Aix Marseille Université

NNT : 0000AIXM0000

THÈSE DE DOCTORAT

Soutenue à Aix-Marseille Université
le 9 décembre 2024 par

Rabja Maria POPALL

Microbial primary production in the serpentinite-hosted Prony Bay hydrothermal field, an Early Earth analog

Discipline
Sciences de l'environnement

Spécialité
Océanographie

École doctorale
ED 251 – Sciences de l'environnement

Laboratoire/Partenaires de recherche
Institut Méditerranéen d'Océanologie

Composition du jury

Mylène HUGONI Maître de conférences, UCBL-MAP	Rapporteur
Ludwig JARDILLIER Professeur, UPSaclay-IDEEV	Rapporteur
Barbara SCHOEPP-COTHENET Directeur de recherche, CNRS-BIP	Examinateur
Matthew SCHRENK Professeur associé, MSU	Examinateur
Roy PRICE Professeur adjoint, SBU	Examinateur
Philippe CUNY Professeur, AMU-MIO	Président du jury
Gaël ERAUSO Professeur, AMU-MIO	Directeur de thèse
Anne POSTEC Maître de conférences, AMU-MIO	Co-directeur de thèse

Mediterranean Institute of Oceanography

Affidavit

I, undersigned, Rabja Maria Popall, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific supervision of Gaël Erauso and Anne Postec, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Marseille, 13 October 2024



Cette œuvre est mise à disposition selon les termes de la Licence Creative Commons Attribution - Pas d'Utilisation Commerciale - Pas de Modification 4.0 International.

Liste de publications et participation aux conférences

Liste des publications réalisées dans le cadre du projet de thèse

1. Popall RM, Postec A, Lecoeuvre A, Quéméneur M and Erauso G (2023) Metabolic challenges and key players in serpentinite-hosted microbial ecosystems. Front. Microbiol. 14:1197823. doi: 10.3389/fmicb.2023.1197823
2. Popall RM, Hennart M, Marre S, Peyret P, Postec A and Erauso G. Carbon fixation and assimilation by autotrophic and heterotrophic foundation species in the serpentinite-hosted Prony Bay. In preparation
3. Popall RM, Roland A, Davidson S, Combet-Blanc Y, Postec A and Erauso G. Cultivating hydrogenotrophic microbial communities from the serpentinite-hosted Prony Bay on different carbon sources. In preparation

Participation aux conférences au cours de la période de thèse

1. Popall RM, Roland A, Davidson S, Combet-Blanc Y, Postec A, Erauso G. Cultivating microbial communities from the serpentinite-hosted Prony Bay on different carbon sources. 31ème congrès de l'ED251. Aix-en-Provence, France. May 2024. Poster presentation
2. Popall RM, Lecoeuvre A, Postec A, Erauso G. Fixation du carbone par les communautés microbiennes du système serpentinisé de la Baie de Prony, Nouvelle-Calédonie. Conférence nationale d'Exobiologie 2023. Grenoble, France. November 2023. Oral presentation
3. Popall RM, Lecoeuvre A, Postec A, Erauso G. Fixation du carbone dans les communautés microbiennes hydrogénotrophes du système hydrothermal serpentinisé de la Baie de Prony, Nouvelle-Calédonie. 11ème congrès de l'Association Francophone d'Écologie Microbienne. Carqueiranne, France. October 2023. Oral presentation
4. Popall RM, Lecoeuvre A, Estoup P, Quéméneur M, Davidson S, Combet-Banc Y, Marre S, Peyret P, Postec A, Erauso G. Hydrogenotrophic microbial enrichments from an alkaline hydrothermal spring driven by serpentinization, Prony Bay, New

Caledonia. Extremophiles 2022. Loutraki, Greece. September 2022. Preparation of oral presentation

5. Popall RM, Lecoeuvre A, Estoup P, Quéméneur M, Davidson S, Combet-Banc Y, Marre S, Peyret P, Postec A, Erauso G. Cultivated hydrogenotrophic microbial consortia from an alkaline hydrothermal spring in a serpentinization context, the Prony Bay, New Caledonia. Astrobiology Science Conference 2022. Atlanta, GA, USA (videoconference). May 2022. Oral presentation
6. Popall RM, Lecoeuvre A, Estoup P, Quéméneur M, Davidson S, Combet-Banc Y, Marre S, Peyret P, Postec A, Erauso G. Cultivated hydrogenotrophic microbial consortia from an alkaline hydrothermal spring in a serpentinization context, the Prony Bay, New Caledonia. Junior Scientists Microbiology Meeting of Marseille 2022. Marseille, France. May 2022. Oral presentation

Participation à la communication grand public

1. Annalisa Plaitano (2023) A la recherche des origines de la vie. Cosinus 262, 42-43. Article on research topic assessed in the frame of the PhD project
2. Serpentinisation & Origine de la vie - Le Film (2024). Directed by Marc Daréoux. Premiere at Festival EXPLORE on May 28, 2024, at Cinéma La Baleine, Marseille, France. Documentary on research performed in the frame of the PhD project, interview on assessed research questions

Abstract

Serpentinization, the hydration of mantle rocks into serpentinites, has been occurring since there has been liquid water on Earth. Producing reduced fluids enriched in organic molecules and geological formations with compartments and mineral catalysts, it is central to "metabolism first" theories on the origin of life. The microbial ecosystems developing on the byproducts of serpentinization might thus illuminate one of the biggest questions in biology but are poorly understood because they grow in the absence of CO_2. The ultrabasic milieu of serpentinizing fluids precipitates and immobilizes dissolved inorganic carbon, preventing microbial primary production as we know it.

Four carbon compounds have been suggested to replace CO_2 in a serpentinization context: bicarbonate, as well as abiotically produced formate, acetate and glycine. Here, we tested whether these compounds are used by the serpentinite-hosted community of the coastal Prony Bay hydrothermal field. In the first part of the thesis, we applied a metagenomic approach and identified potential primary producers along a land-ocean gradient that can perform bicarbonate fixation and acetate assimilation. In the second part, we cultivated Prony Bay's anaerobic marine community under hydrogenotrophic conditions and found that bicarbonate, acetate and formate support the enrichment of specific taxonomic groups. We propose that the base of the trophic network includes bicarbonate and formate fixing autotrophs, as well as heterotrophs establishing a local supply of CO_2 by oxidizing abiogenic acetate.

Keywords: serpentinization, alkaline hydrothermal system, alkaliphile, hydrogenotroph, origin of life.

Résumé

La serpentinisation, l'hydratation des roches du manteau terrestre en serpentinites, a lieu depuis qu'il y a de l'eau liquide sur Terre. Produisant des fluides réduits enrichis en molécules organiques, des compartiments et des catalyseurs minéraux, elle est au cœur des théories du “metabolism first” sur l'origine de la vie. Les écosystèmes microbiens indigènes pourraient donc éclairer l'une des plus grandes questions de la biologie, or ils sont peu compris car ils se développent en l'absence de CO_2. Le milieu ultrabasique des fluides serpentinisés précipite le carbone inorganique dissous, empêchant la production primaire microbienne comme on la connaît. Quatre composés carbonés ont été suggérés pour remplacer le CO_2 : le bicarbonate, puis le formiate, l'acétate et la glycine abiogènes. Pour cette thèse, nous avons testé si ces composés sont utilisés par la communauté microbienne du système serpentinisé de la baie de Prony. D’abord, nous avons utilisé une approche métagénomique qui nous a permis d’identifier les producteurs primaires potentiels le long d'un gradient terre-océan, pouvant fixer le bicarbonate et assimiler l'acétate. Puis, nous avons cultivé la communauté microbienne marine anaérobie dans des conditions hydrogénotrophiques pour constater que le bicarbonate, l'acétate et le formiate favorisent l'enrichissement de groupes taxonomiques spécifiques. Nous proposons que la base de ce réseau trophique comprenne les autotrophes fixant le bicarbonate et le formiate, et les hétérotrophes en approvisionnant localement du CO_2 par oxydation de l'acétate abiogène.

Mots clés : serpentinisation, système hydrothermal alcalin, alcaliphile, hydrogénotrophe, origine de la vie.

Synthèse en langue française

CHAPITRE 1 : introduction

Contexte

La question de l'origine de la vie est l'une des plus fondamentales de la biologie et touche de nombreuses autres disciplines. L'histoire de l'énigme de l'origine de la vie a été influencée par de nombreux débats aussi bien politiques que religieux, ce qui souligne l'importance du sujet.

Après la réfutation de la génération spontanée par Louis Pasteur et l'émergence de théories alternatives telles que l'hypothèse des cosmozoaires, Alexander Ivanovich Oparin a suggéré dans la première moitié du 20ème siècle que la première forme de vie était un hétérotrophe se nourrissant de composés organiques d'origine abiotique. Cette hypothèse était conforme à l'idée de John Burdon Sanderson Haldane de "soupe primitive" et à plusieurs expériences biochimiques démontrant la synthèse abiotique de composés organiques. L'idée d'Oparin a été contestée par la théorie du "gene first", qui suppose que le matériel génétique est apparu avant les réactions métaboliques. Ces deux approches restent jusqu'à aujourd'hui les principaux concepts de ce domaine de recherche.

Le point de vue d'Oparin a été repris plus tard par la théorie du "metabolism first", qui maintient l'accent sur le métabolisme plutôt que sur la génétique, mais propose une origine autotrophe plutôt qu'hétérotrophe de la vie. Cette théorie suppose que la vie a commencé par une série de réactions chimiques évoluant vers un métabolisme primitif auto-entretenu, entraîné par un déséquilibre électrochimique continu et catalysé par des minéraux contenant des métaux imitant les enzymes modernes. Michael Russel a suggéré que les systèmes hydrothermaux serpentinisés sont des candidats idéaux pour l'émergence de la vie. Les forts gradients d'oxydoréduction qui se développent entre le fluide hydrothermal et l'eau de mer pourraient avoir été exploités par des protocellules grâce à une force motrice protonique rudimentaire, et plusieurs minéraux pourraient avoir catalysé des réactions primitives.

L'étude des systèmes serpentinisés en tant qu'analogues potentiels de la Terre primitive s'est considérablement développée au cours des dernières décennies et a été fortement influencée par la découverte du champ hydrothermal de Lost City dans l'océan Atlantique profond. Cependant, des environnements terrestres et côtiers serpentinisés ont été signalés bien plus tôt. Dans les années 1980, le champ hydrothermal peu profond de Prony Bay a été découvert à l'extrémité sud du massif de péridotite de Nouvelle-Calédonie. En 2010, sa pertinence scientifique a été pleinement reconnue et une équipe de chercheurs français, calédoniens et américains a commencé à étudier ses caractéristiques géochimiques, puis microbiologiques. La baie de Prony présente des sites terrestres, intertidaux et sous-marins. Elle peut donc être interprétée comme un système de transition entre les champs hydrothermaux terrestres et les champs hydrothermaux profonds serpentinisés. Au cours des quinze dernières années, la biodiversité et la distribution spatiale des bactéries et des archées colonisant les cheminées

hydrothermales ont été décrites et plusieurs souches ont été isolées. Cependant, le fonctionnement métabolique du réseau trophique de la baie de Prony reste énigmatique. En tant qu'analogue potentiel de la Terre primitive, les caractéristiques métaboliques des communautés microbiennes de la baie de Prony pourraient nous éclairer sur les limites de la vie moderne et sur l'émergence de formes de vie primitives.

L'état de l'art

Pour se faire une première idée des conditions préalables au métabolisme microbien que la serpentinisation établit, nous avons réalisé une revue de la littérature. Nous y avons résumé la façon dont le milieu serpentinisé est censé limiter ou stimuler l'activité métabolique, synthétisé les informations sur les groupes taxonomiques colonisant des systèmes serpentinisés dont le métabolisme est connu et nous avons discuté de façon critique de la méthodologie des recherches disponibles afin d'optimiser l'approche expérimentale de cette thèse. La revue a été publiée dans Frontiers in Microbiology le 24 juillet 2023 et sera brièvement décrite ici.

La serpentinisation est l'altération géochimique des roches ultramafiques qui ont été soulevées et exposées à l'eau. L'hydratation de la péridotite en serpentinite, brucite et magnétite produit de grandes quantités de gaz hydrogène (H_2) et de fluides hydrothermaux réduits. Ces fluides peuvent catalyser la réaction de H_2 avec le carbone dérivé du manteau pour produire du méthane (CH_4) et de petites molécules organiques dans les processus abiotiques de Sabatier et de Fischer-Tropsch. En même temps, la serpentinisation crée un milieu ultrabasique avec des valeurs de pH dépassant 10. Cela conduit à la précipitation de carbonates, formant des cheminées hydrothermales au fil du temps et éliminant le carbone inorganique dissous (DIC) de l'environnement.

Les composés produits par la serpentinisation peuvent favoriser la croissance de communautés microbiennes chimiolithotrophes. On trouve des écosystèmes microbiens dans divers environnements serpentinisés terrestres, marins peu profonds et profonds. Cependant, leur mode de survie reste peu clair, car la serpentinisation crée des conditions très contraignantes pour la vie. Tout d'abord, le pH ultrabasique pose un problème énergétique fondamental au niveau cellulaire, car il inverse le gradient de pH transmembranaire qui régit généralement tous les processus cellulaires. Par conséquent, les organismes colonisant des systèmes serpentinisés présentent des adaptations spéciales telles que des mécanismes de concentration des protons ou la capacité à utiliser les gradients de sodium pour exploiter l'énergie métabolique. Le donneur d'électrons thermodynamiquement le plus efficace disponible dans les environnements de serpentinite est H_2, mais CH_4, le monoxyde de carbone (CO) et les espèces de soufre réduites peuvent également être utilisés comme source d'énergie. Les accepteurs d'électrons sont fournis de l'extérieur et comprennent l'oxygène (O_2) dans les environnements de surface, le nitrate dans la subsurface peu profonde et le sulfate dans la subsurface anoxique profonde. En particulier dans la subsurface profonde, une grande partie de la communauté anaérobie peut ne pas présenter de chaîne de transport d'électrons *bona fide*, mais effectuer la fermentation de divers composés organiques.

Deuxièmement, le pH élevé réduit la biodisponibilité des nutriments essentiels. Il s'agit notamment du phosphore et potentiellement de l'azote, mais surtout du DIC qui est éliminé du système par la précipitation des carbonates. Tous les écosystèmes connus dépendent du DIC comme source primaire de carbone pour la base du réseau trophique, et la manière dont les

communautés colonisant des systèmes serpentinisés peuvent survivre sans DIC n'est pas claire. Quatre composés ont été suggérés pour remplacer le dioxyde de carbone (CO_2) en tant que sources primaires de carbone : le bicarbonate, qui peut être redissous du carbonate de calcium par certains organismes, le formiate, l'acétate et la glycine. Ces trois derniers peuvent être produits par des procédés de type Fischer-Tropsch et Strecker et pourraient donc faciliter la production de biomasse à partir de composés d'origine abiotique.

Les défis métaboliques posés par la serpentinisation suggèrent la présence de groupes taxonomiques spécialisés jouant un rôle important dans le réseau trophique. La communauté des oxydants H_2 semble être dominée par des *Gammaproteobacteria*. Cela inclut une proportion importante de *Serpentinimonas*, qui est l'un des taxons les plus communément associés à la serpentinisation. L'oxydation aérobie du méthane est principalement réalisée par des bactéries, la méthanotrophie anaérobie et la méthanogénèse exclusivement par des archées telles que *Methanobacteriales* et *Methanosarcinales.* Ces dernières sont probablement endémiques aux environnements de serpentinisation et comprennent deux phylotypes distincts : The Cedars *Methanosarcinales* (TCMS) et Lost City *Methanosarcinales* (LCMS). La communauté des fermenteurs est presque entièrement dominée par des *Clostridia*, dont plusieurs nouvelles espèces ont été isolées de la baie de Prony. L'oxydation du CO, l'oxydation du soufre et la réduction du sulfate sont réalisées par une plus grande diversité de groupes taxonomiques. Notamment, la communauté des réducteurs de sulfate comprend des espèces *Desulfovibrionales* et *Dethiobacter*, qui peuvent être très abondantes dans les environnements de serpentinisation. En ce qui concerne l'absorption du carbone, il pourrait être particulièrement intéressant d'étudier de nouveaux phyla candidats tels que *Ca. Bipolaricaulota, Ca.* NPL-UPA2 et *Ca. Lithacetigena*.

L'étude des communautés colonisant les systèmes serpentinisés est soumise à des biais méthodologiques, et surtout, la grande majorité des études sont basées sur la métagénomique. Ces approches offrent un aperçu du potentiel génétique d'une communauté, mais ne permettent pas d'accéder à l'activité métabolique dans la plupart des cas. Des preuves expérimentales sont donc nécessaires pour confirmer les hypothèses *in silico*. Comme il est difficile de recréer en laboratoire les conditions de la serpentinisation, la complémentarité des approches *in silico* et *in vivo* constitue une lacune importante de la recherche.

Objectifs

Pour comprendre le fonctionnement des communautés microbiennes colonisant des systèmes serpentinisés, la base du réseau trophique revêt une importance particulière. Le problème le plus important concernant la production primaire dans les environnements serpentinisés est probablement la limitation de la DIC. L'objectif de cette thèse est d'identifier les sources de carbone primaire alimentant la base du réseau trophique dans les communautés microbiennes de la baie de Prony. Nous testons le potentiel des communautés microbiennes de la baie de Prony à utiliser le bicarbonate, le formiate, l'acétate et la glycine comme source de carbone primaire. Bien que l'origine des trois derniers dans la baie de Prony ne soit pas entièrement établie, nous supposons pour cette thèse qu'ils sont (au moins partiellement) dérivés de réactions abiotiques. Nous émettons l'hypothèse que les quatre composés facilitent la croissance des espèces autotrophes et hétérotrophes qui soutiennent le développement des écosystèmes microbiens. Dans les environnements actuels, la base du réseau trophique est

généralement constituée d'autotrophes qui produisent des substances organiques complexes à partir du CO_2 pour les niveaux trophiques supérieurs. Le bicarbonate et le formiate peuvent être transformés en CO_2 à l'intérieur de la cellule et constituent donc des sources potentielles de carbone autotrophe. Dans des conditions d'appauvrissement en DIC, les hétérotrophes pourraient toutefois jouer un rôle tout aussi fondamental en transformant le carbone organique d'origine abiotique, tel que l'acétate et la glycine, en CO_2 (comme le suggère la théorie des espèces fondatrices de Lang et de ses collègues). Cela permettrait le développement d'une communauté secondaire d'autotrophes qui n'est pas en mesure de se développer directement à partir du bicarbonate ou du formiate. Dans ce contexte, nous évaluons d'une part, lesquelles des quatre sources de carbone putatives sont utilisées par les communautés microbiennes de la baie de Prony, et d'autre part, quels groupes taxonomiques sont impliqués dans le métabolisme de ces composés carbonés en tant qu'espèces fondatrices autotrophes ou hétérotrophes putatives. Pour décrire les consortia fondateurs de la baie de Prony aussi précisément que possible, nous combinons l'analyse métagénomique de l'ADN environnemental avec la culture de communautés microbiennes anaérobies hydrogénotrophes sur chacun des quatre composés carbonés.

CHAPITRE 2 : matériel et méthodes

Récupération des échantillons

Cette thèse porte sur trois sites différents de la baie de Prony : le site terrestre de la Rivière des Kaoris (RdK), le site intertidal du Bain des Japonais (BdJ) et le site sous-marin de l'Aiguille de Prony (ST07). Le chapitre trois (la métagénomique) évalue les trois sites, le chapitre quatre (la culture) exclusivement ST07. Les concentrations en DIC mesurées dans le fluide hydrothermal de la baie de Prony sont constamment inférieures à 1 mmol L^{-} 1. Le pH du fluide hydrothermal est en moyenne de 10 et la température de 32°C.

Des échantillons de substrat et de cheminée hydrothermale de RdK et BdJ ont été prélevés dans des bouteilles en verre. Les échantillons de cheminée hydrothermale de ST07 ont été collectés dans des sacs étanches, qui ont été laissés en place jusqu'à ce que l'eau de mer ait été complètement remplacée par le fluide hydrothermal. La couche externe de tous les fragments de cheminée hydrothermale a été enlevée. Ensuite, les parties intérieures de cheminée ont été immergées dans le fluide hydrothermal du site concerné et placées sous atmosphère N_2. Les échantillons destinés à l'extraction de l'ADN ont été congelés dans du N_2 liquide et conservés à -80°C. Les échantillons destinés à la culture ont été réduits en bouillie dans une chambre anaérobie, aliquotés en sous-échantillons de 35-40 g et conservés sous atmosphère N_2 à 4°C.

La métagénomique

Le troisième chapitre est basé sur l'analyse métagénomique de l'ADN environnemental de RdK (un échantillon de 2014), BdJ (deux échantillons de 2011 et 2014) et ST07 (deux échantillons de 2011 et 2014). L'extraction de l'ADN a été réalisée en 2016 en utilisant une approche basée sur le phénol-chloroforme en combinaison avec le « bead beating ». Le séquençage shotgun du génome entier a été réalisé sur un système Illumina HiSeq 3000. Après le contrôle de la qualité des reads, des assemblages *de novo* indépendants ont été réalisés à

l'aide de metaSPAdes. Par la suite, un ensemble de génomes assemblés de métagénome (MAG) a été établi. Le binning initial a été réalisé avec MetaBAT2, MaxBin2 et CONCOCT. Pour obtenir des résultats optimaux, nous avons ensuite procédé à un raffinement consensuel des bin avec metaWRAP bin_refinement. Les seuils de complétude et de contamination des MAG ont été fixés à 50 % et 10 % respectivement.

Dans la première partie du chapitre 3, les MAG des cinq métagénomes différents sont caractérisés et comparés les uns aux autres. Les régions codantes des protéines ont été prédites à l'aide de Bakta (intégrant Pyrodigal). Les séquences d'acides aminés ont été utilisées pour calculer l'état d'oxydation moyen des protéines de chaque métagénome. Pour contextualiser nos données, nous avons également calculé l'état d'oxydation moyen des protéines des organismes aérobies, microaérophiles et anaérobies enregistrés dans la base des données BacDive. Ensuite, nous avons construit un arbre phylogénomique incluant tous nos MAG et des séquences provenant d'autres écosystèmes serpentinisées à l'aide de GTDB-tk. Nous avons également calculé l'abondance relative des MAG dans leurs échantillons respectifs avec CoverM.

Dans la deuxième partie du chapitre, nous explorons la capacité métabolique des MAG à utiliser le bicarbonate, le formiate, l'acétate et la glycine comme source de carbone. L'absorption de ces quatre composés carbonés repose sur des protéines transporteuses qui facilitent leur passage à travers la membrane cellulaire. En tant que sources de carbone autotrophes, le bicarbonate et le formiate sont ensuite transformés en CO_2 par l'intermédiaire de l'anhydrase carbonique et de la formiate déshydrogénase, respectivement. Le CO_2 ainsi produit peut ensuite passer par une voie de fixation du carbone pour produire de l'acétyl coenzyme A (acétyl-CoA). En tant que sources de carbone hétérotrophes, l'acétate et la glycine sont directement assimilés à l'acétyl-CoA par l'intermédiaire de l'acétyl-coA synthétase (alternativement acétate kinase et phosphate acétyltransférase) ou de la glycine réductase, respectivement. Nous avons recherché dans nos MAG les gènes codant pour les transporteurs correspondants et les variantes de l'anhydrase carbonique, de la formate déshydrogénase, de l'acétyl-coA synthétase, de l'acétate kinase, de la phosphate acétyltransférase et de la glycine réductase à l'aide de kofam_scan, qui recherche dans les séquences protéiques d'entrée des identifiants d'orthologues KEGG (KO) basés sur des profils de « hidden Markow models » (HMM).

