29, 31] Investigation of nitrogen fixation and subsequent assimilation are thus key targets for optimization of biological-materials hybrids that utilize *X. autotrophicus*. Nitrogenase proteins that catalyze the fixation of N_2 to NH_3 were found to be generally upregulated in the hybrid, suggesting an increased capacity to perform nitrogen fixation in the presence of electrochemical water-splitting (Fig. 2c). Specifically, the nitrogenase proteins NifB, NifE, NifN, NifV, NifQ, NifX, and NifZ were all upregulated while only NifD was found to be downregulated. While NifD is necessary for nitrogen fixation to occur as it is a structural component of the catalytic complex of the MoFe nitrogenase, [32] most of the other Nif proteins listed are also required for nitrogen fixation and diazotrophic growth in N_2-fixing microorganisms. [33-38] The fate of NH_3 generated by this N_2 fixation is largely controlled by its incorporation into glutamine and glutamate from which essential amino acids and other nitrogen-containing biomolecules are derived. [39] In most bacteria, nitrogen is assimilated following this route by the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle. [40, 41] The cycle iterates through the incorporation of ammonia into glutamine by GS and the addition of 2-oxoglutarate to glutamine by GOGAT to form glutamate. As central components of nitrogen metabolism, glutamine and 2-oxoglutarate are known to regulate nitrogen assimilation in α-proteobacteria such as *X. autotrophicus* where they function as complementary indicators of intracellular nitrogen; heightened glutamine levels indicate excess nitrogen while heightened 2-oxoglutarate indicates a lack of nitrogen. [42] In addition to the GS/GOGAT pathway, glutamate dehydrogenase (GDH) is commonly used by nitrogen-fixing bacteria to catalyze the conversion of ammonia and 2-oxoglutarate to glutamate. [43] Under hybrid conditions, GS was observed to be downregulated while GOGAT was upregulated, indicating increased glutamine levels and thus a larger intracellular nitrogen. This observation was supported by decreased levels of 2-oxoglutarate (Fig. 3c), and upregulation of GDH, further supporting an excess nitrogen balance favorable to ammonia assimilation. GDH has been reported to be preferentially expressed when excess ammonia accumulates in the cell,[44] making its upregulation in this study also suggest an increased rate of nitrogen fixation in hybrid growth conditions.

Differences in the central carbon metabolism include an increased abundance of glycerate-3-phosphate and acetyl-CoA under hybrid conditions. The central carbon metabolism involves CO_2 fixation by the Calvin cycle and oxidation of resulting multi-carbon metabolites by the tricarboxylic acid (TCA) cycle is also a prime target for understanding adaptations of *X. autotrophicus* to electrochemical water splitting. Of particular interest, we observed an increased abundance of glycerate-3-phosphate under hybrid conditions. (Fig. 3c). As the direct product of carbon dioxide fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) in the Calvin Cycle, this finding suggests an increased rate of carbon dioxide fixation.[45] The increased abundance of both glycerate-3-phosphate and acetyl-CoA (Fig. 3c) together suggest an effective energy supply under hybrid conditions, as major metabolic fates of both molecules end in the oxidative TCA cycle.[46] The downregulation of 2-oxoglutarate, which also participates in the TCA cycle, may be explained by its role as a key substrate in an upregulated nitrogen assimilation pathway.

The redox and energy balances of the cell were maintained across the electron transport chain and including hydrogenase, while ATPase expression was increased. ATP synthase, which couples proton translocation to ATP generation, was found to be upregulated under hybrid conditions (Fig. 2c). This finding suggests an increased capacity for ATP production that may particularly benefit the ATP-consuming nitrogen assimilation via GOGAT during nitrogen fixation.[47] The cytochromes in the electron transport chain were expressed similarly across conditions (Supplementary Data Table S1), indicating retained utilization of dominant electron transfer pathways. Similarly, hydrogenase proteins that are responsible for converting reducing equivalents from H_2 into bio-accessible redox species were consistently regulated. While the presence of extracellular redox-active molecules such as riboflavin has been reported to induce changes in the metabolism of the bacteria *Sporomusa ovata*,[17] which also derives reducing equivalents from H_2, no such metabolic rewiring has been reported for *X. autotrophicus.* Still, it is worth noting that two of the three ferredoxins detected by proteomics were significantly upregulated under hybrid conditions, hinting at a more reduced intracellular state. The connection between the different method of supplying reducing equivalents and the observed metabolic changes thus merits further study.

