significant *I. hospitalis* metabolites suggest that *I. hospitalis* may be capable of more complex carbohydrate metabolism than has been anticipated. The LC–MS data on carbohydrate intermediates suggest that glucose or other carbohydrate storage pathways may be present in *I. hospitalis,* and could serve to maintain proper cellular osmotic control as well as to satisfy other metabolic needs.

4 Conclusion

This study is the first metabolomics analysis of the *I. hospitalis* and *N. equitans* interaction. This work also emphasizes the benefits of using both LC–MS and NMR approaches, as more complete characterization of the metabolome of *I. hospitalis* and *N. equitans* was obtained. Through integration of our LC–MS and NMR metabolite profiles with previously published proteomics data, we have been able to map several key metabolic pathways that appear to modulate *I. hospitalis-N equitans* interspecies interactions. This study has highlighted key metabolic changes occurring when *I. hospitalis* is grown in co-culture with *N. equitans*. Although *I. hospitalis* is able to grow in the presence of *N. equitans*, our data suggests that there is a significant metabolic cost for the host organism. *N. equitans* seems to consume a large fraction of the small molecule pool of *I. hospitalis*, producing a sharp decrease in the energy status of the host. We cannot rule out that in their natural environment, the interactions between these two organisms with severely reduced genomes may confer an ecological advantage in the larger meta-microbial community, but the results of this study suggest that, at least under the laboratory conditions used here, *N. equitans* imposes a significant metabolic energy strain on *I. hospitalis*.

The combination of NMR and MS for untargeted metabolomics analysis has proven to be very powerful and complementary. Often times, the two methods yielded similar metabolite identification with comparable fold change measured by both for the different *I. hospitalis* and *I. hospitalis–N equitan*s samples. In some cases, metabolite identification was only possible using either LC–MS or NMR but not both, making the use of both analytical platforms a necessity. Furthermore, the additional sensitivity of LC–MS for low abundance molecules combined with the ability of NMR to obtain quantitative metabolite concentrations (as opposed to relative concentrations) demonstrate the highly advantageous application of both techniques to untargeted metabolite profiling of challenging microbial systems.

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Conflict of interest The authors declare no conflict of interest.

Compliance with ethical requirements This article does not contain any studies with human or animal subjects.

References

Boucher, Y., Kamekura, M., & Doolittle, W. F. (2004). Origins and evolution of isoprenoid lipid biosynthesis in archaea. *Molecular Microbiology, 52*(2), 515–527. doi:10.1111/j.1365-2958.2004.03992.x.

Burghardt, T., Junglas, B., Siedler, F., Wirth, R., Huber, H., & Rachel, R. (2009). The interaction of *Nanoarchaeum equitans* with *Ignicoccus hospitalis*: Proteins in the contact site between two cells. *Biochemical Society Transactions, 37*, 127–132. doi:10.1042/BST0370127.

Chenomx NMR Suite 7.0. (2010). Chenomx Inc.

Giannone, R. J., Huber, H., Karpinets, T., Heimerl, T., Küper, U., Rachel, R., et al. (2011). Proteomic characterization of cellular and molecular processes that enable the *Nanoarchaeum equitans–Ignicoccus hospitalis* relationship. *PLoS One, 6*(8), e22942. doi:10.1371/journal.pone.0022942.

Giannone, R. J., Wurch, L. L., Heimerl, T., Martin, S., Yang, Z., Huber, H., Reinhard, R., Hettich, R.L., Podar, M. (2014). Life on the edge: functional genomic response of *Ignicoccus hospitalis* to the presence of *Nanoarchaeum equitans*. *The ISME Journal*, 1–14. Jul 11 doi:10.1038/ismej.2014.112 [Epub ahead of print].

Godde, J. S. (2012). Breaking through a phylogenetic impasse: a pair of associated archaea might have played host in the endosymbiotic origin of eukaryotes. *Cell & Bioscience, 2*(29), 1–11. doi:10.1186/2045-3701-2-29.

Heinemann, J., Hamerly, T., Maaty, W. S., Movahed, N., Steffens, J. D., Reeves, B. D., et al. (2014). Expanding the paradigm of thiol redox in the thermophilic root of life. *Biochimica et Biophysica Acta, 1840*(1), 80–85. doi:10.1016/j.bbagen.2013.08.009.

Huber, H., Burggraf, S., Mayer, T., Wyschkony, I., Rachel, R., & Stetter, K. O. (2000). *Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic Archaea, represented by two new species, *Ignicoccus islandicus* sp nov and *Ignicoccus pacificus* sp nov. and Ignicoccus pacificus sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *50*, 2093–2100. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11155984.

Huber, H., Gallenberger, M., Jahn, U., Eylert, E., Berg, I. A, Kockelkorn, D., et al., (2008). A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum *Ignicoccus hospitalis*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(22), 7851–7856. doi:10.1073/pnas.0801043105.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C., & Stetter, K. O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature, 417*(6884), 63–67. doi:10.1038/417063a.

Huber, H., Hohn, M. J., Stetter, K. O., & Rachel, R. (2003). The phylum Nanoarchaeota: Present knowledge and future

perspectives of a unique form of life. *Research in Microbiology, 154*(3), 165–171. doi:10.1016/S0923-2508(03)00035-4.

Huber, H., Küper, U., Daxer, S., & Rachel, R. (2012). The unusual cell biology of the hyperthermophilic Crenarchaeon *Ignicoccus hospitalis*. *Antonie van Leeuwenhoek, 102*, 203–219. doi:10.1007/s10482-012-9748-5.