Pour identifier les voies de fixation du carbone dans les MAG avec des gènes d'absorption du bicarbonate et du formiate, nous avons fusionné les bases de données KEGG et MetaCyc afin de constituer une collection d'identifiants KO pour chaque version de voie. Les structures finales des voies ont été encodées dans un script bioinformatique analysant les séquences d'entrée pour les identifiants KO respectifs via kofam_scan et calculant un score de complétude de voie pour chaque MAG.

Outre les gènes de l'absorption du carbone et les voies de fixation du carbone, nous avons recherché dans nos MAG les familles de gènes jouant un rôle clé dans le métabolisme de H_2, CO, CH_4, de l'azote, du soufre et de O_2 à l'aide de kofam_scan.

Enfin, toutes les informations métaboliques ont été synthétisées pour identifier les MAG avec un ensemble complet de gènes permettant la fixation ou l'assimilation d'une des quatre sources de carbone putatives, identifiant ainsi les espèces fondatrices autotrophes et hétérotrophes potentielles dans les différents échantillons.

La culture

Le quatrième chapitre est basé sur la culture de consortia microbiens de ST07 dans des conditions anoxiques et hydrogénotrophiques. Les échantillons ont été collectés lors d'une campagne de terrain en 2022 et le travail de laboratoire a été partiellement réalisé au cours d'un stage de master de 6 mois qui a été supervisé dans le cadre de cette thèse.

Quatre conditions de culture différentes ont été établies, chacune avec une seule source de carbone : soit le bicarbonate, le formate, l'acétate ou la glycine. Chaque condition de culture a été testée deux fois, ce qui a donné un total de huit cultures qui ont été maintenues pendant 25 à 26 jours. Les consortia microbiens cultivés ont été comparés les uns aux autres en ce qui concerne la croissance cellulaire, la consommation de la source de carbone et des accepteurs d'électrons, la composition et la diversité de la communauté.

Les expériences de culture ont été réalisées dans des bioréacteurs anaérobies. La plate-forme était constituée de deux bioréacteurs en verre à double enveloppe d'un volume effectif de 1,5 L, chacun étant placé sur une balance pour mesurer le volume des liquides contenus. Le milieu de culture était agité par des hélices axiales à 450 ou 500 rpm, respectivement, permettant un transfert de masse de gaz équivalent pour les deux bioréacteurs. La température a été mesurée à l'aide d'un capteur et régulée automatiquement à 35 ± 2 °C avec de l'eau chauffée dans un bain puis pompée à travers la double enveloppe. De même, le potentiel d'oxydoréduction et le pH ont été mesurés à l'aide d'un capteur et le pH a été régulé à 10,5 ± 0,5 par ajout automatique ou manuel de NaOH. Les deux bioréacteurs ont été alimentés par un flux continu de gaz N_2 et H_2 à raison de 5 ml min^{-1} chacun. Le taux d'entrée du gaz était contrôlé par deux débitmètres massiques. Le flux de gaz sortant a été condensé dans un refroidisseur d'eau en verre à 4°C pour éviter la perte de volume de liquide du bioréacteur. La composition du flux de gaz sortant a été mesurée sur un système de chromatographie en phase gazeuse en aval avec un détecteur catharométrique et une sonde CO_2. Les échantillons ont été prélevés au moyen d'un tube immergé dans le milieu de culture et fermé par un fermoir à l'extérieur du bioréacteur. L'agitation, le pH, la température et l'apport de gaz ont été automatiquement régulés par une boucle locale. Le système a été surveillé et contrôlé à l'aide des logiciels BatchPro et SOPRANE.

Le milieu de culture était basé sur la composition minérale du fluide hydrothermal naturel de Prony. Du thiosulfate et du sulfate 5 mM ont été ajoutés comme accepteurs d'électrons. 5 mM d'acétate de sodium, de formiate de sodium, de glycine ou de bicarbonate de sodium ont été ajoutés comme seule source de carbone en fonction des conditions de culture. Le milieu a été pompé à travers un tube stérile dans le bioréacteur autoclavé, en passant à travers un filtre acrodisque (0,2 µm) pour la stérilisation. Ensuite, le milieu a été agité à grande vitesse sous un flux important de gaz N_2 jusqu'à ce que l'O_2 soit complètement éliminé.

L'inoculation a été effectuée en ouvrant légèrement le couvercle du bioréacteur sous un débit élevé de gaz N_2 et une agitation très lente pour minimiser la diffusion d'air dans le milieu de culture, et à côté d'une flamme pour garantir la stérilité.

Un échantillon a été prélevé dans chaque bioréacteur tous les 2 ou 3 jours, le premier directement après l'inoculation et le dernier juste avant la fin de l'expérience. Une aiguille stérile a été fixée à l'extrémité du tube d'échantillonnage et insérée dans un tube Hungate anoxique hermétiquement fermé avant d'ouvrir le fermoir. Ensuite, une seringue rincée au gaz

N_2 a été insérée dans le tube de Hungate et le piston a été tiré vers le haut pour créer une pression négative, provoquant le transfert du milieu de culture du bioréacteur dans le tube. Un sous-échantillon de 4 ml a été fixé avec 2% de formaldéhyde pour la microscopie à fluorescence et conservé à 4°C. Un sous-échantillon de 14 ml a été centrifugé à 7000 rpm et 4°C pendant 20 min pour précipiter un culot cellulaire à partir de l'échantillon de culture. Le culot a été conservé à -80°C pour l'extraction de l'ADN. Le surnageant a été conservé à -20°C pour la chromatographie liquide haute performance (HPLC) et la chromatographie ionique (IC).

La consommation de formiate et d'acétate a été mesurée par HPLC en utilisant une phase mobile d'eau ultrapure avec 5 mM H_2SO_4 éluée à un taux de 0,6 ml min^{-1}. La colonne a été maintenue à 45°C. La détection des pics a été effectuée à l'aide d'un détecteur UV et d'un réfractomètre. La consommation de sulfate et de thiosulfate a été mesurée avec IC en utilisant une phase mobile d'eau pure avec 9 mM de Na_2CO_3 éluée à 1 ml min^{-1}. La colonne d'échange d'anions a été maintenue à 45°C. Un suppresseur ampérométrique a été placé avant le détecteur de conductivité afin d'améliorer la détection des pics. La supervision de la machine et l'analyse du signal ont été effectuées avec le logiciel Chromeleon.

Le sous-échantillon fixé au formaldéhyde a été dilué dans un milieu de croissance stérile et filtré sur des filtres en polycarbonate blanc (porosité de 0,2 μm et diamètre de 25 mm) à l'aide d'une pompe à vide. Pour chaque échantillon, au moins deux filtres ont été préparés. Les filtres ont été colorés avec une solution de 3,15 μM de 4′,6-Diamidin-2-phenylindol (DAPI). Ensuite, les filtres ont été analysés sous un microscope OLYMPUS BX-61 avec un filtre à 372 nm. Les cellules ont été comptées dans 15 champs aléatoires et ces comptages ont été moyennés entre les réplicats afin d'établir une courbe de croissance pour chaque culture.

Pour analyser la composition et la diversité des huit consortia microbiens cultivés, nous avons effectué une analyse basée sur l'amplicon du gène de l'acide ribonucléique ribosomique 16S (ARNr 16S). L'ADN a été extrait des culots récupérés lors de l'étape de centrifugation, à l'aide du kit DNAeasy PowerSoil Pro. La région V3-V4 du gène ARNr 16S a été amplifiée à l'aide d'une paire d'amorces bactériennes et d'une paire d'amorces archéennes et séquencée sur un système Illumina MiSeq. Après un contrôle de la qualité des reads dans DADA2, les variants de séquence d'amplicon (ASV) ont été reconstruits et assignés taxonomiquement à l'aide de la base de données SILVA. L'ensemble des données a été normalisé à un nombre de reads soigneusement sélectionné, puis analysé quant à sa diversité. Plusieurs indices de diversité alpha ont été calculés et comparés entre les conditions de culture, et une matrice de distance a été construite pour évaluer l'influence de la source de carbone sur la structure de la communauté des différents consortia microbiens. Enfin, l'abondance relative des ASV par culture a été calculée à trois moments d'échantillonnage pour évaluer l'enrichissement de groupes taxonomiques uniques (espèces autotrophes et hétérotrophes putatives) dans chaque condition de culture.

CHAPITRE 3 : la métagénomique

L'état d'oxydation moyen des échantillons a diminué depuis les sites terrestres influencés par la lumière et l'air jusqu'aux sites sous-marins plus anoxiques, à l'exception de ST0714 qui a probablement été contaminé par de l'eau de mer pendant le processus d'échantillonnage et qui

peut donc être interprété comme représentatif de la zone de mélange entre le fluide hydrothermal et l'eau de mer. Tous les autres échantillons présentaient une communauté classique colonisant des systèmes serpentinisés, qui passait de *Gammaproteobacteries* aérobies et microaérophiles telles que les *Burkholderiales* à des anaérobies telles que les *Desulfovibrionales*, les *Methanocellales* et les *Bipolaricaulia* dans ST0711. La distribution des MAG dans l'arbre phylogénétique n'était pas spécifique à un site ou à un système, ce qui suggère que la baie de Prony ne constitue pas une niche écologique isolée. Dans les clades phylogénétiques, cependant, les MAG de tous les sites de la baie de Prony formaient des microclusters.

Les communautés microbiennes de tous les sites dépendaient de l'acétate et du bicarbonate comme sources primaires de carbone. Il est peu probable que l'assimilation de la glycine joue un rôle à Prony, car seuls quelques MAG à faible abondance présentaient une glycine réductase et aucun d'entre eux un transporteur de glycine. La glycine réductase a été détectée dans plusieurs autres écosystèmes serpentinisées. Cependant, elle est incorporée dans la voie de la glycine réductrice et, bien qu'elle puisse assimiler la glycine exogène, elle peut également traiter la glycine endogène produite à partir du bicarbonate ou du formiate en tant qu'intermédiaire dans la fixation du carbone.

De même, la fixation du formiate ne contribue probablement pas de manière significative au bilan carbone des communautés de la baie de Prony échantillonnées. Peu de MAG peu abondants présentaient un transporteur de formiate ou une déshydrogénase de formiate, et aucun d'entre eux ne présentait les deux protéines. L'utilisation signalée du formiate comme source de carbone n'est pas cohérente. En particulier à Lost City, il a été proposé que le formate alimente la méthanogénèse autotrophe dans les biofilms dominés par LCMS. Cela implique probablement des relations symbiotiques, étant donné que les LCMS ne présentent pas de transporteurs de formiate ou de gènes de déshydrogénase de formiate. Il est intéressant de noter que presque tous les MAG de la baie de Prony peu abondants présentant un transporteur de formiate appartiennent aux *Methylococcales*.

Contrairement à la glycine et au formate, le transporteur d'acétate actP et surtout l'acétyl-coA synthétase étaient présents dans un nombre considérable de MAG. Des gènes d'assimilation de l'acétate ont été documentés dans les métagénomes de plusieurs environnements serpentinisées. Si l'acétate peut être produit de manière abiotique, il est probablement aussi produit par acétogenèse et fermentation. La contribution réelle des organismes assimilateurs d'acétate à la base du réseau trophique est donc difficile à établir et nécessiterait des analyses plus détaillées du fluide hydrothermal de Prony.

Outre l'acétate, une proportion relativement importante de MAG à forte abondance comportait le transporteur de bicarbonate sbtA et une anhydrase carbonique gamma. Le bicarbonate est très abondant dans les systèmes à serpentinite, ce qui en fait une source de carbone primaire idéale pour les organismes capables de le redissoudre des cheminées hydrothermales carbonées. On a observé que *Serpentinimonas*, associé à l'ordre des *Burkholderiales*, formait des agrégats sur des particules de carbonate de calcium en culture. On a émis l'hypothèse que ces agrégats diminuent le pH dans les micro-environnements et concentrent localement le CO_2. Il est probable que d'autres groupes taxonomiques ont évolué vers la même stratégie de fixation du carbone pour s'adapter à la limitation du DIC dans les environnements serpentinisés.

Cinq taxons fondateurs autotrophes et hétérotrophes potentiels possédant un ensemble complet de gènes d'absorption du bicarbonate ou de l'acétate ont pu être identifiés. Il ne s'agit que d'aérobies et d'anaérobies facultatifs, à l'exception d'un *Desulfobulbales* MAG peu abondant provenant de ST0714. Nous n'avons donc échantillonné que des espèces fondatrices de l'environnement de surface.

Le premier taxon putatif de la fondation de surface est un organisme aérobie assimilateur d'acétate associé aux *Cyanobacteriia* (genre RDYZ01). Il a été démontré que l'acétate peut favoriser la croissance des cyanobactéries en tant que source de carbone supplémentaire dans des conditions de faible luminosité, en supprimant l'activité photosynthétique et en renforçant le métabolisme glycolytique. Deux autres espèces putatives de fondations de surface présentaient un patrimoine génétique mixotrophe, avec des gènes d'absorption de l'acétate et du bicarbonate. Une *Rhodobacterales* MAG aérobie de BdJ11 était capable d'oxyder le sulfure et le thiosulfate. Il a été suggéré que le thiosulfate pourrait fournir un "regain d'énergie" supplémentaire pour la production d'adénosine triphosphate (ATP) et l'assimilation du CO_2 dans des conditions hyperalcalines. En outre, ce MAG peut probablement passer à un mode de vie chimio-autotrophe en fixant le bicarbonate par oxydation du sulfure ou du thiosulfate. À côté du MAG *Rhodobacterales*, un MAG *Burkholderiales* de RdK14 présentait un ensemble complet de gènes d'absorption de l'acétate et du bicarbonate. La composition métabolique de cet organisme était étonnamment diversifiée : il présentait non seulement des cytochromes mais aussi des gènes pour la respiration du nitrate, du nitrite et du sulfite, ainsi que la capacité d'utiliser le CO, le sulfure et l'H_2 comme source d'énergie. Outre ces taxons mixotrophes, trois autres MAG présentaient des gènes d'absorption du bicarbonate. Il s'agit de deux organismes associés aux *Xanthomonadales* de BdJ11 (genre *Silanimonas*) et de ST0711 (famille *Wenzhouxiangellaceae*). Le premier était capable d'oxyder le sulfure, tandis que le second ne présentait aucun gène permettant d'utiliser une source d'énergie lithotrophe. Il est donc peu probable qu'elle contribue à la fixation primaire du carbone dans la baie de Prony. La dernière espèce autotrophe putative de fondation était associée à la gammaprotéobactérie UBA7966 (UBA7966 sp003485715) et capable d'utiliser à la fois le thiosulfate et l'hydrogène comme source d'énergie. Curieusement, tous les fixateurs de bicarbonate proposés ont obtenu des complétudes élevées pour le cycle CBB, mais il leur manquait l'enzyme RuBisCo.

CHAPITRE 4 : la culture

L'inoculum utilisé pour les expériences de culture était dominé par plusieurs groupes taxonomiques que l'on trouve couramment dans la baie de Prony et dans d'autres environnements serpentinisés : *Acetoanaerobium*, *Desulfovibrio* et *Truepera*. Aucun de ces taxons n'était significativement enrichi dans aucune des conditions de culture, ce qui suggère que ces taxons marqueurs dépendent de sources de carbone autres que le bicarbonate, le formiate, l'acétate ou la glycine.

Toutes les sources de carbone testées ont facilité la croissance et le développement des cultures, à l'exception de la glycine. Le consortium microbien maintenu sur la glycine présentait un taux de croissance très faible et les scores de diversité et d'homogénéité sont restés constamment élevés, ce qui suggère qu'il n'y a pas eu d'enrichissement significatif des groupes taxonomiques spécialisés dans l'absorption de ce composé carboné. Cela a été confirmé par la

composition taxonomique des consortia, qui n'a pas beaucoup changé au fil du temps et est restée similaire à la communauté naturelle. Il est probable que la glycine soit thermodynamiquement moins favorable que les autres sources de carbone, car sa concentration dans le système naturel devrait être extrêmement faible.

Une croissance significative a été observée dans les cultures cultivées sur formiate, bicarbonate et surtout acétate, accompagnée d'une forte diminution de la diversité et d'homogénéité, suggérant un enrichissement de groupes taxonomiques spécifiques sur ces sources de carbone. Ceci est confirmé par l'évolution de la composition taxonomique des cultures après la deuxième et la troisième semaine d'incubation.

Les consortia cultivés sur l'acétate et le formiate étaient fortement dominés par *Roseibaca*. Les membres de ce genre sont des photohétérotrophes anoxygéniques et leur couleur rose est due à leurs pigments des bactériochlorophylles. Curieusement, *les Roseibaca* sont classés comme aérobies stricts, mais les concentrations d'O_2 sont restées bien inférieures à 0,5 % pendant toute la période d'incubation dans toutes les conditions de culture. Comme les expériences ont été réalisées dans des bioréacteurs en verre transparent, la lumière ambiante aurait pu favoriser la croissance de phototrophes oxygénés fournissant localement de l'O_2. Cependant, aucune présence significative de tels taxons n'a été observée. Certains membres de *Roseibaca* pourraient donc être des anaérobies facultatifs. Les pigments bactériochlorophyll utilisés dans la photosynthèse anoxygène peuvent absorber la lumière à des longueurs d'onde proches de l'infrarouge et faciliter la photosynthèse dans les eaux profondes ou les sédiments (Hanada, 2016). En outre, l'apparition de taxons normalement strictement aérobies dans des fluides serpentinisés anoxiques est un phénomène relativement courant et a été suggéré pour conduire à la spéciation localisée de populations étroitement apparentées. Outre *Roseibaca*, les consortia cultivés sur acétate et formate comprenaient une proportion significative de *Meiothermus*. Les cultures alimentées avec du formiate présentaient en outre un léger enrichissement en *Thioalkalimicrobium*, la bactérie la plus susceptible d'oxyder le thiosulfate, ainsi qu'un enrichissement à moyen terme en *Acetoanaerobium*. Cette dernière a probablement fermenté les composés organiques produits par le consortium fondateur au cours de la première semaine.

Les consortia cultivés sur bicarbonate étaient fortement dominés par le genre *Meiothermus*. Comme *Roseibaca*, *Meiothermus* a été décrit comme un hétérotrophe strictement aérobie ; cependant, il a été démontré que les populations de subsurface peuvent subir une spéciation parapatrique et développer des traits d'autotrophes facultativement anaérobies. Il est donc probable que la population de *Meiothermus* cultivée sur bicarbonate se soit développée de manière chimio-autotrophe en produisant du CO_2 avec H_2 comme donneur d'électrons anaérobie. Outre *Meiothermus,* un enrichissement significatif des *Hydrogenophaga* chimio-autotrophes*,* un groupe frère du genre marqueur *Serpentinimonas*, ainsi qu'un enrichissement des *Roseibaca* ont pu être observés. Alors que *les Roseibaca* sont généralement classés comme photohétérotrophes, leur croissance constante et significative sur le bicarbonate pourrait suggérer que nous avons enrichi un nouveau génotype photoautotrophe. En outre, le genre *Halomonas* était dominant au cours de la deuxième semaine du premier cycle expérimental. Ce genre étant hétérotrophe, il est peu probable qu'il ait utilisé le bicarbonate comme source de carbone.

La communauté des archées initiale se composait presque exclusivement du groupe ANME-3, qui appartient aux *Methanosarcinales* (95,31 % d'identité de séquence avec LCMS). Ce groupe n'a pas été enrichi mais est resté stable pendant toute la durée des expériences de culture sur toutes les sources de carbone, avec une prédominance si forte que la composition taxonomique archéen n'a pas varié de manière significative entre les sources de carbone. Outre le maintien des *Methanosarcinales*, un léger enrichissement des *Woesearchaeales* a été observé sur le formiate, le bicarbonate et surtout l'acétate lors de la première série d'expériences. La composition taxonomique des archées peut refléter une relation symbiotique émergente entre les *Methanosarcinales* et les *Woesearchaeales*. *Les Woesearchaeales* sont probablement incapables de mener une vie indépendante et il a été suggéré qu'elles établissent des relations symbiotiques avec les méthanogènes. Jusqu'à présent, seuls les génotypes hétérotrophes sont connus. Il est possible que notre souche *Woesearchaeales* soit capable d'oxyder l'acétate en CO_2 et H_2 pour un partenaire *Methanosarcinales*. Dans la deuxième série expérimentale, un enrichissement remarquable des *Methanomicrobiales* a été observé sur le bicarbonate vers la fin de la culture (81,44% d'identité de séquence ASV avec TCMS).

La croissance prononcée sur l'acétate soutient la théorie des espèces fondatrices proposée par Lang et ses collègues. Dans les bioréacteurs alimentés en acétate, le consortium hétérotrophe fondateur était fortement dominé par le photohétérotrophe anoxygène *Roseibaca*, ce qui suggère que ce métabolisme joue un rôle plus important dans la subsurface (peu profonde) des environnements serpentinisés que ce que l'on supposait auparavant. Outre *Roseibaca*, le consortium comprenait le génotype hétérotrophe de *Meiothermus*. Ensemble, *Roseibaca* et *Meiothermus* peuvent produire du CO_2 à partir de l'acétate pour divers autotrophes du premier niveau trophique de l'écosystème naturel.

A côté de ce consortium fondateur hétérotrophe putatif, nous avons cultivé des consortia fondateurs autotrophes potentiels sur le bicarbonate et le formiate. Sur le bicarbonate, il s'agissait du génotype autotrophe *Meiothermus* et du *Hydrogenophaga* hydrogénotrophe. Il est probable que le même génotype *Meiothermus* fait partie de la base du réseau trophique cultivé sur formate, aux côtés de l'autotrophe oxydant le thiosulfate *Thioalkalimicrobium* et potentiellement d'un nouveau génotype photoautotrophe de *Roseibaca*. Ensemble, ces autotrophes fixant le bicarbonate et le formiate pourraient produire des composés organiques pour les hétérotrophes du premier niveau trophique tels que *Acetoanaerobium* et *Halomonas*.

Un cas particulier de ce cycle du carbone entre hétérotrophes et autotrophes constitue la relation entre méthanogènes et méthanotrophes : Les méthanotrophes et les méthanogènes acétoclastiques pourraient fournir du CO_2 aux méthanogènes autotrophes et vice versa. Les *Methanomicrobiales* pourraient effectuer la méthanogenèse avec du bicarbonate comme source de carbone autotrophe, constituant ainsi une espèce fondatrice autotrophe. Les *Methanosarcinales* prédominantes, quant à elles, dépendent probablement du CO_2 qui est soit produit par un symbiote *Woesearchaeales*, soit libéré localement par d'autres membres de la communauté.

CHAPITRE 5 : discussion et perspectives

La métagénomique et la culture étaient complémentaires non seulement d'un point de vue méthodologique, mais aussi en termes de résultats : alors que l'analyse métagénomique a

permis de comprendre la base du réseau trophique dans les environnements de surface de la baie de Prony, les expériences de culture ont permis d'accéder aux communautés anaérobies de la subsurface peu profonde. En combinant les deux approches, nous sommes en mesure de proposer une ébauche de la communauté fondatrice près de la surface serpentinisée, en surmontant la limitation du DIC. Les deux approches suggèrent que l'écosystème de la baie de Prony ne dépend pas de la glycine comme source primaire de carbone. Les communautés de surface ont pu métaboliser le bicarbonate et l'acétate, et les communautés cultivées en subsurface peu profonde ont pu se développer en plus sur le formiate.