CONCLUSIONS

The results presented in this work suggest that microorganisms introduced into biological-inorganic hybrid systems respond with significant alterations to their metabolism. In the case of *X. autotrophicus* partnered with electrochemical water-splitting, metabolic changes under these conditions included increased expression of nitrogen fixation and ammonia assimilation pathways as well as carbon dioxide fixation products which may contribute to increased growth and associated biomass accumulation for biofertilizer production. Further study of the intracellular and extracellular redox balance of *X. autotrophicus* under these conditions would yield more detailed insight for the future design of hybrid systems that seek to maximize N_2 and CO_2 fixation. This research also showcases the utility of multi-omics studies as a promising tool for obtaining a deeper fundamental understanding of the interface between microorganisms and materials.

ASSOCIATED CONTENT

Supporting Information

The supporting information contains a detailed materials and methods section for experiments performed as well as data files and other supplemental information related to the research presented in the main text.

Author Information

Z.S. conceptualized the project, developed the hybrid systems and conducted the majority of the experiments; S.O. and Z.S. conducted the metabolomic experiments and data analysis under the supervision of J.O.P.; X.G. helped establish the electrochemical setup; Jihui Sha and Z.S. conducted the proteomic experiments and data analysis under the supervision of J.A.W.; Jingwen Sun and Z.S. wrote the code for data analysis; Y.X. assisted in data analysis for proteomic and metabolic experiments; Z.S. wrote the first draft of the manuscript; C.L. supervised the project; All authors provided input and edits to the final manuscript.

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Notes

The authors declare no competing financial interest.

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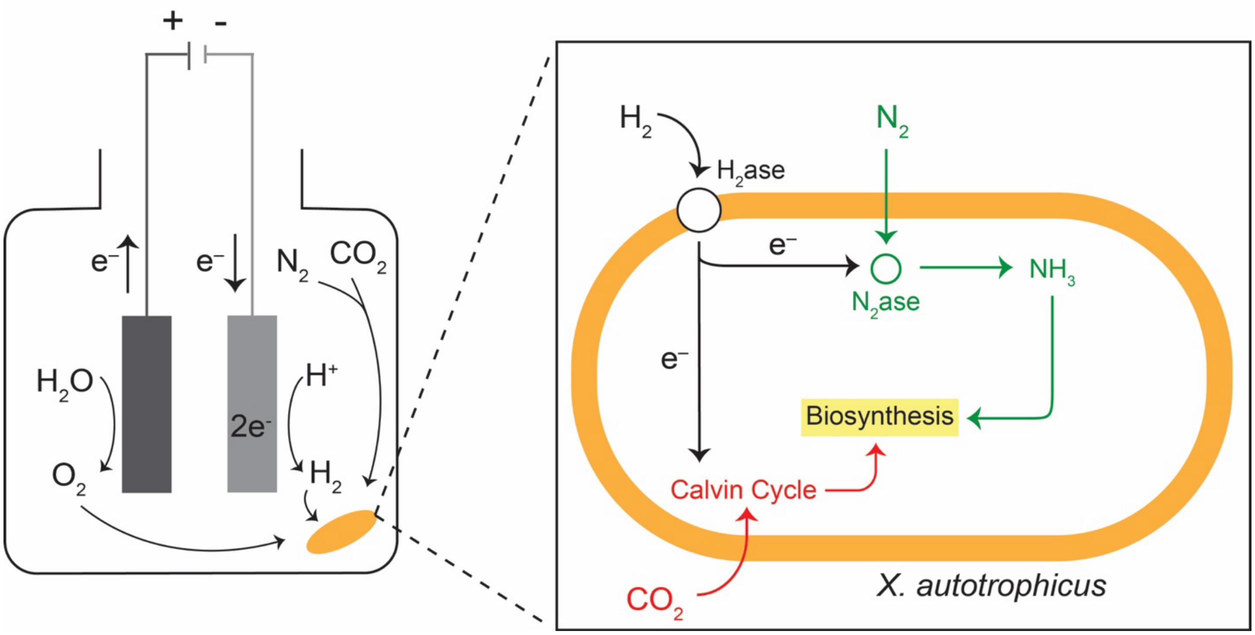