Jahn, U., Gallenberger, M., Paper, W., Junglas, B., Eisenreich, W., Stetter, K. O., et al. (2008). *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: New insights into a unique, intimate association of two archaea. *Journal of Bacteriology, 190*(5), 1743–1750. doi:10.1128/JB.01731-07.

Jahn, U., Huber, H., Eisenreich, W., Hügler, M., & Fuchs, G. (2007). Insights into the autotrophic CO_2 fixation pathway of the archaeon *Ignicoccus hospitalis*: Comprehensive analysis of the central carbon metabolism. *Journal of Bacteriology, 189*(11), 4108–4119. doi:10.1128/JB.00047-07.

Jahn, U., Summons, R., Sturt, H., Grosjean, E., & Huber, H. (2004). Composition of the lipids of *Nanoarchaeum equitans* and their origin from its host *Ignicoccus* sp. strain KIN4/I. *Archives of Microbiology, 182*, 404–413. doi:10.1007/s00203-004-0725-x.

Junglas, B., Briegel, A., Burghardt, T., Walther, P., Wirth, R., Huber, H., et al. (2008). *Ignicoccus hospitalis* and *Nanoarchaeum equitans*: ultrastructure, cell-cell interaction, and 3D reconstruction from serial sections of freeze-substituted cells and by electron cryotomography. *Archives of Microbiology, 190*, 395–408. doi:10.1007/s00203-008-0402-6.

Karp, P. D., Ouzounis, C. A, Moore-Kochlacs, C., Goldovsky, L., Kaipa, P., Ahrén, D., et al. (2005). Expansion of the BioCyc collection of pathway/genome databases to 160 genomes. *Nucleic Acids Research*, *33*(19), 6083–6089. doi:10.1093/nar/gki892.

Karp, P. D., & Paley, S. (1996). Integrated access to metabolic and genomic data. *Journal of Computational Biology*, *3*(1), 191–212. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8697237.

Karp, P. D., Paley, S. M., Krummenacker, M., Latendresse, M., Dale, J. M., Lee, T. J., et al. (2010). Pathway tools version 13.0: Integrated Software for pathway/genome informatics and systems Biology. *Briefings In Bioinformatics, 11*(1), 40–79. doi:10.1093/bib/bbp043.

Karp, P. D., Paley, S., & Romero, P. (2002). The pathway tools software. *Bioinformatics, 18*, S1–S8.

Küper, U., Meyer, C., Müller, V., Rachel, R., & Huber, H. (2010). Energized outer membrane and spatial separation of metabolic processes in the hyperthermophilic Archaeon *Ignicoccus hospitalis*. *Proceedings of the National Academy of Sciences of the United States of America, 107*(7), 3152–3156. doi:10.1073/pnas.0911711107.

Moran, U., Phillips, R., & Milo, R. (2010). SnapShot: key numbers in biology. *Cell*, *141*(7), 1262–1262.e1. doi:10.1016/j.cell.2010.06.019.

Paley, S. M., Latendresse, M., & Karp, P. D. (2012). Regulatory network operations in the Pathway Tools software. *BMC Bioinformatics, 13*, 243. doi:10.1186/1471-2105-13-243.

Paper, W., Jahn, U., Hohn, M. J., Kronner, M., Näther, D. J., Burghardt, T., et al. (2007). *Ignicoccus hospitalis* sp. nov., the host of "*Nanoarchaeum equitans*". *International Journal of Systematic and Evolutionary Microbiology, 57*, 803–808. doi:10.1099/ijs.0.64721-0.

Podar, M., Anderson, I., Makarova, K. S., Elkins, J. G., Ivanova, N., Wall, M. A., et al. (2008). A genomic analysis of the archaeal system *Ignicoccus hospitalis–Nanoarchaeum equitans*. *Genome Biology, 9*, R158. doi:10.1186/gb-2008-9-11-r158.

Promega Corporation. (2012). BacTiter-Glo Microbial Cell Viability Assay.

Sun, J., Zhang, S., Chen, J., & Han, B. (2012). Metabolic profiling of Staphylococcus aureus cultivated under aerobic and anaerobic conditions with ^{1}H NMR-based nontargeted analysis. *Canadian Journal of Microbiology, 718*(17), 709–718. doi:10.1139/W2012-046.

Thauer, R. K., Jungermann, K., & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriology Reviews, 41*(1), 100–180.

Tredwell, G. D., Edwards-Jones, B., Leak, D. J., & Bundy, J. G. (2011). The development of metabolomic sampling procedures for Pichia pastoris, and baseline metabolome data. *PLoS ONE, 6*(1), e16286. doi:10.1371/journal.pone.0016286.

Waters, E., Hohn, M. J., Ahel, I., Graham, D. E., Adams, M. D., Barnstead, M., et al. (2003). The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. *Proceedings of the National Academy of Sciences of the United States of America, 100*(22), 12984–12988. doi:10.1073/pnas.1735403100.

Weljie, A. M., Newton, J., Mercier, P., Carlson, E., & Slupsky, C. M. (2006). Targeted profiling: Quantitative analysis of ^{1}H NMR metabolomics data. *Analytical Chemistry, 78*(13), 4430–4442. doi:10.1021/ac060209g.

Wishart, D. S. (2008). Quantitative metabolomics using NMR. *Trends in Analytical Chemistry, 27*(3), 228–237. doi:10.1016/j.trac.2007.12.001.

Wu, X.-H., Yu, H.-L., Ba, Z.-Y., Chen, J.-Y., And, H.-G. S., & Han, B.-Z. (2010). Sampling methods for NMR-based metabolomics of *Staphylococcus aureus*. *Biotechnology Journal, 5*(1), 75–84.