L'identification du bicarbonate, du formiate et de l'acétate comme sources potentielles de carbone confirme l'importance des espèces fondatrices aussi bien autotrophes qu'hétérotrophes. Les espèces autotrophes de surface comprennent les *Xanthomonadales* gammaprotéobactériennes fixant le bicarbonate et l'UBA7966. Les espèces autotrophes de subsurface peu profonde comprennent les gammaprotéobactéries *Hydrogenophaga* (fixation du bicarbonate) et *Thiolkalimicrobium* (fixation du formiate), ainsi qu'un génotype *Meiothermus* récemment proposé (principalement fixation du bicarbonate) et un génotype photautotrophe putatif de *Roseibaca* (principalement fixation du formiate). En outre, une espèce fondatrice de subsurface peu profonde méthanogène fixant le bicarbonate et associée aux *Methanomicrobiales* a été identifiée. Les espèces fondatrices hétérotrophes assimilant l'acétate comprennent un taxon de *Cyanobacteriia* dans les environnements de surface et les génotypes standard de *Meiothermus* ainsi que de *Roseibaca* dans la subsurface peu profonde. En outre, une symbiose potentielle entre une souche *Woesearchaeales* oxydant l'acétate et un membre de *Methanosarcinales* étroitement lié au phylotype Lost City a été observée lors d'expériences de culture anaérobie. Dans l'environnement de surface, nous avons également identifié deux espèces fondatrices mixotrophes qui présentaient le patrimoine génétique nécessaire pour fixer le bicarbonate et assimiler l'acétate : les *Rhodobacterales* (qui incluent le genre *Roseibaca*) et les *Burkholderiales*. Alors que deux génotypes distincts ont été décrits pour *Meiothermus*, il est possible que le taxon *Roseibaca* que nous avons cultivé soit en fait mixotrophe au lieu d'avoir divergé en génotypes distincts. Les espèces fondatrices identifiées dépendent d'espèces de soufre réduites, de H_2 et de la lumière comme sources d'énergie, et de O_2 ainsi que d'espèces d'azote oxydées comme accepteurs d'électrons.

Bien que les communautés de surface et de subsurface peu profonde décrites fassent partie intégrante de l'écosystème de la baie de Prony, elles ne sont pas entièrement représentatives de la communauté colonisant des systèmes serpentinisés à ramifications profondes qui constitue un analogue potentiel de la Terre primitive. Pour approfondir les questions de recherche liées à l'origine de la vie, il est nécessaire d'évaluer la communauté de subsurface à une plus grande échelle que celle à laquelle nous avons eu accès ici. Cela implique une révision des techniques utilisées dans cette thèse, ainsi qu'un élargissement potentiel du pool de sources potentielles de carbone primaire pour lesquelles les membres de la communauté sont testés.

Pour accéder à la subsurface profonde grâce à la métagénomique, il faudrait non seulement cibler une plus grande diversité de taxons bactériens, mais aussi récupérer davantage de séquences archéennes qui sont typiquement associées aux environnements profonds serpentinisés. Cet objectif pourrait être atteint en optimisant les protocoles de collecte d'échantillons et d'extraction d'ADN. Les expériences de culture devraient être menées dans des bioréacteurs opaques afin d'exclure la lumière comme source d'énergie. En outre, le

protocole expérimental doit être adapté pour éclairer la "boîte noire du bioréacteur", c'est-à-dire pour exclure la possibilité que les taxons fondateurs identifiés soient enrichis par les produits métaboliques d'autres membres de la communauté. Pour ce faire, il est possible d'effectuer un sondage des isotopes stables de l'ADN (DNA-SIP), des cultures axéniques et d'évaluer les réponses transcriptionnelles des communautés aux différentes conditions de culture. Ce dernier objectif peut être atteint par la métatranscriptomique, l'ADN complémentaire (cDNA) ou des approches de capture de gènes. En outre, le matériel carboné doit être éliminé de l'inoculum afin d'éviter l'introduction de bicarbonate dans toutes les conditions de culture (dans les expériences présentées ici, ce biais est compensé par le fait que les consortia cultivés sur bicarbonate présentaient des taux de croissance inférieurs à ceux des consortia cultivés sur formiate et acétate). L'utilisation d'échantillons de fluides hydrothermaux purs, tant pour la métagénomique que pour la culture, serait une possibilité de s'attaquer à la communauté de la subsurface profonde.

Ces deux approches se limitent à un petit extrait de la communauté naturelle qui n'est probablement pas représentatif de l'écosystème dans son ensemble. Cela pose des problèmes, notamment en ce qui concerne la subsurface profonde, car on sait peu de choses sur la dimension et la constitution de ce type d'environnement en général. Concernant la métagénomique, l'échelle de l'écosystème peut être visée en évaluant la matière noire fonctionnelle et en reconstruisant *de novo* les familles de protéines. Concernant la culture, une représentation plus précise de la communauté naturelle pourrait être obtenue dans des microcosmes.

Outre la minimisation des biais méthodologiques, l'identification des espèces fondatrices de la subsurface profonde pourrait nécessiter l'étude d'autres sources de carbone et types de métabolisme. Les expériences menées dans le cadre de la recherche sur l'origine de la vie ont révélé une gamme relativement large de composés organiques pouvant être produits de manière abiotique en laboratoire. Bien qu'il ne soit pas prouvé que ces réactions puissent se produire dans l'environnement naturel, elles pourraient hypothétiquement alimenter des espèces fondatrices fermentaires dans la subsurface profonde. En outre, la prévalence des phototrophes dans nos consortia de fondations hypothétiques est remarquable. Il a été suggéré que les phototrophes utilisant l'infrarouge colonisent la subsurface profonde des cheminées hydrothermales en utilisant le rayonnement thermique émis par les fluides chauds ; il pourrait donc être intéressant d'examiner la présence potentielle de phototrophes dans la subsurface profonde de la baie de Prony.

L'identification d'espèces fondatrices hétérotrophes et autotrophes dans un système potentiellement analogue à la Terre primitive suscite le débat sur l'origine hétérotrophe ou autotrophe de la vie. La théorie hétérotrophe implique que les composés organiques sont apparus avant le métabolisme primordial. La théorie de la "soupe primitive" a dominé le domaine de la recherche pendant longtemps et reste aujourd'hui l'un des principaux concepts, mais elle présente des faiblesses fondamentales. C'est pourquoi de nombreuses approches sont actuellement centrées sur une origine autotrophe de la vie, en se concentrant sur les minéraux contenant des métaux de transition qui pourraient avoir catalysé des réactions de fixation du carbone autotrophes auto-entretenues lors de l'émergence de la vie. Tous ces minéraux se trouvent dans des environnements serpentinisés et pourraient également expliquer l'origine des premières voies hétérotrophes. Les métabolismes du carbone et des hydrates de carbone

impliquent des voies qui sont potentiellement anciennes et comprennent des réactions qui peuvent être catalysées sans enzymes. Il s'agit notamment de la voie de Wood-Ljundahl, du cycle rTCA et de la gluconéogenèse, de la glycolyse et de la voie des pentoses phosphates. En outre, les métalloenzymes ne sont pas seulement importantes dans le métabolisme du carbone et des hydrates de carbone, mais aussi dans le métabolisme énergétique lithotrophe et la fermentation impliquant des hydrogénases NiFe et FeFe, qui étaient présentes dans nos consortia microbiens de surface et de subsurface peu profonde. La bifurcation électronique basée sur la flavine constitue un cas particulier de bioénergétique lors de l'émergence de la vie.

Alors que de nombreux chercheurs considèrent qu'une origine autotrophe de la vie est plus plausible qu'une origine hétérotrophe aujourd'hui, les résultats présentés dans cette thèse suggèrent que non seulement les autotrophes, mais aussi les hétérotrophes jouent un rôle fondamental dans le réseau trophique d'un système qui peut être interprété comme un analogue de la Terre primitive. L'évaluation de la subsurface de la baie de Prony pourrait fournir des résultats plus clairs sur cette question à l'avenir.

Acknowledgements

Firstly, I would like to thank the members of my jury for taking the time to evaluate the work presented here, and the members of my PhD committee for providing valuable advice throughout the years.

Un très grand merci à mes directeur.rices de thèse. Gaël, merci pour les nombreuses opportunités et portes ouvertes, pour la confiance, pour m'avoir poussée à toujours continuer à poser des questions et à aller plus loin. Un merci tout particulier pour m'avoir donné l'opportunité de participer à une campagne de terrain, ce dont je rêvais depuis le début de mes études. Anne, merci d'avoir toujours eu une main tendue et une oreille attentive, pour ton soutien au laboratoire que j'ai beaucoup apprécié et pour la relecture. C'est grâce à vous que j'ai beaucoup appris ces trois dernières années.

Merci à toute l'équipe de MICROPRONY, vous m'avez tous.tes aidée d'une manière différente et j'ai beaucoup apprécié chacun.e - je suis heureuse d'avoir pu travailler avec vous. Je remercie tout particulièrement Mélanie, Agathe et Sylvain, sans qui cette thèse n'aurait pas été possible.

Thank you to everyone who has supported the work presented here, by collecting samples and participating in field work (special thanks to Roy Price and Jacopo Pasotti, who also provided the photos that illustrate this thesis), taking the time to discuss experimental designs, teaching me laboratory and bioinformatic techniques, answering my questions, engaging in inspiring conversations on science and beyond, and sometimes giving me personal advice.

Enfin, mille mercis aux “filles du labo” pour avoir été là tout au long de ces années, pour l'expérience partagée, les conseils (Aina, Elsa, vous m'avez sauvé la vie) et pour les moments que nous avons passés ensemble dans et en dehors du labo.

Mama, Papa, als ich beschloss, Meeresbiologin zu werden, habt ihr nicht versucht, mich vom Gegenteil zu überzeugen, sondern euch mit mir zusammengesetzt und einen Plan gemacht. Ich werde diesen Sommernachmittag nie vergessen, an dem ich verstanden habe, dass ihr meine Träume bedingungslos unterstützt. Danke, dass ihr nachgefragt und zugehört habt, dass ihr mir einen sicheren Ort geboten habt, an den ich immer zurückkehren konnte, und dass ihr mich all die Jahre sanft angeleitet habt, wenn ich es brauchte. Ich habe eure Unterstützung nie als selbstverständlich angesehen; diese Dissertation ist euch gewidmet.

Danke an Raban und Johanna, Rasmus mit Marie und den Rest meiner Familie, dass ihr immer für mich da wart und an meinen Erfahrungen teilgenommen habt. An diesem einen Festivalmorgen, an dem wir nach einer langen Nacht bereits in unseren Schlafsäcken lagen und begonnen haben, über die Installation eines widerspenstigen Conda-Pakets zu diskutierten, hättet ihr mir sagen können, ich solle einfach die Klappe halten. Aber das habt ihr nicht getan, und das ist alles, was gesagt werden muss.

I would not have made it to the last paragraph of this thesis without my friends, who listened to endless monologues over the phone and over coffee, reminded me that there is a life outside of science, picked me up when I came home crying, celebrated every small success, recruited

acquaintances to help me out just in case, cheered me on in all of my phases between hating and loving science. Thank you, to each and every one of you – Rike, Sophie, Billy, Amos, Alex, thank you for always being there even if you are far, Bissera, Adam, Ariane, Liza, Tim, Paul, thank you for having been a part of "home", thank you to all of my friends from Germany and Amsterdam, and to everyone who is now part of my life in Marseille. You were so much more important than you think.

Anne-Ying. Quand on s'est rencontrées, je t'ai demandé si tu avais peur que je fasse une thèse et tu m'as dit que je devais arrêter de m'inquiéter pour ça et me préparer à recevoir du soutien. Et finalement, c'est exactement ce qui s'est passé. Merci pour les rêves partagés, les rires, les larmes, les cadeaux du matin, les gâteaux au chocolat et la bouffe incroyable (je n'allais pas l'avouer mais tu as raison - je n'aurais mangé que du pain et du chocolat ces derniers mois si tu n'avais pas été là) et, surtout, pour les racines.

Table of contents

List of figures..... 27
List of tables..... 32
List of abbreviations..... 33

CHAPTER 1: Introduction..... 36

1.1 Background..... 37
1.1.1 The search for the origin of life..... 37
1.1.2 Taking a closer look at the serpentinite-hosted Prony Bay..... 38
1.2 State of the Art..... 40
1.2.1 Preface..... 40
1.2.2 Article..... 41
1.3 Objectives..... 54
1.4 References..... 56

CHAPTER 2: Material and methods..... 62

2.1 Preface..... 63
2.2 Sample recovery..... 64
2.2.1 Study site..... 64
2.2.2 Sampling procedure..... 65
2.3 The metagenomic approach..... 67
2.3.1 Introduction to metagenomics..... 67
2.3.2 The metagenomic workflow..... 68
2.3.3 Benefits and drawbacks of the metagenomic approach..... 71
2.4 The cultivation approach..... 73
2.4.1 Introduction to the cultivation of anaerobic extremophiles..... 73
2.4.2 The cultivation workflow..... 74
2.4.2.1 The bioreactor platform..... 75
2.4.2.2 The growth medium..... 76
2.4.2.3 Inoculation..... 76
2.4.2.4 The sampling procedure..... 76
2.4.2.5 Chromatography..... 77
2.4.2.6 Cellular growth..... 77
2.4.2.7 Excursion to the metagenomic approach: Targeted gene analysis..... 78
2.4.3 Benefits and drawbacks of the cultivation approach..... 78
2.5 References..... 80

CHAPTER 3: The metagenomic approach..... 85

3.1 Preface..... 86
3.2 Manuscript..... 87

3.3 Figures and tables..... 110
3.4 Supplementary material..... 117

CHAPTER 4: The cultivation approach..... 126

4.1 Preface..... 127
4.2 Manuscript..... 128
4.3 Figures..... 146
4.4 Supplementary material..... 152

CHAPTER 5: Discussion and perspectives..... 157

5.1 Preface..... 158
5.2 Foundation species from the surface and shallow subsurface..... 159
5.3 Tracking down foundation species from the deep subsurface..... 161
5.3.1 Minimizing methodological bias..... 161
5.3.1.1 Metagenomics: Optimization of sample collection and DNA extraction..... 161
5.3.1.2 Cultivation: Illuminating the bioreactor "black box" 162
5.3.1.3 Extrapolating results to the ecosystem scale..... 164
5.3.2 Looking into alternative carbon sources and metabolic types..... 165
5.4 Links to the origin of life..... 167
5.5 References..... 170

List of figures

CHAPTER 1: Introduction

Figure 1-1. The biogeochemical environment of marine serpentinite-hosted systems. (A) Production of compounds of interest in microbial metabolism. Reactions are color-coded with solid arrows showing abiotic processes, empty arrows showing biotic processes, and striped arrows indicating external supply. The metabolic potential of shown compounds as an energy source, electron acceptor, carbon source, or other nutrient source is indicated with different shapes. Note that compound concentrations, external supply, temperature, and pH are strongly site dependent. In continental serpentinizing systems, travertines form instead of chimneys. (B) Redox potential of available electron acceptors along a gradient from the oxic surface to the anoxic interior of the chimney wall. Adapted from Boyd et al. (2014) 42

Figure 1-2. Hypothesized primary carbon sources in serpentinite-hosted systems. Different carbon sources are color-coded. Hypothesized abiotic reactions are indicated with numbers and autotrophic or heterotrophic metabolism of the respective carbon source with letters. Square shapes indicate that the respective carbon source is an organic molecule, oval shapes that it is an inorganic molecule.....55

CHAPTER 2: Material and Methods

Figure 2-1. Study site. (A) Location of Prony Bay at the Southern coast of New Caledonia. (B) Location of the three sites discussed in this thesis at Prony Bay. Maps adapted from Google Earth: Image © 2024 TerraMetrics, Image © 2024 Airbus, Image © 2024 CNES / Airbus. Data SIO, NOAA, U.S. Navy, NGA, GEBCO. Photos from © Jacopo Pasotti, 2022 and © Roy Price, 2022.....64

Figure 2-2. Metagenomic workflow. Sample characterization steps are highlighted in green, the functional analysis in blue.....71

Figure 2-3. Anaerobic fed-batch bioreactor used in cultivation experiments. (A) Scheme detailing technical set-up (B) Photo of a bioreactor in use.....75

CHAPTER 3: The metagenomic approach

Figure 3-1. Average protein oxidation states of sample MAG sequences and reference sequences from the Bacterial Diversity Metadatabase classified as "aerobic", "microaerophilic" or "anaerobic" 110

Figure 3-2. Taxonomic composition per sample showing metagenome-assembled genomes (MAGs) with a relative abundance > 1%. MAGs with a relative abundance < 1% are grouped under "other". MAGs were assigned to orders according to GTDB. Classes shared by at least two orders are indicated with abbreviations: Pac for *Paceibacteria*; Gamma for *Gammaproteobacteria*; Dei for *Deinococci*; Chl for *Chlorobia*; Cyano for *Cyanobacteriia*; Bip for *Bipolaricaulia*. The percentages of unmapped reads are the following: BdJ11: 64%. BdJ14: 63%. RdK14: 42%. ST0711: 59%. ST0714: 82%. MAG identifiers from top to bottom are as follows (note that MAGs were reconstructed from individual assemblies, hence their numbering is not coherent between samples): BdJ11: Bin66, Bin30, Bin47, Bin41, Bin27, Bin26 and Bin46, Bin52, Bin58. BdJ-14: Bin62, Bin7, Bin57, Bin31 and Bin39, Bin33, Bin50, Bin51, Bin9, Bin65, Bin52, Bin66. RdK14: Bin98, Bin78, Bin113, Bin49, Bin2, Bin16, Bin97, Bin44, Bin12, Bin17, Bin76 and Bin92. ST0711: Bin16, Bin11, Bin15, Bin14, Bin12, Bin6. ST07-14: Bin33 and Bin46, Bin53.....111

Figure 3-3. Presence and absence of A) transporters and B) primary assimilation enzymes in the most abundant metagenome-assembled genomes (relative abundance >1%) per sample. The depicted proteins facilitate the primary uptake bicarbonate, formate, acetate or glycine as putative carbon sources. Genes were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109. Where no genes are specified, all available enzyme subdomains (all must be present) or alternatives (one of them must be present) were taken into account.....112

Figure 3-4. Carbon fixation pathway completeness in the most abundant metagenome-assembled genomes (relative abundance > 1%) per sample. Completeness scores were calculated based on custom HMM profiles and are given in percent. A) Distribution of pathway completeness scores in each sample. B) Pathway completeness across metagenome-assembled genomes in each sample.....113

Figure 3-5. Presence and absence of key metabolic genes in the most abundant metagenome-assembled genomes (relative abundance > 1%) per sample. Genes playing a crucial role in the metabolism of hydrogen, carbon monoxide, methane, nitrogen, sulfur or oxygen were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109.....114

Figure 3-6. Proposed metabolism of bicarbonate-fixing autotrophic and acetate-assimilating heterotrophic foundation species in Prony Bay surface environments. The order of MAGs with a complete set of uptake genes is given next to the corresponding carbon source. Enzymes and pathways that are absent or poorly represented in the assessed metagenomes are faded out.

Enzyme commission number sequences occurring in multiple carbon fixation pathways are color-coded.....115

Supplementary Figure 3-1. Phylogenomic tree including all MAGs from this study and reference sequences from other Prony Bay metagenomes (stars in red shades), as well as other terrestrial (circles in pink shades) and marine (triangles in purple shades) serpentinite-hosted systems. MAGs from this study with a relative abundance > 1% in their respective sample are highlighted in pink. Details of reference sequences are given in Supplementary Table 2. The tree is based on the bacterial marker gene set from GTDB R214 and rooted at midpoint.....119

Supplementary Figure 3-2. Relative abundance of A) MAGs assigned to orders according to GTDB and B) 16S rRNA sequences assigned to orders according to SILVA-138. Orders with < 1% relative abundance are grouped under "other". Classes shared by at least two orders are indicated with abbreviations: Pac for *Paceibacteria* or *Parcubacteria*; Gamma for *Gammaproteobacteria*; Bac for *Bacteroidia*; Dei for *Deinococci*; Chl for *Chlorobia*; Cyano for *Cyanobacteriia*; Bip for *Bipolaricaulia*. Orders that occur only in the MAG or the 16S rRNA sequence data set are marked with stars. In A) note that the percentages of unmapped, thus unclassified, reads are the following: BdJ11: 64%. BdJ-14: 63%. RdK14: 42%. ST07-11: 59%. ST0714: 82%. MAG identifiers from top to bottom are as follows (note that MAGs were reconstructed from individual assemblies, hence their numbering is not coherent between samples): BdJ-11: Bin66, Bin30, Bin47, Bin41, Bin27, Bin26 and Bin46, Bin52, Bin58. BdJ14: Bin62, Bin7, Bin57, Bin31 and Bin39, Bin33, Bin50, Bin51, Bin9, Bin65, Bin52, Bin66. RdK-14: Bin98, Bin78, Bin113, Bin49, Bin2, Bin16, Bin97, Bin44, Bin12, Bin17, Bin76 and Bin92. ST0711: Bin16, Bin11, Bin15, Bin14, Bin12, Bin6. ST0714: Bin33 and Bin46, Bin53.....120

Supplementary Figure 3-3. Presence and absence of A) transporters and B) primary assimilation enzymes in all metagenome-assembled genomes per sample. The depicted proteins facilitate the primary uptake of bicarbonate, formate, acetate or glycine as putative carbon sources. Genes were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109. Where no genes are specified, all available enzyme subdomains (all must be present) or alternatives (one of them must be present) were taken into account.....121

Supplementary Figure 3-4. Carbon fixation pathway completeness across all metagenome-assembled genomes per sample. Completeness scores were calculated based on custom HMM profiles and are given in percent.....122

Supplementary Figure 3-5. Presence and absence of key metabolic genes in all metagenome-assembled genomes per sample. Genes playing a crucial role in the metabolism of hydrogen, carbon monoxide, methane, nitrogen, sulfur or oxygen were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109.....123

Supplementary Figure 3-6. Blastn results of the UBA148 (also called *Methanocellales*) MAG from ST0711 against the The Cedars *Methanocellales* MAG Met12 (GenBank accession CP017966.2), the Lost City *Methanosarciniales* MAG-838 (GenBank accession

GCA_021129915.1) and The Cedars *Methanosarciniales* MAG Met13 (GenBank accession GCA_036677945.1). Outer to inner tracks show (1) Forward CDS (2) Reverse CDS (3) Met13 (4) MAG-838 (5) Met12 (6) GC content (7) GC skew.....124

CHAPTER 4: The cultivation approach

Figure 4-1. Culture development in the first experimental run (cultures Ac1, Fo1, G1, Bc1) (A) and in the second run (cultures Ac2, Fo2, G2, Bc2) (B). The number of cells was counted via DAPI-staining and fluorescence microscopy. The concentration of acetate and formate in the respective consortia was determined by high-performance liquid chromatography. The net increase of cells and the net decrease of acetate and formate (shown as inserts) were calculated as the difference between the first and last sampling timepoint.....146

Figure 4-2. Alpha diversity and evenness calculated to the observed number of ASVs, the Shannon index and the Pielou index in bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). Samples from both experimental runs are combined.....147

Figure 4-3. Aitchison distance matrices (k = 5) constructed on bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (blue) or glycine (purple). Distance matrices were constructed using a non-metric multidimensional scaling on log-ratio transformed data.....148