Figure 1. Fixation of CO_2 and N_2 by *Xanthobacter autotrophicus* under electrochemical water-splitting. H_2 and O_2 gas generated by electrochemical water-splitting are utilized along with N_2 and CO_2 to drive central metabolic pathways including anabolic biosynthesis pathways. H_2ase, hydrogenase; N_2ase, nitrogenase.

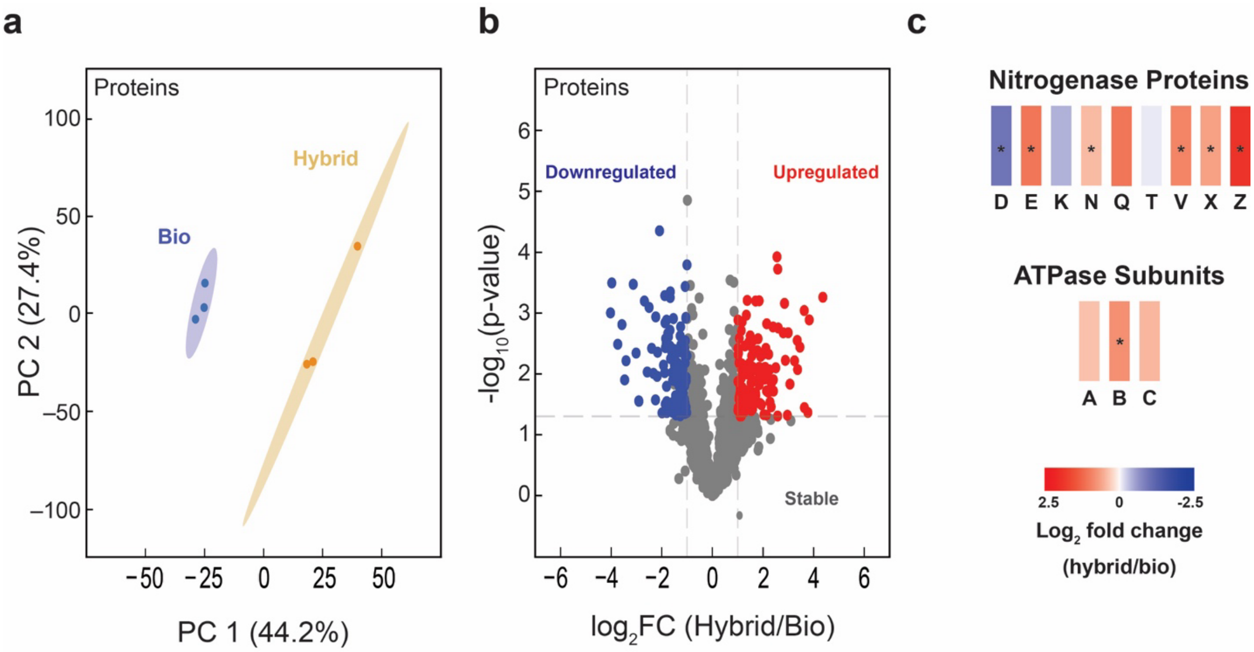

Figure 2. Altered proteome of *X. autotrophicus* grown under electrochemical water-splitting conditions. (a) Principal component analysis score plot and (b) volcano plot representing all detected proteins in the presence ("hybrid") or absence ("bio") of electrochemical water-splitting. Significant differences in expression used for the volcano plot were decided by hybrid/bio fold change values > 1.5 or < 0.66 with $p < 0.05$. Colored numbers represent the number of proteins of each regulation type. (c) Heat map of individual nitrogenase subunits D, E, K, N, Q, T, V, X, & Z and ATPase subunits A, B, & C. Log_2FC = Log_2-transformed fold change.