Figure 4-4. Top ASVs driving significant effects of carbon source, sampling timepoint and experimental run on sample variation in bacterial (A) and/or archaeal (B) sequences. Different ASVs are color-coded according to the lowest taxonomic level to which they were assigned. Coefficients for the respective group of variable were calculated in permutational multivariate analysis of variance tests; corresponding F values, p values and R^2 value are given on the top right of each graph. Positive coefficients indicate an effect of the respective ASV on sample dissimilarity, negative coefficients indicate an effect on sample similarity. Key taxonomic groups are highlighted with symbols to improve the readability of the graph.....149

Figure 4-5. Taxonomic composition of bacterial (A) and archaeal (B) microbial consortia grown on acetate (Ac), bicarbonate (Bc), formate (Fo) or glycine (G) in the first (1) and second (2) experimental run over time. For each carbon source and experimental run, the taxonomic composition of up to three different timepoints is depicted, including the first day (t0), an intermediate day and the last day (t25 or t26) of the culture. In addition, the taxonomic composition of the inoculum used in the respective experimental run (INOC) is shown. For bacterial sequences, only genera with a relative abundance > 3% are depicted. For archaeal sequences, all orders are shown. Note that ANME-3 is a genus of the archaeal order *Methanosarcinales* (both depicted in the same color) which was also amplified by the bacterial

primers. Key taxonomic groups are emphasized with symbols to improve readability of the graph.....150

Figure 4-6. Proposed metabolic network of the cultivable Prony Bay hydrothermal community. A) Metabolic potential of enriched key taxa according to the references cited in the main text. B) Hypothesized trophic relationships. Putative foundation species are shown next to metabolic categories, with symbols indicating all culture conditions they were enriched in. Note the following abbreviations: SAO for syntrophic acetate oxidation, Bc for bicarbonate, Fo for formate, Ac for acetate.....151

Supplementary Figure 4-1. Sulfate and thiosulfate consumption in the first (A) and second (B) experimental run in the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). The respective net decrease of sulfate or thiosulfate concentration, calculated as the difference between the first and last timepoint measured, is illustrated with barplots.....152

Supplementary Figure 4-2. Rarefaction curves for bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). Bacterial sequences were normalized to 11420 reads, which corresponds to the minimal sample size, archaeal sequences to 721 reads. The rarefaction depth is indicated with a red vertical line.....153

Supplementary Figure 4-3. Stressplots of the non-metric multidimensional scaling matrices calculated for bacterial (A) and archaeal (B) sequences.....154

Supplementary Figure 4-4. Percentage of oxygen in the bioreactor gas phase during (A) the first and (B) the second experimental run. In the second replicate of the culture grown on bicarbonate and glycine, data acquisition was temporally interrupted due to a technical problem. In the cultures grown on formate, the oxygen peaks correspond to sampling timepoints. Initial and final oxygen peaks in all cultures correspond to the startup and shutdown of the bioreactors and do not influence the samples discussed in the article.....155

CHAPTER 5: Discussion and perspectives

Figure 5-1. Hypothesized foundation species consortia in Prony Bay's surface (metagenomic approach) and subsurface (cultivation approach) environment. Carbon source, electron donors and electron acceptors used by each putative foundation taxon are depicted with color-coded symbols. Hypotheses on deep-surface foundation species are shown in black boxes.....160

List of tables

CHAPTER 1: Introduction

Table 1-1. Taxonomic survey for critical metabolic functions in all types of serpentinite-hosted environments, specifying the methodological approach including metagenome-assembled genomes (MAGs) and single cell amplified genomes (SAGs).....44

CHAPTER 2: Material and Methods

Table 2-1. Physicochemical characteristics of the endmember fluid at the Rivière des Kaoris (RdK), the Bain des Japonais (BdJ) and the Aiguille the Prony (ST07) sites.....65

CHAPTER 3: The metagenomic approach

Table 3-1. Characterization of MAGs that feature both a transporter and an assimilation enzyme for the same carbon source and potentially play a key role in primary carbon uptake in the respective samples. Carbon fixation pathway completeness is given in percent.....116

Supplementary Table 3-1. Quality of metagenome-assembled genomes with a relative abundance > 1% in their respective sample.....117

Supplementary Table 3-2. BioProject accession numbers of sequences from the National Center for Biotechnology Information database that were included in the phylogenomic tree.....118

List of abbreviations (in alphabetical order)

16S rRNA	16S ribosomal ribonucleic acid
3HP	3-hydroxypropionate cycle
Acetyl-coA	Acetyl coenzyme A
ANOVA	Analysis of variance
AOM	Anaerobic methane oxidizers
ASV	Amplicon-sequence variant
ATP	Adenosine triphosphate
BacDive	Bacterial metadatabase
BdJ	Bain des Japonais (intertidal sampling site)
$CaCO_3$	Calcium carbonate
CBB	Calvin-Benson-Bassham cycle
cDNA	Complementary DNA
CH_4	Methane
CO	Carbon monoxide
CO_2	Carbon dioxide
DAPI	4′,6-Diamidin-2-phenylindol
DC/HB	Dicarboxylate-hydroxybutyrate cycle
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DNA-SIP	DNA stable isotope probing
EC	Enzyme commission number
GTDB	Genome Taxonomy database
H_2	Hydrogen
HMM	Hidden Markov model
HP/HB	Hydroxypropionate-hydroxybutyrate cycle
HPLC	High-performance liquid chromatography
IC	Ion chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG ortholog identifier
LCMS	Lost City Methanosarcinales
MAG	Metagenome-assembled genome
N_2	Nitrogen
NAD, NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NH_3	Ammonia
NMDS	Non-metric multidimensional scaling
O_2	Oxygen
ORP	Oxidation-reduction potential
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
RdK	Rivière des Kaoris (terrestrial sampling site)
RNA	Ribonucleic acid

rTCA	Reductive tricarboxylic acid cycle
RuBisCo	Ribulose-bisphosphate carboxylase
SAG	Single cell amplified genome
SAO	Syntrophic acetate oxidation
SSU rRNA	Small subunit ribosomal ribonucleic acid
ST07	Aiguille de Prony (submarine sampling site)
TCMS	The Cedars Methanosarcinales (Methanocellales)
WGS	Whole genome shotgun sequencing
ΔpH	Transmembrane pH gradient
Δψ	Transmembrane electrical potential



CHAPTER 1
Introduction

1.1 Background

1.1.1 The search for the origin of life

How did life on Earth begin? This simple question is not only the most fundamental problem in biology but touches on many other disciplines. Theories on the origin of life have been put forward and rejected under the influence of philosophical, religious and even political debates, which underlines the broad significance of the subject (Lazcano, 2010).

The scientific history of the origin of life enigma starts with Louis Pasteur's disproval of spontaneous generation (Pasteur, 1860). In a deeply catholic society, Pasteur's results were revolutionary as they questioned the divine creation of life; this was all the more the case as they coincided with Charles Darwin's publication of "On the Origin of Species". From there, alternative theories on the emergence of life have been put forward, some more daring than others. For example, proponents of the "cosmozoa" theory bypassed the heated clerical discourse by "outsourcing" the origin of life into space and suggesting it was transported to Earth on comets (Lazcano, 2010). This early theory is recalled by the recent discovery of prebiotic organic material on meteorites (Oba et al., 2020). A clearer direction was finally given by the emergence of biochemistry as a scientific discipline. Alexander Ivanovich Oparin suggested that the first life form was a heterotroph feeding on abiotically derived organic compounds, which was in line with John Burdon Sanderson Haldane's idea of a "primitive soup" (Haldane, 1929; Oparin, 1938). Oparin and Haldane's hypotheses were supported by the experiments of Friedrich Wöhler, Adolph Strecker, Alexander Michailowitsch Butlerow and others, who had indeed succeeded in producing organics from an abiotic source using different energy sources (Wöhler, 1828; Strecker, 1850; Butlerow, 1861). Later, the famous Miller-Urey experiments went ahead to demonstrate the formation of amino acids in a hydrogen (H_2), methane (CH_4) and ammonia (NH_3) rich medium, and the Fischer-Tropsch process was shown to produce hydrocarbons from carbon monoxide (CO) and H_2 (Fischer and Tropsch, 1923; Miller and Urey, 1959). Although today many assume that the prebiotic Earth's atmosphere was rather neutral than reducing and enriched in carbon dioxide (CO_2), the abiotic synthesis of organic compounds continues to be successful in laboratory experiments (Cleaves et al., 2008). Oparin and Haldane's hypotheses were contested by the "gene first" theory, which arose shortly after Rosalind Franklin, Raymond Gosling, James Watson and Francis Crick discovered the double-helix deoxyribonucleic acid (DNA). Hermann Joseph Muller suggested that life began with genetic material, focusing on information storage and replication (hence Darwinian selection) rather than bioenergetics and growth (Muller, 1922, 1926). Muller's ideas evolved with the discovery that ribonucleic acid (RNA) could have preceded DNA, and eventually gave way to the "RNA world" hypothesis (Neveu et al., 2013). As much as Oparin and Muller's debate was fueled by the Cold War, it still marks opposite cornerstones of the research field today (Lazcano, 2010; Nitschke et al., 2024).

Oparin's perspective was later picked up by the "metabolism first" theory, which maintains the focus on metabolism over genetics but proposes an autotrophic instead of heterotrophic

origin of life. The fundamental idea behind "metabolism first" approaches is that life started as a series of chemical reactions evolving into a self-sustaining primitive metabolism. Following the second law of thermodynamics, this would have required a continuous source of energy driving these biochemical reactions. All modern organisms harness energy from electrochemical disequilibria, and advocates of an autotrophic origin of life mostly assume that this can be extrapolated to primitive metabolism (Muchowska et al., 2020). In line with this idea, Harold Joseph Morowitz proposed that life could have only emerged after the formation of bilayer vesicles, allowing to yield energy via the translocation of protons across a primitive membrane (Morowitz, 1992). Furthermore, "metabolism first" approaches imply the search for primitive enzymes catalyzing prebiotic reactions. Most modern enzymes have inorganic metal clusters in their active centers. It is thus a plausible assumption that primitive metabolic reactions relied on metal-containing minerals such as greigite, mackinawite, pyrite and fougerite (Duval et al., 2021). The most important hypotheses in this context include Günter Wächtershäuser's iron-sulfur world, which proposes that autotrophic carbon fixation was catalyzed on the surface of iron-sulfide minerals in volcanic hydrothermal vents at the beginning of life, and Michael Russel's alkaline hydrothermal vent theory (Wächtershäuser, 1992; Russell and Hall, 1997).

Contrary to Wächtershäuser, Russel proposes that life emerged in serpentinite-hosted hydrothermal vents. These hydrothermal systems are lesser known than their volcanic counterparts and differ in their geochemical environment, which is characterized by the slow hydration of exposed ultramafic rock. This process of serpentinization produces highly reduced hydrothermal fluids enriched in H_2 and hydroxide ions. The resulting hyperalkaline pH promotes the precipitation of dissolved inorganic carbon (DIC) from the fluid, forming carbonaceous hydrothermal chimneys over time. These structures are very porous, and according to Russel, ideal candidates for protocells (Russell et al., 2010). The pores might function as microreactors separating the H_2-rich hydrothermal fluid from the CO_2-rich ambient seawater and harvest energy from the resulting electrochemical gradient via a rudimentary proton-motive force. Catalyzed by chimney-bound metals such as iron and nickel, as well as sulfur, these protocells could have maintained abiotic reactions producing simple organic compounds that slowly evolved into the first metabolic pathways (Russell and Ponce, 2020). In an attempt to bridge the gap with the "RNA world" hypothesis, Russel suggests that such simple organic compounds could have developed into RNA molecules in the protected interior of the chimney pores (Russell et al., 2013). This is backed up by the relatively mild temperature in alkaline hydrothermal vents, which renders the survival of fragile RNA molecules more likely than in a volcanic setting.

1.1.2 Taking a closer look at the serpentinite-hosted Prony Bay

While biochemists are attempting to determine the transition between abiotic and metabolic reactions, biological research is mostly focused on understanding the functioning of modern extremophile ecosystems that are potential Early Earth analogs. The study of serpentinite-hosted environments has developed significantly over the past few decades and was greatly influenced by the discovery of Lost City in the beginning of the 21st century (Kelley et al.,

2001). Lost City became the first known deep-sea alkaline hydrothermal field and sparked much interest in the scientific community. However, serpentinite-hosted systems had already been reported much earlier in terrestrial ("ophiolitic") and coastal environments, as will be detailed in section 1.2. In the 1980s, Launay and Fontes (1985) described multiple terrestrial and intertidal hyperalkaline sources in Prony Bay at the southern end of the peridotite massif of New Caledonia, but their publication remained largely unnoticed. It was only after the discovery of Lost City that the scientific relevance of this system was fully recognized. A bathymetric survey of the seafloor revealed multiple submarine hydrothermal vents at depths of up to 50 m, which led to a first spot sampling in 2010, then a multi-disciplinary campaign in 2011 and a more detailed description of the Prony Bay's geochemistry. While the terrestrial, intertidal and submarine vents generally feature characteristics similar to ophiolitic systems and Lost City respectively, Prony Bay is unique in that hydrothermal fluids of meteoric origin are discharged in a marine environment (Monnin et al., 2014). It might thus be interpreted as a transition system between terrestrial and deep-sea serpentinite-hosted hydrothermal fields.

In the past 15 years, a collaboration between French, New Caledonian and US-American researchers has continued the effort to characterize not only the geochemistry, but also the microbiology of the system. The biodiversity and spatial distribution of bacteria and archaea colonizing the hydrothermal chimneys has been described (Quéméneur et al., 2014, 2021b, 2021a, 2023; Postec et al., 2015; Frouin et al., 2018), highlighting organisms using H_2 as their metabolic energy source (Mei et al., 2016b). Several bacterial strains were isolated from these communities, which up until today count among the only anaerobic serpentinite-hosted organisms that have ever been brought into culture (Ben Aissa et al., 2014, 2015; Mei et al., 2014, 2016a, 2022; Bes et al., 2015; Postec et al., 2021). Furthermore, first attempts were made to uncover their metabolic traits, highlighting carbonate mineralization and a catabolic pathway to overcome phosphorous limitation (Pisapia et al., 2017; Frouin et al., 2022).

While Prony Bay's biodiversity is relatively well described, the metabolic functioning of the trophic network remains unclear. The conditions in serpentinite-hosted systems do not support the metabolism of most modern organisms. In line with the "metabolism first" theory, the metabolic traits of microbial communities adapted to this environment might not only provide valuable insights into the limits of life today, but also into the emergence of primitive life forms (Barge and Price, 2022). This is the central notion of this thesis.

1.2 State of the art

1.2.1 Preface

To develop an initial idea on the preconditions that serpentinization establishes for microbial metabolism, a literature review was performed. This review gives a summary on the serpentinite-hosted environment and how it is expected to limit or drive metabolic activity. Furthermore, we synthesize information on all serpentinite-hosted taxonomic groups whose metabolism has previously been characterized. The methodology of available research is critically discussed in an attempt to optimize the experimental approach of the thesis.

Contributions made in the frame of this thesis include the design of the study with revisions from the PhD supervisors, the conduction of the literature review and the writing of the manuscript. The results were published as a minireview in Frontiers in Microbiology on 24 July 2023.

frontiers | Frontiers in Microbiology

TYPE Mini Review
PUBLISHED 24 July 2023
DOI 10.3389/fmicb.2023.1197823

Check for updates

OPEN ACCESS

EDITED BY
William J. Brazelton,
The University of Utah, United States

REVIEWED BY
Daniel Colman,
Montana State University, United States
Leah Trutschel,
University of Cincinnati, United States

*CORRESPONDENCE
Gaël Erauso
✉ gael.erauso@mio.osupytheas.fr

RECEIVED 31 March 2023
ACCEPTED 29 June 2023
PUBLISHED 24 July 2023

CITATION
Popall RM, Postec A, Lecoeuvre A, Quéméneur M and Erauso G (2023) Metabolic challenges and key players in serpentinite-hosted microbial ecosystems. *Front. Microbiol.* 14:1197823. doi: 10.3389/fmicb.2023.1197823

COPYRIGHT
© 2023 Popall, Postec, Lecoeuvre, Quéméneur and Erauso. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Metabolic challenges and key players in serpentinite-hosted microbial ecosystems

Rabja Maria Popall, Anne Postec, Aurélien Lecoeuvre, Marianne Quéméneur and Gaël Erauso*

Aix-Marseille Univ, Univ Toulon, CNRS, IRD, MIO, Marseille, France

Serpentinite-hosted systems are amongst the most challenging environments for life on Earth. Serpentinization, a geochemical alteration of exposed ultramafic rock, produces hydrothermal fluids enriched in abiotically derived hydrogen (H_2), methane (CH_4), and small organic molecules. The hyperalkaline pH of these fluids poses a great challenge for metabolic energy and nutrient acquisition, curbing the cellular membrane potential and limiting electron acceptor, carbon, and phosphorous availability. Nevertheless, serpentinization supports the growth of diverse microbial communities whose metabolic make-up might shed light on the beginning of life on Earth and potentially elsewhere. Here, we outline current hypotheses on metabolic energy production, carbon fixation, and nutrient acquisition in serpentinizing environments. A taxonomic survey is performed for each important metabolic function, highlighting potential key players such as H_2 and CH_4 cycling *Serpentinimonas*, *Hydrogenophaga*, *Methanobacteriales*, *Methanosarcinales*, and novel candidate phyla. Methodological biases of the available data and future approaches are discussed.

KEYWORDS

serpentinization, alkaline hydrothermal system, alkaliphile, hydrogenotroph, lithotroph, submarine alkaline vent theory, origin of life

1. Introduction

The beginnings of life remain one of the most outstanding scientific issues and have been dubbed the "black hole at the heart of biology" (Lane, 2015). One of the central requirements for living systems is a continuous physicochemical disequilibrium driving biological activity (Russell et al., 1988). In marine alkaline hydrothermal systems, strong electrochemical gradients develop between the ultrabasic hydrothermal fluid rising from the deep subsurface, and the seawater. These gradients are maintained across the porous hydrothermal chimney wall, which can be compared to an osmotic membrane. It is hypothesized that this rudimentary proton motive force has driven chimney nanopores to develop into protocells at the emergence of life (Russell et al., 2010; Sojo et al., 2016). Modern ecosystems at hydrothermal vents might thus provide a glimpse into very early microbial life forms.

Most alkaline hydrothermal systems are formed in environments where mantle rocks have been tectonically uplifted and exposed, either above sea level or on the seafloor. Contact with water initiates serpentinization, a geochemical alteration of the ultramafic rock. This process

yields large amounts of hydrogen (H_2) and constitutes one of the most important sources of H_2 on Earth (Reaction 1) (Truche et al., 2020).

$$\underset{\text{Olivine}}{(MgFe)_2SiO_4} + H_2O \rightarrow \underset{\text{Serpentinite}}{Mg_3Si_2O_5(OH)_4} + \underset{\text{Brucite}}{Mg(OH)_2} + \underset{\text{Magnetite}}{Fe_3O_4} + H_2 \quad (1)$$

The oxidation of ferrous iron in olivine or pyroxene to ferric iron and magnetite by water creates reducing conditions. Catalyzed by minerals, this facilitates abiotic reactions of the produced H_2 with mantle-derived carbon dioxide (CO_2) or carbon monoxide (CO) (McCollom and Seewald, 2001). In Sabatier (Reaction 2) and Fischer-Tropsch (Reaction 3) type processes, methane (CH_4) and small organic molecules [$C_n H(2n+2)$] are enriched in the hydrothermal fluid (Barbier et al., 2020):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

$$(3n+1)H_2 + nCO_2 \rightarrow C_nH_{2n+2} + 2nH_2O \quad (3)$$

The serpentinization reaction produces hydrothermal fluids with pH values commonly surpassing 12. In these ultrabasic conditions, the carbonate equilibrium is permanently shifted from CO_2 to carbonate species, removing most dissolved inorganic carbon (DIC) from the environment. Upon reaching the rock surface, much of the carbonate precipitates with fluid-derived calcium. Over time, calcium carbonate amalgamates with brucite and forms the chimneys or travertines typical for serpentinizing environments (Barnes et al., 1978; Früh-Green et al., 2004; McCollom and Seewald, 2013) (Figure 1A).

2. Serpentinite-hosted ecosystems

The products of serpentinization can support chemosynthetic microbial ecosystems growing independently from sunlight. Such serpentinite-hosted ecosystems are found in marine and continental environments, with hydrothermal fluids originating from marine, meteoric and/or groundwater sources.

Most marine serpentinizing ecosystems are located along rather slow-spreading mid-ocean ridges, where continuous tectonic activity facilitates frequent exposure of ultramafic rock (Schrenk et al., 2013; Albers et al., 2021). The most prominent example is the Lost City hydrothermal field near the Mid-Atlantic ridge (e.g., Kelley et al., 2005). A similar system, the Old City hydrothermal field, has recently been discovered along the Southwest Indian ridge (Lecoeuvre et al., 2021). Several other marine sites host mixed-type ecosystems that feature characteristics of both alkaline and acidic hydrothermal vents, such as the Rainbow (Flores et al., 2011), Logatchev (Perner et al., 2007), Ashadze (Fabri et al., 2011) and Kairei fields (Nakamura et al., 2009).

The most well-studied continental systems include the Samail ophiolite in Oman (Rempfert et al., 2017; Kraus et al., 2021), the Tablelands (Bay of Islands) ophiolite in Newfoundland (Brazelton et al., 2013), the Cabeço de Vide aquifer in Portugal (Tiago and Veríssimo, 2013), the Leka ophiolite complex in Norway (Daae et al., 2013), the Italian Gruppo di Voltri (Quéméneur et al., 2015), the Zambales ophiolite in the Philippines (Woycheese et al., 2015), The Cedars (Suzuki et al., 2013; Kohl et al., 2016) and Coast Range (Twing et al., 2017) ophiolites on the West Coast of the United States, the Chimaera (Tekirova) ophiolite in Turkey (Neubeck et al., 2017), the tropical Santa Elena ophiolite in Costa Rica (Crespo-Medina et al., 2017), the Del Puerto ophiolite in California (Blank et al., 2009) and the Troodos ophiolite in Cyprus (Rizoulis et al., 2016).

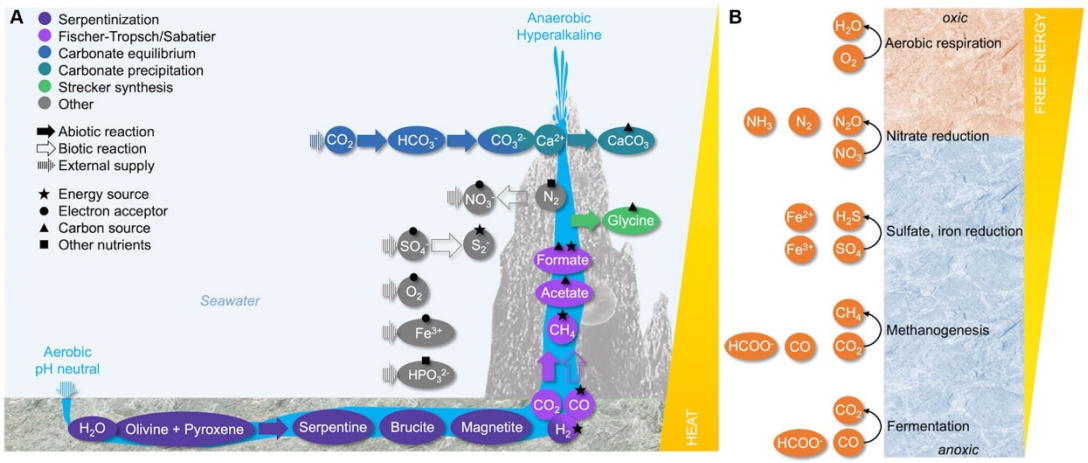


FIGURE 1
The biogeochemical environment of marine serpentinite-hosted systems. **(A)** Production of compounds of interest in microbial metabolism. Reactions are color-coded with solid arrows showing abiotic processes, empty arrows showing biotic processes, and striped arrows indicating external supply. The metabolic potential of shown compounds as an energy source, electron acceptor, carbon source, or other nutrient source is indicated with different shapes. Note that compound concentrations, external supply, temperature, and pH are strongly site dependent. In continental serpentinizing systems, travertines form instead of chimneys. **(B)** Redox potential of available electron acceptors along a gradient from the oxic surface to the anoxic interior of the chimney wall. Adapted from Boyd et al. (2014).

Terrestrial and marine serpentinizing systems are likely to differ in environmental variables such as salinity and the compounds available for microbial metabolism, which might influence the ecosystem's overall functioning. An exciting transition site between terrestrial and marine serpentinite-hosted ecosystems is the Prony Bay Hydrothermal field located on the Southern Coast of New Caledonia, South Pacific (Launay and Fontes, 1985; Monnin et al., 2014). Prony Bay features several venting sites along a gradient from land to sea with a maximum depth of 50 m. The Prony Bay springs are fed by meteoric water, implying a strong salinity gradient between the hydrothermal fluid and ambient seawater (Monnin et al., 2014; Postec et al., 2015). Inversely, the Ney Springs system in Northern California features marine-type hydrothermal fluids in a continental context (Trutschel et al., 2022). Prony Bay is geochemically and microbiologically reflective of both ophiolitic and deep marine sites (Quéméneur et al., 2014; Postec et al., 2015; Frouin et al., 2018, 2022; Trutschel et al., 2022).

3. Metabolic strategies of serpentinite-hosted ecosystems

Serpentinite-hosted ecosystems are subjected to very challenging environmental conditions. The elevated pH poses a fundamental energetic problem on the cellular level, as it inverts the transmembrane pH gradient that typically drives all cellular processes. Furthermore, the high pH also reduces the bioavailability of electron acceptors, carbon, and other macronutrients (McCollom and Seewald, 2013; Schrenk et al., 2013). The microbial adaptations to these challenges are essential to understand life in a serpentinization context.

3.1. The challenge of maintaining bioenergetics

3.1.1. Maintaining pH homeostasis and a proton motive force

The central challenge for life in hyperalkaline conditions revolves around maintaining intracellular pH homeostasis and, more critically, conserving an electrochemical proton gradient across the cell membrane, which is the main driving force of the cell. The so-called proton motive force has two components: A transmembrane pH gradient (ΔpH), which is usually alkaline inside the cell relative to the outside, and a transmembrane electrical potential ($\Delta\psi$), which is negative as long as the inner membrane surface is negatively charged. Maintaining a circum-neutral intracellular pH is crucial to ensure the stability of nucleic acids and proteins. In hyperalkaline conditions, however, the ΔpH is inverted and very low due to H^+ limitation outside of the cell, as the concentration of H^+ decreases by 10^{-4} at pH 11 compared to pH 7. This reduces the proton motive force and jeopardizes pH homeostasis (Krulwich et al., 2011 and references therein). Most alkaliphiles employ mechanisms increasing the efficiency of H^+ uptake while maintaining a high $\Delta\psi$, which is also essential for pH homeostasis. This is achieved via K^+/H^+ and Na^+/H^+ antiporters with high H^+ affinity, such as the Mrp complex in alkaliphilic *Bacillus* spp. (Ito et al., 2017). Those antiporters contribute to creating a transmembrane Na^+ gradient, generating a sodium motive force that requires specialized sodium-F_1F_0-ATP synthases or Na^+-dependent respiratory complexes (Krulwich et al., 2011; Kuhns et al., 2020). Based on varying Na^+ concentrations, it is likely that these mechanisms differ between terrestrial and marine serpentinization-influenced site microorganisms. In low salt environments, alkaliphiles might excrete Na^+ via V_1V_0-ATPases to maintain a sodium motive force (Suzuki et al., 2014; Ohlsson et al., 2019). Other alkaliphiles such as *Serpentinimonas* spp. isolated from The Cedars maintain a proton motive force using specialized H^+ binding F type ATPases (Hicks et al., 2010; Suzuki et al., 2014).

3.1.2. Energy conservation

The membrane potential generated via H^+ or Na^+ translocation depends on the redox potential of electron donors and acceptors. Serpentinization yields a range of reduced compounds that can serve as metabolic energy sources, most importantly H_2 and CH_4 (Figure 1A) (Boyd et al., 2014). The relative concentration of these gases varies significantly between sites (Etiope et al., 2011; Monnin et al., 2014), rendering generalized statements on a primary source of electrons provided by serpentinization difficult. While methanotrophs are more easily detected than hydrogenotrophs in many serpentinite-hosted environments (e.g., Brazelton et al., 2006; Kraus et al., 2021) (Table 1), the energetic potential of H_2 oxidation greatly surpasses the oxidation potential of CH_4. The detection of hydrogenotrophs may be limited by the methodological approach, as the metabolic potential to oxidize H_2 cannot be predicted from 16S rRNA sequences (Brazelton et al., 2012, 2022). Accordingly, metagenomic surveys show that many organisms in all types of serpentinite-hosted systems feature [FeFe]- and [NiFe]-hydrogenases (Brazelton et al., 2012; Mei et al., 2016; Kraus et al., 2021; Lecoeuvre et al., 2021; Frouin et al., 2022) (Table 1).

Another potential electron source in serpentinizing systems is constituted by compounds not directly created by serpentinization such as reduced sulfur species (Sabuda et al., 2020; Trutschel et al., 2022) and CO (Brazelton et al., 2012; Morrill et al., 2014; Fones et al., 2019) (Table 1). While the oxidation potential of CO is very low, the ability to use this energy source may provide a valuable ecological advantage. In surface exposed serpentinization-influenced waters, light constitutes an additional energy source used by cyanobacterial phototrophs (e.g., Kamran et al., 2020) (Table 1).

While serpentinization provides an abundance of electron donors, the availability of terminal electron acceptors is limited, especially in terrestrial serpentinizing systems, and mainly derived from the ambient environment (Figure 1A). Oxygen represents a very potent electron acceptor on the chimney or travertine surface. With increasing proximity to the reduced hydrothermal endmember, however, the availability of oxygen or alternative electron acceptors sharply decreases. The microbial community near the oxic-anoxic interphase may use nitrate (Frouin et al., 2022), even though data on nitrate reduction is scarce. In addition, organisms from the Troodos and The Cedars ophiolites have been shown to reduce metals such as iron or magnetite (Rizoulis et al., 2016; Rowe et al., 2017) (Table 1). Towards the anoxic interior of the hydrothermal carbonate chimneys or in deep ophiolite groundwaters, sulfate reduction is a dominant metabolic strategy in all types of serpentinite-hosted systems (Brazelton et al., 2006; Tiago and Veríssimo, 2013; Postec et al., 2015; Glombitza et al., 2021) (Table 1 and Figure 1B).

To deal with electron acceptor limitation, many microbes also perform fermentation of sugars, simple organic acids and amino

TABLE 1 Taxonomic survey for critical metabolic functions in all types of serpentinite-hosted environments, specifying the methodological approach including metagenome-assembled genomes (MAGs) and single-cell amplified genomes (SAGs).

Metabolism	Taxon	Site	Method	References
Hydrogen oxidation	Bacteria_Pseudomonadota_Alphaproteobacteria[1]	The Cedars (shallow waters)	MAGs	Suzuki et al. (2017)
	Bacteria_Pseudomonadota_Gammaproteobacteria	The Cedars (shallow waters)	MAGs; 16S rRNA	Suzuki et al. (2013, 2017)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Betaproteobacteriales (formerly Betaproteobacteria)[2]	The Cedars (shallow waters)	MAGs	Suzuki et al. (2017)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Hydrogenophaga[3]	The Tablelands, Lost City (oxic/anoxic interface); Voltri; The Cedars (shallow waters); Prony Bay; Zambales	MAGs; 16S rRNA	Brazelton et al. (2012, 2017), Suzuki et al. (2013), Frouin et al. (2018), Woycheese et al. (2015)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Serpentinimonas[4]	Voltri; The Cedars (shallow waters/non-specified)	16S rRNA; cultivation	Quéméneur et al. (2015), Suzuki et al. (2014, 2017), Brazelton et al. (2017), Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Serpentinimonas_S. raichei[5]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Serpentinimonas_S. barnesii[6]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Serpentinimonas_S. maccroryi[7]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_ Cupriavidus_C. necator (formerly *Ralstonia eutropha*)[8]	The Tablelands, Lost City (oxic/anoxic interface)	MAGs	Brazelton et al. (2012)
	Bacteria_Former class Deltaproteobacteria	The Cedars (shallow waters)	MAGs; 16S rRNA	Suzuki et al. (2013, 2017)
	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales[9]	Voltri (deep subsurface)	MAGs	Brazelton et al. (2017)
	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales_Desulfonatronaceae_ Desulfonatronum[10]	Prony Bay	16S rRNA	Postec et al. (2015), Mei et al. (2016)
	Bacteria_BacillotaB_Desulfotomaculia_Desulfotomaculales_ Desulfotomaculaceae_ Desulfotomaculum_D. alkaliphilum[11]	Lost City	16S rRNA	Brazelton et al. (2006, 2010)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium_Lineage type I	Voltri; Samail (surface waters); Prony Bay	MAGs, SAGs, 14C labelling	Quéméneur et al. (2015, 2021, 2023), Fones et al. (2021)
Aerobic methane oxidation	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium_M. alkalithermotolerans strain DSM102889	La Crouen	Cultivation	Mei et al. (2022)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Methylococcales_Methylococcaceae[12]	Voltri (shallow subsurface mixing zone)	16S rRNA, MAGs, 13C labelling	Brazelton et al. (2017)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Methylococcales_Methylococcaceae_ Methylococcus	Samail	16S rRNA	Kraus et al. (2021)
	Bacteria_Pseudomonadota_Alphaproteobacteria_Rhizobiales_Beijerinckiaceae_ Methylosinus	Voltri	16S rRNA	Quéméneur et al. (2015)

(Continued)

TABLE 1 (Continued)

Metabolism	Taxon	Site	Method	References
Methanogenesis/ anaerobic methane oxidation	Archaea_Halobacteriota_Methanomicrobia_Methanomicrobiales	Santa Elena	16S rRNA (MAGs)	Crespo-Medina et al. (2017)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales	Santa Elena; Voltri	16S rRNA (MAGs)	Crespo-Medina et al. (2017), Quéméneur et al. (2015)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae[13]	Voltri (deep subsurface)	16S rRNA, MAGs, 13C labelling	Brazelton et al. (2017)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium	Samail (subsurface); Voltri; Zambales	16S rRNA; MAGs; SAGs; 13C labelling; 14C labelling	Kraus et al. (2021), Brazelton et al. (2017), Fones et al. (2021), Woycheese et al. (2015), Quéméneur et al. (2015)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium_M. alcaliphilum strain DSM3387	Del Puerto	16S rRNA	Blank et al. (2009)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium_M. alkalithermotolerans strain DSM102889	La Crouen	Cultivation	Mei et al. (2022)
	Archaea_Halobacteriota_Methanosarcinia_Methanosarcinales	Santa Elena; Voltri	16S rRNA (MAGs)	Crespo-Medina et al. (2017), Quéméneur et al. (2015), Suzuki et al. (2013)
	Archaea_Halobacteriota_Methanosarcinia_Methanosarcinales_LCMS phylotype[14]	Lost City (Brazelton et al. (2006): High-temperature); Prony Bay (intertidal and submarine); Old City	16S rRNA, MAGs; 13C labelling	Schrenk et al. (2004), Brazelton et al. (2006, 2011), Frouin et al. (2018), Lecoeuvre et al. (2021)
	Archaea_Halobacteriota_Methanosarcinia_Methanosarcinales_TCMS phylotype	The Cedars; Prony Bay (intertidal and submarine); Old City	16S rRNA, MAGs	Suzuki et al. (2013), Frouin et al. (2018), Lecoeuvre et al. (2021)
	Archaea_Halobacteriota_Syntropharchaeia_ANME-1	Lost City (low temperature); Santa Elena; Cabeço de Vide	16S rRNA (MAGs)	Brazelton et al. (2006), Crespo-Medina et al. (2017), Tiago and Veríssimo (2013)
Formate consumption	Bacteria_Pseudomonadota_Gammaproteobacteria_Methylococcales_Methylococcaceae[12]	Voltri (shallow subsurface mixing zone)	MAGs	Brazelton et al. (2017)
	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales[9]	Voltri (deep subsurface)	MAGs	Brazelton et al. (2017)
	Bacteria_Ca. Lithacetigena[15]	The Cedars, Hakuda Happo hot springs	MAGs	Nobu et al. (2022)
	Bacteria_Ca. Bipolaricaulota (OP1/MSBL6)[17]	Lost City	MAGs	Brazelton et al. (2022)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_ Methanobacterium_Lineage type II	Samail (subsurface)	MAGs, SAGs, 14C labelling	Fones et al. (2021)
Acetate consumption	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales[9]	Voltri (deep subsurface)	MAGs	Brazelton et al. (2017)
	Archaea_Halobacteriota_Methanosarcinia_Methanosarcinales_LCMS phylotype[14]	Lost City	MAGs	Brazelton et al. (2011)

(Continued)

10.3389/fmicb.2023.1197823

TABLE 1 (Continued)

Metabolism	Taxon	Site	Method	References
Glycine consumption	Bacteria_Ca. Lithacetigena[15]	The Cedars, Hakuda Happo hot springs	MAGs	Nobu et al. (2022)
Calcium carbonate consumption	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Serpentinimonas[4]	The Cedars	Cultivation	Suzuki et al. (2014)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Serpentinimonas_S. raichei[5]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Serpentinimonas_S. barnesii[6]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Serpentinimonas_S. maccroryi[7]	The Cedars	Cultivation	Bird et al. (2021)
	Bacteria_ NPL-UPA2 clade[18]	The Cedars, Prony Bay, Lost City	MAGs	Suzuki et al. (2018), Brazelton et al. (2022)
	Bacteria_Ca. Bipolaricaulota (OP1/MSBL6)[17]	Lost City	MAGs	Brazelton et al. (2022)
CO oxidation	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Hydrogenophaga[3]	The Tablelands, Lost City (oxic/anoxic interface)	MAGs; 16S rRNA, 13C labelling	Brazelton et al. (2012), Morrill et al. (2014)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Burkholderiales_Burkholderiaceae_Cupriavidus_C. necator (formerly *Ralstonia eutropha*)[8]	The Tablelands, Lost City (oxic/anoxic interface)	MAGs	Brazelton et al. (2012)
	Bacteria_Actinobacteriota_Ca. Hakubanella thermoalkaliphilus	Hakuda Happo hot springs	16S rRNA, SAGs	Merino et al. (2020)
	Archaea_Hadesarchaea (formerly SAGMEG)	Prony Bay	16S rRNA	Postec et al. (2015)
Sulfide/sulfur oxidation	Bacteria_Pseudomonadota_Gammaproteobacteria_Betaproteobacteriales (formerly Betaproteobacteria)[2]	Cabeço de Vide	16sS rRNA (DGGE)	Tiago and Veríssimo (2013)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Thiomicrospirales_Thiomicrospiraceae_Hydrogenovibrio (formerly Thiomicrospira)	Lost City (low temperature)	16S rRNA	Brazelton et al. (2006)
	Bacteria_Pseudomonadota_Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae	Ney Springs	MAGs, Cultivation	Trutschel et al. (2022)
	Bacteria_Pseudomonadota_Gammaproteobacteria_Pseudomonadales_Halomonadaceae_Halomonas	Ney Springs	MAGs, Cultivation	Trutschel et al. (2022)

(Continued)

10.3389/fmicb.2023.1197823

TABLE 1 (Continued)

Metabolism	Taxon	Site	Method	References
Sulfate reduction	Bacteria_BacillotaA_Clostridia[16]	Cabeço de Vide; The Cedars (deep subsurface)	16S rRNA (DGGE)	Tiago and Veríssimo (2013), Suzuki et al. (2013)
	Bacteria_BacillotaB_Desulfotomaculia_Desulfotomaculales_ Desulfotomaculaceae_ Desulfotomaculum_D. alkaliphilum[11]	Lost City	16S rRNA	Brazelton et al. (2006, 2010)
	Bacteria_BacillotaD_Dethiobacteria_Dethiobacterales_Dethiobacteraceae_Dethiobacter	The Tablelands, The Cedars, Cabeço de Vide, Prony Bay, Zambales		Brazelton et al. (2012), Suzuki et al. (2013), Tiago and Veríssimo (2013), Postec et al. (2015), Woycheese et al. (2015), Crespo-Medina et al. (2017), Trutschel et al. (2022), Twing et al. (2017)
	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales[9]	Voltri	MAGs	Brazelton et al. (2017)
	Bacteria_Desulfobacterota_Desulfovibrionia_Desulfovibrionales_Desulfonatronaceae_ Desulfonatronum[10]	Prony Bay	16S rRNA	Postec et al. (2015), Mei et al. (2016)
	Bacteria_Nitrospirota_Thermodesulfovibrionia_Thermodesulfovibrionales_ Thermodesulfovibrionaceae	Samail (subsurface fluids)	16S rRNA	Rempfert et al. (2017)
Nitrogen fixation	Bacteria_Pseudomonadota_Alphaproteobacteria_Azospirillales_Azospirillaceae_ Azospirillum	Voltri	16S rRNA	Quéméneur et al. (2015)
	Archaea_Methanobacteriota_Methanobacteria_Methanobacteriales_Methanobacteriaceae[13]	Voltri (deep subsurface)	16S rRNA, MAGs	Brazelton et al. (2017)
	Archaea_Halobacteriota_Methanosarcinia_Methanosarcinales_LCMS phylotype[14]	Lost City	MAGs	Brazelton et al. (2011)
Phosphonate catabolism	Bacteria_Proteobacteria_Alphaproteobacteria[1]	Prony Bay, Lost City	MAGs	Frouin et al. (2022)
	Bacteria_Proteobacteria_Gammaproteobacteria_Betaproteobacteriales (formerly Betaproteobacteria)[2]	Coast Range, Voltri, Santa Elena, Cabeço de Vide	MAGs	Frouin et al. (2022)
	Bacteria_ BacillotaA _Clostridia[16]	Prony Bay, Lost City	MAGs	Frouin et al. (2022)
Acetogenesis	Bacteria_Chloroflexota (formerly Chloroflexi)	The Cedars (deep subsurface); Prony Bay (submarine)	MAGs; 16S rRNA	Suzuki et al. (2013, 2017), Frouin et al. (2018)
	Bacteria_ NPL-UPA2 clade[18]	The Cedars; Prony Bay	16S rRNA; MAGs	Postec et al. (2015), Mei et al. (2016), Suzuki et al. (2018)
	Bacteria_BacillotaD_Dethiobacteria_Dethiobacterales_Dethiobacteraceae_Dethiobacter_D. alkaliphilus	The Cedars; Prony Bay; Cabeço de Vide	16S rRNA	Postec et al. (2015), Mei et al. (2016), Suzuki et al. (2013), Tiago and Veríssimo (2013)
	Bacteria_Ca. Bipolaricaulota (OP1/MSBL6)[17]	Samail (subsurface fluids)	MAGs	Colman et al. (2022)

(Continued)

10.3389/fmicb.2023.1197823

TABLE 1 (Continued)

Metabolism	Taxon	Site	Method	References
Fermentation	Bacteria_ BacillotaA _Clostridia	The Tablelands, Lost City	MAGs	Brazelton et al. (2012)
	Bacteria_ BacillotaA _Clostridia_Thermoanaerobacterales_Candidate Division OD1	The Cedars (deep subsurface); Voltri	16S rRNA, MAGs, 13C labelling	Suzuki et al. (2013, 2017), Brazelton et al. (2017)
	Bacteria_ BacillotaA _Clostridia_Lachnospirales_Vallitaleaceae_Vallitalea_V. pronyensis	Prony Bay	Cultivation	Ben Aissa et al. (2014), Mei et al. (2014)
	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Natronincolaceae_Alkaliphilus_A. hydrothermalis	Prony Bay	Cultivation	Ben Aissa et al. (2015)
	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Natronincolaceae_Alkaliphilus_A. serpentinus	Prony Bay	Cultivation	Postec et al. (2021)
	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Natronincolaceae_Alkaliphilus_A. pronyensis	Prony Bay	Cultivation	Postec et al. (2021)
	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Natronincolaceae_Serpentinicella_S. alkaliphila	Prony Bay	Cultivation	Mei et al. (2016)
	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Peptostreptococcaceae_ Acetoanaerobium_A. pronyense	Prony Bay	Cultivation	Bes et al. (2015)
	Bacteria_Bacteroidetes_Bacteroidia_Bacteroidales_ML635J-40	Voltri	16S rRNA, MAGs, 13C labelling	Brazelton et al. (2017)
	Bacteria_BacillotaA_Clostridia_Peptostreptococcales_Peptostreptococcaceae	Ney Springs	MAGs, 16S rRNA	Trutschel et al. (2022)
	Bacteria_BacillotaA_Clostridia_Peptostreptococcales_Tindalliaceae_Tindallia	Ney Springs	MAGs, 16S rRNA	Trutschel et al. (2022)
	Bacteria_BacillotaB_Desulfotomaculia_Desulfotomaculales_ Desulfotomaculaceae_ Desulfotomaculum	The Tablelands, Lost City	MAGs	Brazelton et al. (2012)
	Bacteria_Bacilli_Bacillota_Erysipelotrichales_Erysipelotrichaceae	The Tablelands	MAGs	Brazelton et al. (2012)
Iron reduction	Bacteria_ BacillotaA _Clostridia_Peptostreptococcales_Natronincolaceae_Alkaliphilus	Troodos	Cultivation	Rizoulis et al. (2016)
	Bacteria_Bacillota_Bacilli_Paenibacillales_Paenibacillaceae_Paenibacillus	The Cedars	Cultivation	Rowe et al. (2017)
Photosynthesis	Bacteria_Cyanobacteria_Cyanobacteriia_Leptolyngbyales_Leptolyngbyaceae_Leptolyngbya	Voltri (surface); Del Puerto	16S rRNA	Kamran et al. (2020), Blank et al. (2009)
	Bacteria_Cyanobacteria_Cyanobacteriia_Synechococcales_Synechococcaceae_ Synechococcus	Prony Bay	16S rRNA	Mei et al. (2016)

Taxa appearing in several metabolic groups on the same taxonomic level are assigned with superscript numbers. The taxonomy is based on the Genome Taxonomy Database (GTDB Release 214).

10.3389/fmicb.2023.1197823

acids, including Stickland type reactions (Barker, 1981; Postec et al., 2021). Therefore, a large proportion of the anaerobic serpentinite-hosted community may not feature a *bona fide* electron transport chain with cytochromes or quinones (Table 1). Genomic analysis suggests that many serpentinite-hosted fermenters conserve energy by substrate-level phosphorylation (e.g., in glycolysis), or via bifurcative-confurcative [FeFe] H_2-producing hydrogenases, which balance the reducing equivalents NADH and ferredoxin produced by fermentation (Westphal et al., 2018). This is often associated with the Rnf complex, a respiratory enzyme that catalyzes the oxidation of reduced ferredoxin to the reduction of NAD^+. The negative free energy change of this reaction is used to generate a transmembrane H^+ or Na^+ gradient (Westphal et al., 2018). This system can be considered a primitive respiratory mechanism where the terminal electron acceptor is H^+ (Buckel and Thauer, 2018).