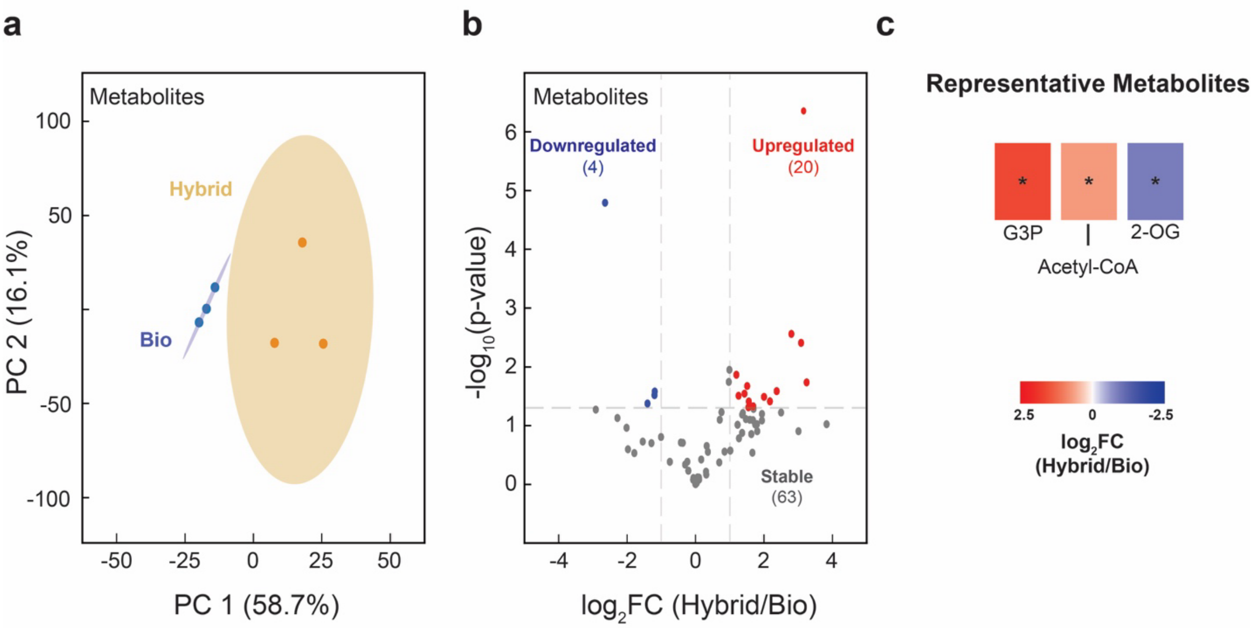

Figure 3. Shift in metabolite balance of *X. autotrophicus* grown under electrochemical water-splitting conditions. (a) Principal component analysis score plot and (b) volcano plot representing all detected metabolites in the presence ("hybrid") or absence ("bio") of electrochemical water-splitting. Significant differences in expression used for the volcano plot were decided by hybrid/bio fold change values > 1.5 or < 0.66 with $p < 0.05$. Colored numbers represent the number of metabolites of each regulation type. (c) Heat map of fold change values for individual metabolites. G3P = glyceraldehyde-3-phosphate ; 2-OG = 2-Oxoglutarate ; FC = fold change ; Log_2FC = Log_2 transformed fold change.

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