3.2. The challenge of coping with nutrient limitation

Next to maintaining energy-yielding reactions, microorganisms in serpentinite-hosted environments must cope with severe nutrient limitation resulting from the decreased solubility of essential macronutrients at high pH (McCollom and Seewald, 2013; Schrenk et al., 2013). Especially relevant for the metabolic functioning of the community are the carbon, nitrogen, and phosphorous sources for primary production (Figure 1A).

3.2.1. Carbon sources and carbon fixation

One of the most significant issues regarding primary production in serpentinite-hosted environments is the absence of DIC, which precipitates as calcium carbonate in hyperalkaline conditions. While calcium carbonate is mostly insoluble and thus unavailable as a carbon source, it has nevertheless been shown to support the growth of some serpentinite-hosted microorganisms. This might be the result of local redissolution into bicarbonate catalyzed by the carbonic anhydrase (Suzuki et al., 2014; Fones et al., 2019; Bird et al., 2021) (Table 1). Alternatively, small organic molecules may serve as primary source of carbon. These include organic acids such as formate and acetate produced in Fischer-Tropsch and Sabatier-type reactions (Barbier et al., 2020; Fones et al., 2021) or via acetogenesis and fermentation (Kohl et al., 2016; Suzuki et al., 2017), as well as amino acids such as glycine produced in Strecker synthesis (Ménez et al., 2018; Nobu et al., 2022) (Figure 1A). The abiotic origin of those organic carbon sources tackles the definition of heterotrophy, which normally refers to the consumption of organic compounds derived from organic sources (Schönheit et al., 2016).

While bicarbonate, formate, acetate, and glycine have been shown to support the growth of microorganisms associated with serpentinization, their metabolic route remains hypothetical. Transferred across the cell membrane via specialized transporters, formate can be oxidized to CO_2 via the formate dehydrogenase in the pH-neutral cytoplasm (Brazelton et al., 2022). Likewise, bicarbonate can be reduced to CO_2 via the carbonic anhydrase (Suzuki et al., 2014; Bird et al., 2021). The produced CO_2 is subsequently introduced to different carbon fixation pathways yielding acetyl-CoA. In serpentinite-hosted environments, the Wood-Ljungdahl pathway, reverse tricarboxylic acid cycle, and Calvin-Benson-Bassham cycle have been confirmed (Seyler et al., 2020). Based on a recent study expanding the phylogenetic range of most carbon fixation pathways, the 3-hydroxypropionate bi-cycle, dicarboxylate/4-hydroxybutyrate cycle, and 3-hydroxypropionate/4-hydroxybutyrate cycle might also be employed (Garritano et al., 2022). Contrary to formate and bicarbonate, glycine can be directly transformed into acetyl-phosphate and subsequently acetyl-coA via the lesser known reductive glycine pathway (Sánchez-Andrea et al., 2020). Genes encoding the glycine reductase are found in metagenomes from Lost City, The Cedars, and the Japanese Hakuba Happo hot springs (Brazelton et al., 2022; Nobu et al., 2022). Also acetate can be directly transformed into acetyl-phosphate and acetyl-CoA, rendering its metabolic route less complex (Rose et al., 1954).

3.2.2. Sources of other nutrients

Serpentinization also decreases the solubility of other macronutrients essential for microbial growth. Inorganic phosphorous is severely limited in serpentinizing environments because it is scavenged by the mineral brucite (Schrenk et al., 2013). A metagenomic survey revealed the high occurrence of genes involved in phosphonate catabolism in serpentinizing sites, suggesting that the microbial community might use phosphonates as an alternative phosphorous source (Frouin et al., 2022). The catabolism of methylphosphonate, the most commonly available phosphonate species in marine environments, may additionally contribute to the global carbon and energy budget in these ecosystems by releasing CH_4 (Frouin et al., 2022). On the contrary, the availability of nitrogen in serpentinizing environments remains controversial. While some authors suggest that concentrations are low (Schrenk et al., 2013), others propose that N_2 and nitrate are readily available to the serpentinite-hosted community (Lang et al., 2013; Rempfert et al., 2023). Potential nitrogen limitation may be alleviated by the fixation of N_2 derived from the endmember fluids or ambient seawater (Morrill et al., 2013; Monnin et al., 2014) (Figure 1A). While a recent study found the associated genetic marker *nifH* in 10 different serpentinite-hosted systems, its overall abundance was low (Frouin et al., 2022).

3.3. Metabolic links to the emergence of life

The biochemical characteristics of serpentinite-hosted ecosystems reinforce the presumed link between serpentinization and the beginnings of life. Serpentinization is an ancient process which likely occurred on early Earth (Russell et al., 2010). The abiotic production of organic acids associated with serpentinization is for instance supported by isotopic signatures (McCollom and Seewald, 2013). Likewise, amino acids such as glycine may be formed abiotically, which is especially interesting in prebiotic chemistry (Aubrey et al., 2009; Ménez et al., 2018). The metabolic use of these compounds is linked to very deep-branching functions, such as the reductive tricarboxylic acid cycle and the Wood-Ljungdahl pathway, which are likely the most ancient carbon fixation pathways on Earth (Sumi and Harada, 2021). Another primordial function preserved in serpentinizing environments is CO oxidation. CO is not only one of the most ancient energy sources exploited in metabolism but is also

suggested to have played a key role in several critical prebiotic reactions (King and Weber, 2007). It may thus constitute a direct link between abiotic and biotic chemistry.

4. Diversity of identified metabolic key players

The specific metabolic challenges posed by serpentinization suggest the presence of specialized taxonomic groups playing an important role in the trophic network. It might be possible that such "core" taxa are relevant in a wide variety of serpentinizing environments, even though the overall community structure can vary significantly in space and time (Suzuki et al., 2013; Postec et al., 2015; Fones et al., 2019; Brazelton et al., 2022; Trutschel et al., 2022).

The community of H_2 oxidizers appears to be dominated by Gammaproteobacteria (Table 1). This includes a major proportion of *Serpentinimonas* (formerly grouped under Betaproteobacteria). *Serpentinimonas* is one of the taxa most commonly associated with serpentinization, and represented by some of the few available isolates from serpentinite-hosted ecosystems (Suzuki et al., 2014; Bird et al., 2021). So far, all of those isolated strains originate from The Cedars. Still, 16S rRNA analysis confirms the presence of *Serpentinimonas* and its sister genus *Hydrogenophaga* in other terrestrial systems, as well as in Prony Bay and Lost City (e.g., Brazelton et al., 2012; Quéméneur et al., 2015; Woycheese et al., 2015; Frouin et al., 2018) (Table 1).

While aerobic methane oxidation is mostly performed by the bacterial *Methylococcales* (Brazelton et al., 2017; Kraus et al., 2021), anaerobic methanotrophy and methanogenesis feature exclusively archaea (Table 1). Hydrogenotrophic methanogens belonging to *Methanobacteriales* are often detected in serpentinite-hosted terrestrial ecosystems (Woycheese et al., 2015; Brazelton et al., 2017; Kraus et al., 2021; Quéméneur et al., 2021, 2023; Mei et al., 2022). In addition, there is a subgroup of *Methanosarcinales* which is probably endemic to serpentinizing environments and includes two distinct phylotypes (e.g., Schrenk et al., 2004; Brazelton et al., 2010; Suzuki et al., 2013). Next to their systems of origin, The Cedars *Methanosarcinales* (TCMS) and Lost City *Methanosarcinales* (LCMS) have been observed in Prony Bay (Frouin et al., 2018) and Old City (Lecoeuvre et al., 2021) (Table 1). However, attempts to culture them have been unsuccessful so far.

The fermenting community seems almost entirely dominated by *Clostridia* (Table 1), of which several novel species have been isolated from Prony Bay (Ben Aissa et al., 2014, 2015; Mei et al., 2014; Bes et al., 2015; Postec et al., 2021).

Other energy yielding metabolic strategies including CO oxidation, sulfur oxidation and sulfate reduction are performed by a broader diversity of taxonomic groups (Table 1). Notably, the community of sulfate reducers includes *Desulfovibrionales* and *Dethiobacter* species, which can be very abundant in serpentinizing environments (Brazelton et al., 2012; Suzuki et al., 2013; Tiago and Veríssimo, 2013; Postec et al., 2015; Woycheese et al., 2015; Mei et al., 2016) (Table 1).

Regarding carbon uptake, it might be especially interesting to further investigate certain candidate phyla that occur in various serpentinizing systems. For example, *Ca. Bipolaricaulota* can use bicarbonate and formate (Brazelton et al., 2022) and plays a role in acetogenesis (Colman et al., 2022). Likewise, *Ca.* NPL-UPA2 grows on bicarbonate (Brazelton et al., 2022) and performs acetogenesis via the Wood-Ljungdahl pathway (Suzuki et al., 2018) (Table 1). The Wood-Ljungdahl pathway is also employed by *Ca. Hakubanella thermoalkaliphilus*, a novel Actinobacteriota from the Hakuba Happo hot springs serpentinizing system (Merino et al., 2020). Finally, *Ca.* Lithacetigena was recently shown to perform glycine reduction (Nobu et al., 2022). These candidate phyla might play an important role in the trophic chain by supplying fixed carbon to the community.

5. Methodological shortcomings and future challenges

While several critical metabolic strategies and taxonomic groups could be identified, the scope of their distribution across serpentinizing ecosystems remains unclear due to methodological biases and shortcomings. Firstly, continental sites have been studied much more extensively than marine ones (Table 1). Our understanding of the latter is almost entirely based on the famous Lost City (Table 1) and most recently Old City (Lecoeuvre et al., 2021), which limits the generalization of findings on marine serpentinizing systems and reduces the meaningfulness of comparison with continental ones. This issue emphasizes the interest of the shallow marine transition field of Prony Bay. Its common characteristics with continental and marine sites may help establish the core metabolic properties of serpentinite-hosted ecosystems. In addition, the study of such shallow fields is facilitated by their geographical accessibility.

Another factor introducing bias is the methodological approach. Most studies rely on metabarcoding and metagenomic techniques (Table 1), which are strongly dependent on the scope of available reference databases. Moreover, the presence of a functional gene does not necessarily signify its activity. However, confirmation of gene expression is rare, notably due to technical difficulties in obtaining quality metatranscriptomes from such environments (Table 1). In addition, there is a lack of experimental evidence complementing bioinformatic hypotheses. Studies attempting to bridge this gap include activity measurements using ^{13}C and ^{14}C labeled substates in microcosms (Brazelton et al., 2011, 2017; Morrill et al., 2014; Fones et al., 2021), as well as isolation of *Serpentinimonas* and *Clostridia* species from cultures (e.g., Suzuki et al., 2014; Postec et al., 2021) (Table 1). Their small number is probably also associated with technical difficulties, including the cultivation of recalcitrant microorganisms such as obligate anaerobic chemolithoautotrophs. While combined omics approaches can provide valuable results, critical metabolic groups will eventually need to be cultivated to confirm their functional role in the ecosystem. This may be facilitated by implementing more sophisticated culture platforms to mimic the conditions associated with serpentinization in the laboratory.

6. Conclusion

Serpentinite-hosted environments are inhabited by microbial communities that cope with energetic challenges and severe nutrient limitation. It can be assumed that a significant proportion of those microorganisms yield energy from H_2 oxidation with electron acceptors derived from external sources or CO_2 degassed from mantle rocks. Calcium carbonate can serve as inorganic carbon source, and

formate, acetate and glycine as organic carbon sources for primary production. Bicarbonate, formate, and glycine may be fixed via different carbon fixation pathways such as the Wood-Ljungdahl pathway, the reverse tricarboxylic acid and Calvin-Benson-Bassham cycles and the reductive glycine pathway. Moreover, the microbial communities might cope with nitrogen and phosphorous limitation by fixing N_2 and breaking down phosphonates. The analysis of functional genes suggests that taxa such as *Gammaproteobacteria*, *Desulfovibrionales*, *Clostridia* and several candidate phyla play a crucial role in the trophic network and that the genera *Serpentinimonas*, *Hydrogenophaga*, and *Methanobacterium* as well as uncultivated *Methanosarcinales*, are characteristic for serpentinizing environments. However, the scope of research on serpentinite-hosted ecosystems needs to be broadened by including a greater diversity of marine and shallow transition sites. In addition, experimental evidence is needed to confirm the metabolic activity of hypothesized key players. A technological advancement of the methodological approach might not only contribute to the understanding of present serpentinite-hosted ecosystems, but also provide insights into the beginning of life on Earth and potentially elsewhere.

Author contributions

GE, AP, and RP: conceptualization. AL and MQ: validation. RP: investigation and writing of original draft. AL, MQ, AP, GE, and RP: review and edit of original draft. GE and AP: supervision. GE: project administration. All authors contributed to the article and approved the submitted version.

Funding

This project was financially supported by the ANR MICROPRONY (N°19-CE02-0020-02), the French Institute of Research for Development (IRD), and a Ph.D. fellowship granted to RP by the Aix-Marseille University's Doctoral School "Sciences de l'Environnement" (ED 251).

Acknowledgments

The authors thank all project partners, including MICROPRONY.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Albers, E., Bach, W., Pérez-Gussinyé, M., McCammon, C., and Frederichs, T. (2021). Serpentinization-driven H2 production from continental break-up to mid-ocean ridge spreading: unexpected high rates at the West Iberia margin. *Front. Earth Sci.* 9:673063. doi: 10.3389/feart.2021.673063

Aubrey, A. D., Cleaves, H. J., and Bada, J. L. (2009). The role of submarine hydrothermal systems in the synthesis of amino acids. *Orig. Life Evol. Biosph.* 39, 91–108. doi: 10.1007/s11084-008-9153-2

Barbier, S., Huang, F., Andreani, M., Tao, R., Hao, J., Eleish, A., et al. (2020). A review of H2, CH4, and hydrocarbon formation in experimental serpentinization using network analysis. *Front. Earth Sci.* 8:209. doi: 10.3389/feart.2020.00209

Barker, H. A. (1981). Amino acid degradation by anaerobic bacteria. *Annu. Rev. Biochem.* 50, 23–40. doi: 10.1146/annurev.bi.50.070181.000323

Barnes, I., O'Neil, J. R., and Trescases, J. J. (1978). Present day serpentinization in New Caledonia, Oman and Yugoslavia. *Geochim. Cosmochim. Acta* 42, 144–145. doi: 10.1016/0016-7037(78)90225-9

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2015). Characterization of Alkaliphilus hydrothermalis sp. nov., a novel alkaliphilic anaerobic bacterium, isolated from a carbonaceous chimney of the Prony hydrothermal field, New Caledonia. *Extremophiles* 19, 183–188. doi: 10.1007/s00792-014-0697-y

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2014). Vallitalea pronyensis sp. nov., isolated from a marine alkaline hydrothermal chimney. *Int. J. Syst. Evol. Microbiol.* 64, 1160–1165. doi: 10.1099/ijs.0.055756-0

Bes, M., Merrouch, M., Joseph, M., Quéméneur, M., Payri, C., Pelletier, B., et al. (2015). Acetoanaerobium pronyense sp. nov., an anaerobic alkaliphilic bacterium isolated from a carbonate chimney of the Prony hydrothermal field (New Caledonia). *Int. J. Syst. Evol. Microbiol.* 65, 2574–2580. doi: 10.1099/ijs.0.000307

Bird, L. J., Kuenen, J. G., Osburn, M. R., Tomioka, N., Ishii, S., Barr, C., et al. (2021). Serpentinimonas gen. Nov., Serpentinimonas raichei sp. nov., Serpentinimonas barnesii sp. nov. and Serpentinimonas maccroryi sp. nov., hyperalkaliphilic and facultative autotrophic bacteria isolated from terrestrial serpentinizing springs. *Int. J. Syst. Evol. Microbiol.* 71:004945. doi: 10.1099/ijsem.0.004945

Blank, J. G., Green, S. J., Blake, D., Valley, J. W., Kita, N. T., Treiman, A., et al. (2009). An alkaline spring system within the Del Puerto ophiolite (California, USA): a Mars analog site. *Planet. Space Sci.* 57, 533–540. doi: 10.1016/j.pss.2008.11.018

Boyd, E. S., Schut, G. J., Peters, J. W., and Adams, M. W. W. (2014). Hydrogen metabolism and the evolution of biological respiration: two separate families of enzymes that oxidize hydrogen and also produce it arose through convergent evolution. *Microbe Mag.* 9, 361–367. doi: 10.1128/microbe.9.361.1

Brazelton, W. J., Ludwig, K. A., Sogin, M. L., Andreishcheva, E. N., Kelley, D. S., Shen, C.-C., et al. (2010). Archaea and bacteria with surprising microdiversity show shifts in dominance over 1,000-year time scales in hydrothermal chimneys. *Proc. Natl. Acad. Sci. USA* 107, 1612–1617. doi: 10.1073/pnas.0905369107

Brazelton, W. J., McGonigle, J. M., Motamedi, S., Pendleton, H. L., Twing, K. I., Miller, B. C., et al. (2022). Metabolic strategies shared by basement residents of the Lost City hydrothermal field. *Appl. Environ. Microbiol.* 88:e0092922. doi: 10.1128/aem.00929-22

Brazelton, W. J., Mehta, M. P., Kelley, D. S., and Baross, J. A. (2011). Physiological differentiation within a single-species biofilm fueled by serpentinization. *mBio* 2, e00127–e00111. doi: 10.1128/mBio.00127-11

Brazelton, W. J., Morrill, P. L., Szponar, N., and Schrenk, M. O. (2013). Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. *Appl. Environ. Microbiol.* 79, 3906–3916. doi: 10.1128/AEM.00330-13

Brazelton, W. J., Nelson, B., and Schrenk, M. O. (2012). Metagenomic evidence for H2 oxidation and H2 production by serpentinite-hosted subsurface microbial communities. *Front. Microbiol.* 2:268. doi: 10.3389/fmicb.2011.00268

Brazelton, W. J., Schrenk, M. O., Kelley, D. S., and Baross, J. A. (2006). Methane- and sulfur-metabolizing microbial communities dominate the Lost City hydrothermal field ecosystem. *Appl. Environ. Microbiol.* 72, 6257–6270. doi: 10.1128/AEM.00574-06

Brazelton, W. J., Thornton, C. N., Hyer, A., Twing, K. I., Longino, A. A., Lang, S. Q., et al. (2017). Metagenomic identification of active methanogens and methanotrophs in serpentinite springs of the Voltri massif, Italy. *PeerJ* 5:e2945. doi: 10.7717/peerj.2945

Buckel, W., and Thauer, R. K. (2018). Flavin-based electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiration with protons (Ech) or NAD+ (Rnf) as electron acceptors: a historical review. *Front. Microbiol.* 9:401. doi: 10.3389/fmicb.2018.00401

Colman, D. R., Kraus, E. A., Thieringer, P. H., Rempfert, K., Templeton, A. S., Spear, J. R., et al. (2022). Deep-branching acetogens in serpentinized subsurface fluids of Oman. *Proc. Natl. Acad. Sci. USA* 119:e2206845119. doi: 10.1073/pnas.2206845119

Crespo-Medina, M., Twing, K. I., Sánchez-Murillo, R., Brazelton, W. J., McCollom, T. M., and Schrenk, M. O. (2017). Methane dynamics in a tropical serpentinizing environment: the Santa Elena ophiolite, Costa Rica. *Front. Microbiol.* 8:916. doi: 10.3389/fmicb.2017.00916

Daae, F. L., Økland, I., Dahle, H., Jørgensen, S. L., Thorseth, I. H., and Pedersen, R. B. (2013). Microbial life associated with low-temperature alteration of ultramafic rocks in the Leka ophiolite complex. *Geobiology* 11, 318–339. doi: 10.1111/gbi.12035

Etiope, G., Schoell, M., and Hosgörmez, H. (2011). Abiotic methane flux from the Chimaera seep and Tekirova ophiolites (Turkey): understanding gas exhalation from low temperature serpentinization and implications for Mars. *Earth Planet. Sci. Lett.* 310, 96–104. doi: 10.1016/j.epsl.2011.08.001

Fabri, M.-C., Bargain, A., Briand, P., Gebruk, A., Fouquet, Y., Morineaux, M., et al. (2011). The hydrothermal vent community of a new deep-sea field, Ashadze-1, 12°58′N on the mid-Atlantic ridge. *J. Mar. Biol. Assoc. U. K.* 91, 1–13. doi: 10.1017/S0025315410000731

Flores, G. E., Campbell, J. H., Kirshtein, J. D., Meneghin, J., Podar, M., Steinberg, J. I., et al. (2011). Microbial community structure of hydrothermal deposits from geochemically different vent fields along the mid-Atlantic ridge: microbial communities of hydrothermal vent deposits. *Environ. Microbiol.* 13, 2158–2171. doi: 10.1111/j.1462-2920.2011.02463.x

Fones, E. M., Colman, D. R., Kraus, E. A., Nothaft, D. B., Poudel, S., Rempfert, K. R., et al. (2019). Physiological adaptations to serpentinization in the Samail ophiolite, Oman. *ISME J.* 13, 1750–1762. doi: 10.1038/s41396-019-0391-2

Fones, E. M., Colman, D. R., Kraus, E. A., Stepanauskas, R., Templeton, A. S., Spear, J. R., et al. (2021). Diversification of methanogens into hyperalkaline serpentinizing environments through adaptations to minimize oxidant limitation. *ISME J.* 15, 1121–1135. doi: 10.1038/s41396-020-00838-1

Frouin, E., Bes, M., Ollivier, B., Quéméneur, M., Postec, A., Debroas, D., et al. (2018). Diversity of rare and abundant prokaryotic phylotypes in the Prony hydrothermal field and comparison with other serpentinite-hosted ecosystems. *Front. Microbiol.* 9:102. doi: 10.3389/fmicb.2018.00102

Frouin, E., Lecoeuvre, A., Armougom, F., Schrenk, M. O., and Erauso, G. (2022). Comparative metagenomics highlight a widespread pathway involved in catabolism of phosphonates in marine and terrestrial serpentinizing. *Ecosystems* 7:e0032822. doi: 10.1128/msystems.00328-22

Früh-Green, G. L., Connolly, J. A. D., Plas, A., Kelley, D. S., and Grobéty, B. (2004). "Serpentinization of oceanic peridotites: implications for geochemical cycles and biological activity" in *Geophysical monograph series*. eds. W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross and S. Craig Cary (Washington, D C: American Geophysical Union), 119–136. doi: 10.1029/144GM08

Garritano, A. N., Song, W., and Thomas, T. (2022). Carbon fixation pathways across the bacterial and archaeal tree of life. *PNAS Nexus* 1:pgac226. doi: 10.1093/pnasnexus/pgac226

Glombitza, C., Putman, L. I., Rempfert, K. R., Kubo, M. D., Schrenk, M. O., Templeton, A. S., et al. (2021). Active microbial sulfate reduction in fluids of serpentinizing peridotites of the continental subsurface. *Commun. Earth Environ.* 2:84. doi: 10.1038/s43247-021-00157-z

Hicks, D. B., Liu, J., Fujisawa, M., and Krulwich, T. A. (2010). F1F0-ATP synthases of alkaliphilic bacteria: lessons from their adaptations. *Biochim. Biophys. Acta BBA – Bioenerg.* 1797, 1362–1377. doi: 10.1016/j.bbabio.2010.02.028

Ito, M., Morino, M., and Krulwich, T. A. (2017). Mrp antiporters have important roles in diverse bacteria and archaea. *Front. Microbiol.* 8:2325. doi: 10.3389/fmicb.2017.02325

Kamran, A., Sauter, K., Reimer, A., Wacker, T., Reitner, J., and Hoppert, M. (2020). Cyanobacterial mats in calcite-precipitating serpentinite-hosted alkaline springs of the Voltri Massif, Italy. *Microorganisms* 9:62. doi: 10.3390/microorganisms9010062

Kelley, D. S., Karson, J. A., Früh-Green, G. L., Yoerger, D. R., Shank, T. M., Butterfield, D. A., et al. (2005). A serpentinite-hosted ecosystem: the Lost City hydrothermal field. *Science* 307, 1428–1434. doi: 10.1126/science.1102556

King, G. M., and Weber, C. F. (2007). Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. *Nat. Rev. Microbiol.* 5, 107–118. doi: 10.1038/nrmicro1595

Kohl, L., Cumming, E., Cox, A., Rietze, A., Morrissey, L., Lang, S. Q., et al. (2016). Exploring the metabolic potential of microbial communities in ultra-basic, reducing springs at the cedars, CA, USA: experimental evidence of microbial methanogenesis and heterotrophic acetogenesis: methanogenesis at the cedars. *J. Geophys. Res. Biogeosci.* 121, 1203–1220. doi: 10.1002/2015JG003233

Kraus, E. A., Nothaft, D., Stamps, B. W., Rempfert, K. R., Ellison, E. T., Matter, J. M., et al. (2021). Molecular evidence for an active microbial methane cycle in subsurface serpentinite-hosted groundwaters in the Samail ophiolite, Oman. *Appl. Environ. Microbiol.* 87, e02068–e02020. doi: 10.1128/AEM.02068-20

Krulwich, T. A., Sachs, G., and Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nat. Rev. Microbiol.* 9, 330–343. doi: 10.1038/nrmicro2549

Kuhns, M., Trifunović, D., Huber, H., and Müller, V. (2020). The Rnf complex is a Na+ coupled respiratory enzyme in a fermenting bacterium, Thermotoga maritima. *Commun. Biol.* 3:431. doi: 10.1038/s42003-020-01158-y

Lane, N. (2015). *The vital question: energy, evolution, and the origins of complex life*. New York City: WW Norton & Company.

Lang, S. Q., Früh-Green, G. L., Bernasconi, S. M., and Butterfield, D. A. (2013). Sources of organic nitrogen at the serpentinite-hosted Lost City hydrothermal field. *Geobiology* 11, 154–169. doi: 10.1111/gbi.12026

Launay, J., and Fontes, J.-C. (1985). Les sources thermales de Prony (Nouvelle Calédonie) et leurs précipités chimiques, exemple de formation de brucite primaire. *Geol. Fr.* 1, 83–100.

Lecoeuvre, A., Ménez, B., Cannat, M., Chavagnac, V., and Gérard, E. (2021). Microbial ecology of the newly discovered serpentinite-hosted Old City hydrothermal field (southwest Indian ridge). *ISME J.* 15, 818–832. doi: 10.1038/s41396-020-00816-7

McCollom, T. M., and Seewald, J. S. (2001). A reassessment of the potential for reduction of dissolved CO 2 to hydrocarbons during serpentinization of olivine. *Geochim. Cosmochim. Acta* 65, 3769–3778. doi: 10.1016/S0016-7037(01)00655-X

McCollom, T. M., and Seewald, J. S. (2013). Serpentinites, hydrogen, and life. *Elements* 9, 129–134. doi: 10.2113/gselements.9.2.129

Mei, N., Postec, A., Bartoli, M., Vandecasteele, C., Wils, L., Gil, L., et al. (2022). Methanobacterium alkalithermotolerans sp. nov., a novel alkaliphilic and hydrogen-utilizing methanogen isolated from an alkaline geothermal spring (La Crouen, New Caledonia). *Int. J. Syst. Evol. Microbiol.* 72. doi: 10.1099/ijsem.0.005554

Mei, N., Postec, A., Monnin, C., Pelletier, B., Payri, C. E., Ménez, B., et al. (2016). Metagenomic and PCR-based diversity surveys of [FeFe]-hydrogenases combined with isolation of alkaliphilic hydrogen-producing bacteria from the serpentinite-hosted Prony hydrothermal field, New Caledonia. *Front. Microbiol.* 7:1301. doi: 10.3389/fmicb.2016.01301

Mei, N., Zergane, N., Postec, A., Erauso, G., Ollier, A., Payri, C., et al. (2014). Fermentative hydrogen production by a new alkaliphilic Clostridium sp. (strain PROH2) isolated from a shallow submarine hydrothermal chimney in Prony Bay, New Caledonia. *Int. J. Hydrog. Energy* 39, 19465–19473. doi: 10.1016/j.ijhydene.2014.09.111

Ménez, B., Pisapia, C., Andreani, M., Jamme, F., Vanbellingen, Q. P., Brunelle, A., et al. (2018). Abiotic synthesis of amino acids in the recesses of the oceanic lithosphere. *Nature* 564, 59–63. doi: 10.1038/s41586-018-0684-z

Merino, N., Kawai, M., Boyd, E. S., Colman, D. R., McGlynn, S. E., Nealson, K. H., et al. (2020). Single-cell genomics of novel actinobacteria with the Wood-Ljungdahl pathway discovered in a serpentinizing system. *Front. Microbiol.* 11:1031. doi: 10.3389/fmicb.2020.01031

Monnin, C., Chavagnac, V., Boulart, C., Ménez, B., Gérard, M., Gérard, E., et al. (2014). Fluid chemistry of the low temperature hyperalkaline hydrothermal system of Prony Bay (New Caledonia). *Biogeosciences* 11, 5687–5706. doi: 10.5194/bg-11-5687-2014

Morrill, P. L., Brazelton, W. J., Kohl, L., Rietze, A., Miles, S. M., Kavanagh, H., et al. (2014). Investigations of potential microbial methanogenic and carbon monoxide utilization pathways in ultra-basic reducing springs associated with present-day continental serpentinization: the tablelands, NL, CAN. *Front. Microbiol.* 5:613. doi: 10.3389/fmicb.2014.00613

Morrill, P. L., Kuenen, J. G., Johnson, O. J., Suzuki, S., Rietze, A., Sessions, A. L., et al. (2013). Geochemistry and geobiology of a present-day serpentinization site in California: the cedars. *Geochim. Cosmochim. Acta* 109, 222–240. doi: 10.1016/j.gca.2013.01.043

Nakamura, K., Morishita, T., Bach, W., Klein, F., Hara, K., Okino, K., et al. (2009). Serpentinized troctolites exposed near the Kairei hydrothermal field, central Indian ridge: insights into the origin of the Kairei hydrothermal fluid supporting a unique microbial ecosystem. *Earth Planet. Sci. Lett.* 280, 128–136. doi: 10.1016/j.epsl.2009.01.024

Neubeck, A., Sun, L., Müller, B., Ivarsson, M., Hosgörmez, H., Özcan, D., et al. (2017). Microbial community structure in a serpentine-hosted abiotic gas seepage at the Chimaera ophiolite, Turkey. *Appl. Environ. Microbiol.* 83, e03430–e03416. doi: 10.1128/AEM.03430-16

Nobu, M. K., Nakai, R., Tamazawa, S., Mori, H., Toyoda, A., Ijiri, A., et al. (2022). Unique H2-utilizing lithotrophy in serpentinite-hosted systems. *ISME J.* 17, 95–104. doi: 10.1038/s41396-022-01197-9

Ohlsson, J. I., Osvatic, J. T., Becraft, E. D., and Swingley, W. D. (2019). Microbial community in hyperalkaline steel slag-fill emulates serpentinizing springs. *Diversity* 11:103. doi: 10.3390/d11070103

Perner, M., Kuever, J., Seifert, R., Pape, T., Koschinsky, A., Schmidt, K., et al. (2007). The influence of ultramafic rocks on microbial communities at the Logatchev hydrothermal field, located 15Â°N on the mid-Atlantic ridge: influence of ultramafic rocks on microbial communities. *FEMS Microbiol. Ecol.* 61, 97–109. doi: 10.1111/j.1574-6941.2007.00325.x

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing

hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Front. Microbiol.* 6:857. doi: 10.3389/fmicb.2015.00857

Postec, A., Quéméneur, M., Lecoeuvre, A., Chabert, N., Joseph, M., and Erauso, G. (2021). Alkaliphilus serpentinus sp. nov. and Alkaliphilus pronyensis sp. nov., two novel anaerobic alkaliphilic species isolated from the serpentinite-hosted Prony Bay hydrothermal field (New Caledonia). *Syst. Appl. Microbiol.* 44:126175. doi: 10.1016/j.syapm.2020.126175

Quéméneur, M., Bes, M., Postec, A., Mei, N., Hamelin, J., Monnin, C., et al. (2014). Spatial distribution of microbial communities in the shallow submarine alkaline hydrothermal field of the Prony Bay, New Caledonia: microbial communities of Prony hydrothermal field. *Environ. Microbiol. Rep.* 6, 665–674. doi: 10.1111/1758-2229.12184

Quéméneur, M., Mei, N., Monnin, C., Postec, A., Guasco, S., Jeanpert, et al. (2023). Microbial taxa related to natural hydrogen and methane emissions in serpentinite-hosted hyperalkaline springs of New Caledonia. *Front. Microbiol.* 14:1196516. doi: 10.3389/fmicb.2023.1196516

Quéméneur, M., Mei, N., Monnin, C., Postec, A., Wils, L., Bartoli, M., et al. (2021). Procaryotic diversity and hydrogenotrophic methanogenesis in an alkaline spring (La Crouen, New Caledonia). *Microorganisms* 9:1360. doi: 10.3390/microorganisms9071360

Quéméneur, M., Palvadeau, A., Postec, A., Monnin, C., Chavagnac, V., Ollivier, B., et al. (2015). Endolithic microbial communities in carbonate precipitates from serpentinite-hosted hyperalkaline springs of the Voltri massif (Ligurian Alps, northern Italy). *Environ. Sci. Pollut. Res.* 22, 13613–13624. doi: 10.1007/s11356-015-4113-7

Rempfert, K. R., Miller, H. M., Bompard, N., Nothaft, D., Matter, J. M., Kelemen, P., et al. (2017). Geological and geochemical controls on subsurface microbial life in the Samail ophiolite, Oman. *Front. Microbiol.* 8:56. doi: 10.3389/fmicb.2017.00056

Rempfert, K. R., Nothaft, D. B., Kraus, E. A., Asamoto, C. K., Evans, R. D., Spear, J. R., et al. (2023). Subsurface biogeochemical cycling of nitrogen in the actively serpentinizing Samail ophiolite, Oman. *Front. Microbiol.* 14:1139633. doi: 10.3389/fmicb.2023.1139633

Rizoulis, A., Milodowski, A. E., Morris, K., and Lloyd, J. R. (2016). Bacterial diversity in the hyperalkaline allas springs (Cyprus), a natural analogue for cementitious radioactive waste repository. *Geomicrobiol J.* 33, 73–84. doi: 10.1080/01490451.2014.961107

Rose, I. A., Grunberg-Manago, M., Korey, S. R., and Ochoa, S. (1954). Enzymatic phosphorylation of acetate. *J. Biol. Chem.* 211, 737–756. doi: 10.1016/S0021-9258(18)71161-7

Rowe, A. R., Yoshimura, M., LaRowe, D. E., Bird, L. J., Amend, J. P., Hashimoto, K., et al. (2017). In situ electrochemical enrichment and isolation of a magnetite-reducing bacterium from a high pH serpentinizing spring. *Environ. Microbiol.* 19, 2272–2285. doi: 10.1111/1462-2920.13723

Russell, M. J., Hall, A. J., Cairns-Smith, A. G., and Braterman, P. S. (1988). Submarine hot springs and the origin of life. *Nature* 336:117. doi: 10.1038/336117a0

Russell, M. J., Hall, A. J., and Martin, W. (2010). Serpentinization as a source of energy at the origin of life: serpentinization and the emergence of life. *Geobiology* 8, 355–371. doi: 10.1111/j.1472-4669.2010.00249.x

Sabuda, M. C., Brazelton, W. J., Putman, L. I., McCollom, T. M., Hoehler, T. M., Kubo, M. D. Y., et al. (2020). A dynamic microbial sulfur cycle in a serpentinizing continental ophiolite. *Environ. Microbiol.* 22, 2329–2345. doi: 10.1111/1462-2920.15006

Sánchez-Andrea, I., Guedes, I. A., Hornung, B., Boeren, S., Lawson, C. E., Sousa, D. Z., et al. (2020). The reductive glycine pathway allows autotrophic growth of *Desulfovibrio desulfuricans*. *Nat. Commun.* 11:5090. doi: 10.1038/s41467-020-18906-7

Schönheit, P., Buckel, W., and Martin, W. F. (2016). On the origin of heterotrophy. *Trends Microbiol.* 24, 12–25. doi: 10.1016/j.tim.2015.10.003

Schrenk, M. O., Brazelton, W. J., and Lang, S. Q. (2013). Serpentinization, carbon, and deep life. *Rev. Mineral. Geochem.* 75, 575–606. doi: 10.2138/rmg.2013.75.18

Schrenk, M. O., Kelley, D. S., Bolton, S. A., and Baross, J. A. (2004). Low archaeal diversity linked to subseafloor geochemical processes at the Lost City hydrothermal field, mid-Atlantic ridge. *Environ. Microbiol.* 6, 1086–1095. doi: 10.1111/j.1462-2920.2004.00650.x

Seyler, L. M., Brazelton, W. J., McLean, C., Putman, L. I., Hyer, A., Kubo, M. D. Y., et al. (2020). Carbon assimilation strategies in ultrabasic groundwater: clues from the integrated study of a serpentinization-influenced aquifer. *mSystems* 5, e00607–e00619. doi: 10.1128/mSystems.00607-19

Sojo, V., Herschy, B., Whicher, A., Camprubí, E., and Lane, N. (2016). The origin of life in alkaline hydrothermal vents. *Astrobiology* 16, 181–197. doi: 10.1089/ast.2015.1406

Sumi, T., and Harada, K. (2021). Kinetics of the ancestral carbon metabolism pathways in deep-branching bacteria and archaea. *Commun. Chem.* 4:149. doi: 10.1038/s42004-021-00585-0

Suzuki, S., Ishii, S., Hoshino, T., Rietze, A., Tenney, A., Morrill, P. L., et al. (2017). Unusual metabolic diversity of hyperalkaliphilic microbial communities associated with subterranean serpentinization at the cedars. *ISME J.* 11, 2584–2598. doi: 10.1038/ismej.2017.111

Suzuki, S., Ishii, S., Wu, A., Cheung, A., Tenney, A., Wanger, G., et al. (2013). Microbial diversity in the cedars, an ultrabasic, ultrareducing, and low salinity serpentinizing ecosystem. *Proc. Natl. Acad. Sci. USA* 110, 15336–15341. doi: 10.1073/pnas.1302426110

Suzuki, S., Kuenen, J. G., Schipper, K., van der Velde, S., Ishii, S., Wu, A., et al. (2014). Physiological and genomic features of highly alkaliphilic hydrogen-utilizing betaproteobacteria from a continental serpentinizing site. *Nat. Commun.* 5:3900. doi: 10.1038/ncomms4900

Suzuki, S., Nealson, K. H., and Ishii, S. (2018). Genomic and in-situ transcriptomic characterization of the candida Monninte phylum NPL-UPL2 from highly alkaline highly reducing serpentinized groundwater. *Front. Microbiol.* 9:3141. doi: 10.3389/fmicb.2018.03141

Tiago, I., and Veríssimo, A. (2013). Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization: microbial and functional diversity of subsurface aquifer. *Environ. Microbiol.* 15, 1687–1706. doi: 10.1111/1462-2920.12034

Truche, L., McCollom, T. M., and Martinez, I. (2020). Hydrogen and abiotic hydrocarbons: molecules that change the world. *Elements* 16, 13–18. doi: 10.2138/gselements.16.1.13

Trutschel, L. R., Chadwick, G. L., Kruger, B., Blank, J. G., Brazelton, W. J., Dart, E. R., et al. (2022). Investigation of microbial metabolisms in an extremely high pH marine-like terrestrial serpentinizing system: Ney Springs. *Sci. Total Environ.* 836:155492. doi: 10.1016/j.scitotenv.2022.155492

Twing, K. I., Brazelton, W. J., Kubo, M. D. Y., Hyer, A. J., Cardace, D., Hoehler, T. M., et al. (2017). Serpentinization-influenced groundwater harbors extremely low diversity microbial communities adapted to high pH. *Front. Microbiol.* 8:308. doi: 10.3389/fmicb.2017.00308

Westphal, L., Wiechmann, A., Baker, J., Minton, N. P., and Müller, V. (2018). The Rnf complex is an energy-coupled transhydrogenase essential to reversibly link cellular NADH and ferredoxin pools in the acetogen *Acetobacterium woodii*. *J. Bacteriol.* 200:e00357. doi: 10.1128/JB.00357-18

Woycheese, K. M., Meyer-Dombard, D. R., Cardace, D., Argayosa, A. M., and Arcilla, C. A. (2015). Out of the dark: transitional subsurface-to-surface microbial diversity in a terrestrial serpentinizing seep (Manleluag, Pangasinan, the Philippines). *Front. Microbiol.* 6:44. doi: 10.3389/fmicb.2015.00044

1.3 Objectives

As all life developing in the system depends on it, the base of the trophic network is of special importance for the serpentinite-hosted microbial community. However, there is a great issue regarding primary production in serpentinite-hosted environments: the precipitation of DIC from the hydrothermal fluid does not only lead to the formation of carbonaceous hydrothermal chimneys, but also removes CO_2 from the environment (Schrenk et al., 2013). All known modern ecosystems use CO_2 as their primary carbon source, and it is unclear how microbial life can survive without it.

The objective of this thesis is to identify alternative primary carbon sources driving the base of the trophic network in Prony Bay microbial communities (Figure 1-2). We test four different carbon compounds which have been proposed to fulfill this role in recent years: bicarbonate, formate, acetate, and glycine (Suzuki et al., 2014; Brazelton et al., 2017; Lang et al., 2018; Nobu et al., 2022). While bicarbonate is an inorganic compound derived from the hydrothermal chimneys, the latter three count among the small organic molecules that can be produced abiotically in a serpentinization-influenced milieu (see the Fischer-Tropsch and Strecker experiments mentioned above). For this thesis, we hypothesize that such reactions do, in fact, take place in Prony Bay. Regarding formate and acetate, this has been tested in several other serpentinite-hosted systems by analyzing the isotopic composition of the hydrothermal fluid. While the results are not always coherent, there is evidence that the assessed pools are at least partially abiotic (Lang et al., 2010; Eickenbusch et al., 2019). This is also supported by the fact that the Fischer-Tropsch reaction is an established process that has been used in industry for many years (Höök et al., 2014; Davis et al., 2018). While there is no isotopic evidence confirming an abiotic origin of glycine in association with serpentinization, a relatively recent study has demonstrated the abiotic production of aromatic amino acids in alkaline hydrothermal vents (Ménez et al., 2018). It is not the subject of this thesis to establish the abiotic origin of formate, acetate and glycine in Prony Bay. This being said, all results presented here need to be interpreted with this open question in mind.

We hypothesize that bicarbonate, formate, acetate and glycine facilitate the growth of autotrophic and heterotrophic foundation species that support the development of microbial communities in serpentinite-hosted systems such as Prony Bay. In present-day environments, the base of the trophic network is usually constituted by autotrophs that produce complex organics from CO_2 for higher trophic levels. Both bicarbonate and formate can be transformed into CO_2 inside of the cell and thus constitute potential autotrophic carbon sources. In DIC-depleted conditions, however, the trophic network might not depend on autotrophic primary producers alone. Lang et al. (2018) suggested that heterotrophs play an equally fundamental role by transforming abiotically derived organic carbon such as acetate and glycine into CO_2. This would allow the development of a secondary community of autotrophs that is unable to directly grow on bicarbonate or formate.

In this context, the following research questions are assessed:

1) Which of the four putative carbon sources are used by the serpentinite-hosted microbial communities of Prony Bay?
2) Which taxonomic groups are involved in the metabolism of those carbon compounds, and potentially contribute as autotrophic or heterotrophic foundation species to the base of the trophic network?

To outbalance their respective drawbacks, we decided to employ two complementary approaches. In the frame of a metagenomic study on environmental DNA from terrestrial, intertidal and submarine Prony Bay sites, the genetic potential of the natural microbial communities was explored. Subsequently, anaerobic hydrogenotrophic microbial communities from one of Prony Bay's submarine sites were grown on each of the four carbon compounds, testing the metabolic activity of the cultivable microbial community. Combining the results from both approaches, we aimed to depict the community's foundation consortia as accurately as possible.

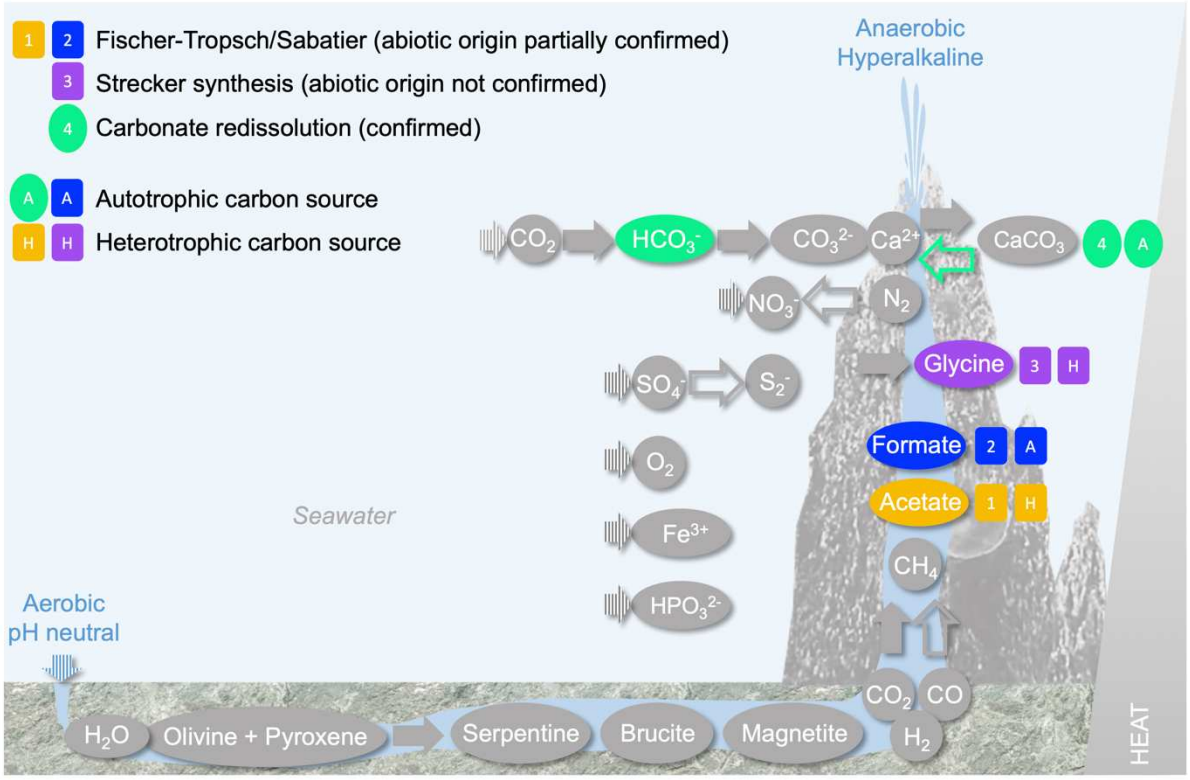


Figure 1-2. Hypothesized primary carbon sources in serpentinite-hosted systems. Different carbon sources are color-coded. Hypothesized abiotic reactions are indicated with numbers and autotrophic or heterotrophic metabolism of the respective carbon source with letters. Square shapes indicate that the respective carbon source is an organic molecule, oval shapes that it is an inorganic molecule

1.4 References

Barge, L. M., and Price, R. E. (2022). Diverse geochemical conditions for prebiotic chemistry in shallow-sea alkaline hydrothermal vents. *Nat. Geosci.* 15, 976–981. doi: 10.1038/s41561-022-01067-1

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2014). Vallitalea pronyensis sp. nov., isolated from a marine alkaline hydrothermal chimney. *Int. J. Syst. Evol. Microbiol.* 64, 1160–1165. doi: 10.1099/ijs.0.055756-0

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2015). Characterization of Alkaliphilus hydrothermalis sp. nov., a novel alkaliphilic anaerobic bacterium, isolated from a carbonaceous chimney of the Prony hydrothermal field, New Caledonia. *Extremophiles* 19, 183–188. doi: 10.1007/s00792-014-0697-y

Bes, M., Merrouch, M., Joseph, M., Quéméneur, M., Payri, C., Pelletier, B., et al. (2015). Acetoanaerobium pronyense sp. nov., an anaerobic alkaliphilic bacterium isolated from a carbonate chimney of the Prony Hydrothermal Field (New Caledonia). *Int. J. Syst. Evol. Microbiol.* 65, 2574–2580. doi: 10.1099/ijs.0.000307

Brazelton, W. J., Thornton, C. N., Hyer, A., Twing, K. I., Longino, A. A., Lang, S. Q., et al. (2017). Metagenomic identification of active methanogens and methanotrophs in serpentinite springs of the Voltri Massif, Italy. *PeerJ* 5, e2945. doi: 10.7717/peerj.2945

Butlerow, A. M. (1861). Bildung einer zuckerartigen Substanz durch Synthese. *Justus Liebigs Ann. Chem.* 120, 295–298.

Cleaves, H. J., Chalmers, J. H., Lazcano, A., Miller, S. L., and Bada, J. L. (2008). A reassessment of prebiotic organic synthesis in neutral planetary atmospheres. *Orig. Life Evol. Biosphere J. Int. Soc. Study Orig. Life* 38, 105–115. doi: 10.1007/s11084-007-9120-3

Davis, S. J., Lewis, N. S., Shaner, M., Aggarwal, S., Arent, D., Azevedo, I. L., et al. (2018). Net-zero emissions energy systems. *Science* 360, eaas9793. doi: 10.1126/science.aas9793

Duval, S., Zuchan, K., Baymann, F., Schoepp-Cothenet, B., Branscomb, E., Russell, M. J., et al. (2021). "5 MINERALS AND THE EMERGENCE OF LIFE," in *Metals, Microbes, and Minerals - The Biogeochemical Side of Life*, eds. P. Kroneck and M. Sosa Torres (De Gruyter), 135–158. doi: 10.1515/9783110589771-011

Eickenbusch, P., Takai, K., Sissman, O., Suzuki, S., Menzies, C., Sakai, S., et al. (2019). Origin of Short-Chain Organic Acids in Serpentinite Mud Volcanoes of the Mariana Convergent Margin. *Front. Microbiol.* 10, 1729. doi: 10.3389/fmicb.2019.01729

Fischer, F., and Tropsch, H. (1923). Über die Herstellung synthetischer Ölgemische (Synthol) durch Aufbau aus Kohlenoxyd und Wasserstoff. *Brennst.-Chem* 4, 276–285.

Frouin, E., Bes, M., Ollivier, B., Quéméneur, M., Postec, A., Debroas, D., et al. (2018). Diversity of Rare and Abundant Prokaryotic Phylotypes in the Prony Hydrothermal Field and Comparison with Other Serpentinite-Hosted Ecosystems. *Front. Microbiol.* 9, 102. doi: 10.3389/fmicb.2018.00102

Frouin, E., Lecoeuvre, A., Armougom, F., Schrenk, M. O., and Erauso, G. (2022). Comparative Metagenomics Highlight a Widespread Pathway Involved in Catabolism of Phosphonates in Marine and Terrestrial Serpentinizing Ecosystems. *mSystems*, e00328-22. doi: 10.1128/msystems.00328-22

Haldane, J. B. S. (1929). Origin of Life. *Ration. Annu.*, 3–10.

Höök, M., Fantazzini, D., Angelantoni, A., and Snowden, S. (2014). Hydrocarbon liquefaction: viability as a peak oil mitigation strategy. *Philos. Trans. R. Soc. Math. Phys. Eng. Sci.* 372, 20120319. doi: 10.1098/rsta.2012.0319

Kelley, D. S., Karson, J. A., Blackman, D. K., Früh-Green, G. L., Butterfield, D. A., Lilley, M. D., et al. (2001). An off-axis hydrothermal vent field near the Mid-Atlantic Ridge at 30° N. *Nature* 412, 145–149. doi: 10.1038/35084000

Lang, S. Q., Butterfield, D. A., Schulte, M., Kelley, D. S., and Lilley, M. D. (2010). Elevated concentrations of formate, acetate and dissolved organic carbon found at the Lost City hydrothermal field. *Geochim. Cosmochim. Acta* 74, 941–952. doi: 10.1016/j.gca.2009.10.045

Lang, S. Q., Früh-Green, G. L., Bernasconi, S. M., Brazelton, W. J., Schrenk, M. O., and McGonigle, J. M. (2018). Deeply-sourced formate fuels sulfate reducers but not methanogens at Lost City hydrothermal field. *Sci. Rep.* 8, 755. doi: 10.1038/s41598-017-19002-5

Launay, J., and Fontes, J.-Ch. (1985). Les sources thermales de Prony (Nouvelle Calédonie) et leurs précipités chimiques, exemple de formation de brucite primaire. *Geol. Fr.* 1, 83–100.

Lazcano, A. (2010). Historical Development of Origins Research. *Cold Spring Harb. Perspect. Biol.* 2, a002089. doi: 10.1101/cshperspect.a002089

Mei, N., Postec, A., Bartoli, M., Vandecasteele, C., Wils, L., Gil, L., et al. (2022). Methanobacterium alkalithermotolerans sp. nov., a novel alkaliphilic and hydrogen-utilizing methanogen isolated from an alkaline geothermal spring (La Crouen, New Caledonia). *Int. J. Syst. Evol. Microbiol.* 72. doi: 10.1099/ijsem.0.005554

Mei, N., Postec, A., Erauso, G., Joseph, M., Pelletier, B., Payri, C., et al. (2016a). Serpentinicella alkaliphila gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from the serpentinite-hosted Prony hydrothermal field, New Caledonia. *Int. J. Syst. Evol. Microbiol.* 66, 4464–4470. doi: 10.1099/ijsem.0.001375

Mei, N., Postec, A., Monnin, C., Pelletier, B., Payri, C. E., Ménez, B., et al. (2016b). Metagenomic and PCR-Based Diversity Surveys of [FeFe]-Hydrogenases Combined with Isolation of Alkaliphilic Hydrogen-Producing Bacteria from the Serpentinite-Hosted Prony Hydrothermal Field, New Caledonia. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.01301

Mei, N., Zergane, N., Postec, A., Erauso, G., Ollier, A., Payri, C., et al. (2014). Fermentative hydrogen production by a new alkaliphilic Clostridium sp. (strain PROH2) isolated from a shallow submarine hydrothermal chimney in Prony Bay, New Caledonia. *Int. J. Hydrog. Energy* 39, 19465–19473. doi: 10.1016/j.ijhydene.2014.09.111

Ménez, B., Pisapia, C., Andreani, M., Jamme, F., Vanbellingen, Q. P., Brunelle, A., et al. (2018). Abiotic synthesis of amino acids in the recesses of the oceanic lithosphere. *Nature* 564, 59–63. doi: 10.1038/s41586-018-0684-z

Miller, S. L., and Urey, H. C. (1959). Organic Compound Synthesis on the Primitive Earth. *Science* 130, 245–251. doi: 10.1126/science.130.3370.245

Monnin, C., Chavagnac, V., Boulart, C., Ménez, B., Gérard, M., Gérard, E., et al. (2014). Fluid chemistry of the low temperature hyperalkaline hydrothermal system of Prony Bay (New Caledonia). *Biogeosciences* 11, 5687–5706. doi: 10.5194/bg-11-5687-2014

Morowitz, H. J. (1992). *Beginnings of Cellular Life: Metabolism Recapitulates Biogenesis*. Binghamton: Yale University Press.

Muchowska, K. B., Varma, S. J., and Moran, J. (2020). Nonenzymatic Metabolic Reactions and Life's Origins. *Chem. Rev.* 120, 7708–7744. doi: 10.1021/acs.chemrev.0c00191

Muller, H. J. (1922). Variation due to change in the individual gene. *Am. Nat.*, 32–50.

Muller, H. J. (1926). The gene as the basis of life. *Proc. 1st Int. Congr. Plant Sci.* Ithaca, 897–921.

Neveu, M., Kim, H.-J., and Benner, S. A. (2013). The "Strong" RNA World Hypothesis: Fifty Years Old. *Astrobiology* 13, 391–403. doi: 10.1089/ast.2012.0868

Nitschke, W., Farr, O., Gaudu, N., Truong, C., Guyot, F., Russell, M. J., et al. (2024). The Winding Road from Origin to Emergence (of Life). *Life* 14, 607. doi: 10.3390/life14050607

Nobu, M. K., Nakai, R., Tamazawa, S., Mori, H., Toyoda, A., Ijiri, A., et al. (2022). Unique H2-utilizing lithotrophy in serpentinite-hosted systems. *ISME J.* doi: 10.1038/s41396-022-01197-9

Oba, Y., Takano, Y., Naraoka, H., Furukawa, Y., Glavin, D. P., Dworkin, J. P., et al. (2020). Extraterrestrial hexamethylenetetramine in meteorites—a precursor of prebiotic chemistry in the inner solar system. *Nat. Commun.* 11, 6243. doi: 10.1038/s41467-020-20038-x

Oparin, A. I. (1938). *The Origin of Life*. New York: McMillan.

Pasteur, L. (1860). Expériences relatives aux générations dites spontanées. *Comptes Rendus Séances L'Académie Sci.* 50, 303–307.

Pisapia, C., Gérard, E., Gérard, M., Lecourt, L., Lang, S. Q., Pelletier, B., et al. (2017). Mineralizing Filamentous Bacteria from the Prony Bay Hydrothermal Field Give New Insights into the Functioning of Serpentinization-Based Subseafloor Ecosystems. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00057

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00857

Postec, A., Quéméneur, M., Lecoeuvre, A., Chabert, N., Joseph, M., and Erauso, G. (2021). Alkaliphilus serpentinus sp. nov. and Alkaliphilus pronyensis sp. nov., two novel anaerobic alkaliphilic species isolated from the serpentinite-hosted Prony Bay Hydrothermal Field (New Caledonia). *Syst. Appl. Microbiol.* 44, 126175. doi: 10.1016/j.syapm.2020.126175

Quéméneur, M., Bes, M., Postec, A., Mei, N., Hamelin, J., Monnin, C., et al. (2014). Spatial distribution of microbial communities in the shallow submarine alkaline hydrothermal field of the Prony Bay, New Caledonia: Microbial communities of Prony Hydrothermal Field. *Environ. Microbiol. Rep.* 6, 665–674. doi: 10.1111/1758-2229.12184

Quéméneur, M., Erauso, G., Bartoli, M., Vandecasteele, C., Wils, L., Gil, L., et al. (2021a). Alkalicella caledoniensis gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from 'La Crouen' alkaline thermal spring, New Caledonia. *Int. J. Syst. Evol. Microbiol.* 71, 004810. doi: 10.1099/ijsem.0.004810

Quéméneur, M., Mei, N., Monnin, C., Postec, A., Guasco, S., Jeanpert, J., et al. (2023). Microbial taxa related to natural hydrogen and methane emissions in serpentinite-hosted hyperalkaline springs of New Caledonia. *Front. Microbiol.* 14, 1196516. doi: 10.3389/fmicb.2023.1196516

Quéméneur, M., Mei, N., Monnin, C., Postec, A., Wils, L., Bartoli, M., et al. (2021b). Procaryotic Diversity and Hydrogenotrophic Methanogenesis in an Alkaline Spring (La Crouen, New Caledonia). *Microorganisms* 9, 1360. doi: 10.3390/microorganisms9071360

Russell, M. J., and Hall, A. J. (1997). The emergence of life from iron monosulphide bubbles at a submarine hydrothermal redox and pH front. *J. Geol. Soc.* 154, 377–402. doi: 10.1144/gsjgs.154.3.0377

Russell, M. J., Hall, A. J., and Martin, W. (2010). Serpentinization as a source of energy at the origin of life: Serpentinization and the emergence of life. *Geobiology* 8, 355–371. doi: 10.1111/j.1472-4669.2010.00249.x

Russell, M. J., Nitschke, W., and Branscomb, E. (2013). The inevitable journey to being. *Philos. Trans. R. Soc. B Biol. Sci.* 368, 20120254. doi: 10.1098/rstb.2012.0254

Russell, M. J., and Ponce, A. (2020). Six "Must-Have" Minerals for Life's Emergence: Olivine, Pyrrhotite, Bridgmanite, Serpentine, Fougerite and Mackinawite. *Life Basel Switz.* 10, 291. doi: 10.3390/life10110291

Schrenk, M. O., Brazelton, W. J., and Lang, S. Q. (2013). Serpentinization, Carbon, and Deep Life. *Rev. Mineral. Geochem.* 75, 575–606. doi: 10.2138/rmg.2013.75.18

Strecker, A. (1850). Ueber die künstliche Bildung der Milchsäure und einen neuen, dem Glycocoll homologen Körper. *Justus Liebigs Ann. Chem.* 75, 27–45. doi: 10.1002/jlac.18500750103

Suzuki, S., Kuenen, J. G., Schipper, K., van der Velde, S., Ishii, S., Wu, A., et al. (2014). Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. *Nat. Commun.* 5, 3900. doi: 10.1038/ncomms4900

Wächtershäuser, G. (1992). Groundworks for an evolutionary biochemistry: The iron-sulphur world. *Prog. Biophys. Mol. Biol.* 58, 85–201. doi: 10.1016/0079-6107(92)90022-X

Wöhler, F. (1828). Über künstliche Bildung des Harnstoffs. *Ann. Phys. Chem.* 88, 253–256.



CHAPTER 2
Materials and methods

2.1 Preface

This chapter gives background information on study site, sample collection and both the metagenomic and the cultivation approach. The respective workflows are summarized, and the benefits and drawbacks of both approaches are discussed. Details on the methodology can be found in the Materials and Methods section of the third and fourth chapter, which correspond to the manuscripts of the two research articles that were produced in the frame of this thesis.

2.2 Sample recovery

2.2.1 Study site

This thesis addresses three different sites at Prony Bay (Figure 2.1 A): The terrestrial Rivière des Kaoris (RdK), the intertidal Bain des Japonais (BdJ) and the submarine Aiguille de Prony (ST07) (Figure 2.1 B). In the third chapter, metagenomes from all three sites are assessed; in the fourth chapter, we focus exclusively on ST07.

The RdK site is located at the mouth of the Kaoris river at the northeastern edge of Prony Bay. The samples used in this thesis were collected on a riverbank with active venting sites not far from an artificially constructed pool that can be visited as a tourist attraction. While RdK is not submerged by seawater and thus discussed as a terrestrial site here, it is important to note that it remains strongly influenced by the marine environment of the bay.

The BdJ site is situated in the northwest of Prony Bay close to the mouth of the Carénage creek. This zone consists of several platforms featuring small, active hydrothermal chimneys that are submerged at high tide and uncovered at low tide.

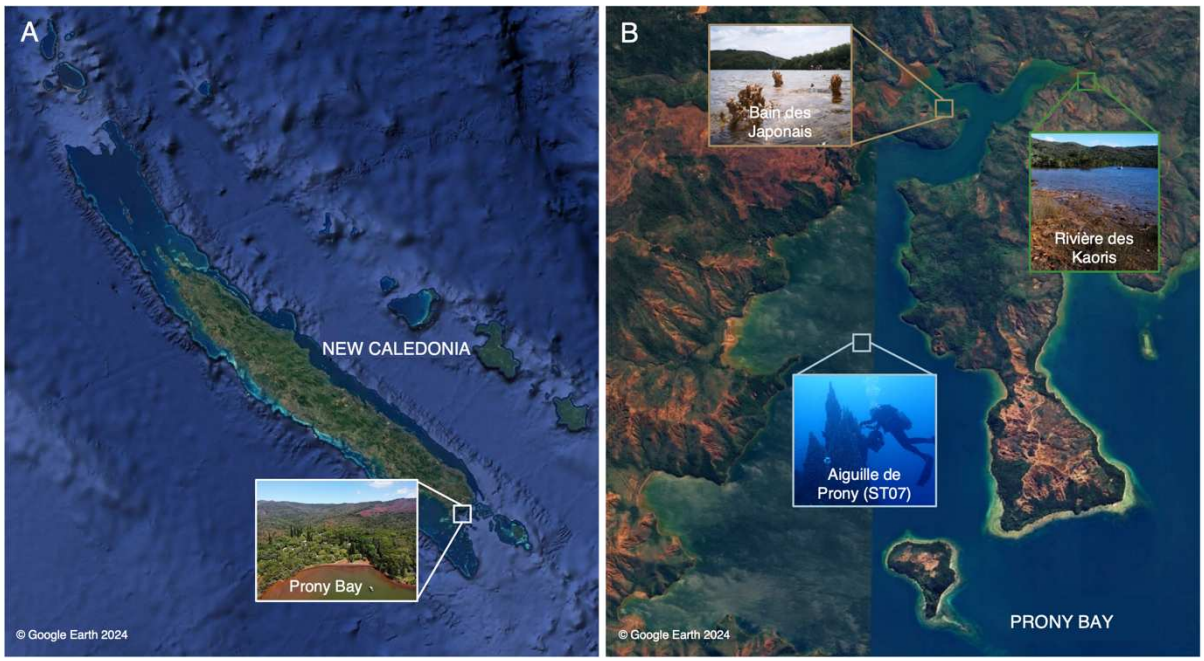


Figure 2-1. Study site. (A) Location of Prony Bay at the southern coast of New Caledonia. (B) Location of the three sites discussed in this thesis at Prony Bay. Maps adapted from Google Earth: Image © 2024 TerraMetrics, Image © 2024 Airbus, Image © 2024 CNES / Airbus. Data SIO, NOAA, U.S. Navy, NGA, GEBCO. Photos from © Jacopo Pasotti, 2022 and © Roy Price, 2022

ST07 is situated in the center of the lagoon at a depth of approximately 45 m depending on the tide. It is a large carbonate massif with a total height of around 38 m. The most active zone is found at a depth of approximately 20 meters, where numerous hydrothermal chimneys discharge hydrothermal fluid. ST07 had initially been described as a coral reef by Launay and Fontes (1985), with its true nature being only revealed 20 years later during the bathymetric survey that led to the rediscovery of Prony Bay (Pelletier, 2005).

Prony Bay's bedrock transitions from peridotite and gabbro in the east to serpentinite in the west. The former are components of the Earth's original mantle and the degree of their serpentinization is currently unknown. The substratum under the sampling sites varies accordingly: While BdJ is situated on fully and ST07 on partially serpentinized rock, RdK marks the transition zone between peridotite and serpentinite (Monnin et al., 2014). For all three sites, the concentration of DIC measured in the endmember fluid is mostly below 1 mmol L^{-1}. The endmember pH averages to 10 and the temperature to 32 °C between sites (Table 2.1) (Monnin et al., 2014, 2021).

Table 2-1. Physicochemical characteristics of the endmember fluid at the Rivière des Kaoris (RdK), the Bain des Japonais (BdJ) and the Aiguille the Prony (ST07) sites

Site	Year	DIC mmol L^{-1}	pH	Temperature °C	Reference
RdK	2011	0.0656 - 0.0662	10.80	31.0	Monnin et al., 2014
	2014	n.m.	10.93	31.1	Monnin et al., 2021
BdJ	2011	0.3097 - 1.7884	8.44 - 11.08	28.2 - 37.6	Monnin et al., 2014
	2014	n.m.	10.88	38.5	Monnin et al., 2021
ST07	2011	0.0001 - 0.0138	9.44 - 10.14	n.m.	Monnin et al., 2014
	2022	n.m.	10.33- 10.73	28.1 - 31.0	Unpublished field data

n.m. = not measured

2.2.2 Sampling procedure

The sampling procedure varies according to the type of analysis; the recovery of rock, fluid and gas samples for geological and chemical assessment will not be further developed here. The analyses presented in this thesis are based on hydrothermal chimney fragments, which served as material for DNA extraction and cultivation experiments.

At RdK, substrate from venting sites was collected with spatulas in Schott bottles. At BdJ, hydrothermal chimneys were recovered using a chisel and a hammer and transferred to Schott bottles containing hydrothermal fluid from the same site. The Schott bottles were then transported to the field laboratory (illustrated on the title page of this chapter), hermetically sealed with butyl rubber stoppers and immediately flushed with nitrogen (N_2) gas to remove oxygen (O_2). At ST07, scientific divers collected the summit part of several hydrothermal chimneys in the most active zone of the carbonate massif at a depth of around 20 m. The chimneys were covered in watertight bags and left in place for several hours until the hydrothermal fluid had flushed out the seawater entirely. They were then sawed off and the bags were hermetically sealed on site before being removed from the water. In the field laboratory, the chimneys were carefully removed from their bags and sawed into slices of

approximately 35 mm thickness. The outer layer was removed with a chisel to isolate the inner parts of the chimney that had not been in contact with seawater.

For DNA extractions, chimney core subsamples were immediately frozen in liquid N_2 and stored at -80 °C as soon as possible. Subsamples for cultivation experiments were transferred into Schott bottles containing hydrothermal fluid from the same site, hermetically sealed with butyl rubber stoppers and flushed with N_2 gas to preserve anaerobic conditions. Back from the field, they were crushed to a slurry in an anaerobic chamber using mortar and pestle. Slurry subsamples of 35 - 40g were stored in hermetically sealed Schott bottles at 4 °C until further use.

All equipment except for the watertight bags and the saw used during dives was autoclaved or flamed with ethanol to ensure sterile conditions both in the field and in the laboratory.

2.3 The metagenomic approach

The third chapter of this thesis is based on the metagenomic analysis of five environmental metagenomes from RdK, BdJ and ST07. The samples were recovered in 2011 and 2014 during the first field campaigns to Prony Bay and remained in storage for several years before metagenomic sequences were recovered in 2016.

2.3.1 Introduction to metagenomics

The rapid development of metagenomics over the past twenty years is closely linked to the advancement of sequencing technologies and the establishment of next generation sequencing (NGS). Today, there are two main approaches: The analysis of amplicons from a specific gene of interest, and the analysis of untargeted gene data produced through whole genome shotgun (WGS) sequencing. Amplicon sequencing is mostly used to assess taxonomic community composition via small subunit ribosomal ribonucleic acid (SSU rRNA) genes. This approach will be further discussed in the fourth chapter, where we use the 16S ribosomal ribonucleic acid (16S rRNA) gene to characterize the cultivated microbial consortia. Untargeted gene data is not limited to a predefined region. This allows the analysis of all information present in a sample (Nam et al., 2023).

After sample recovery, DNA extraction is the first critical step of the metagenomic workflow. Especially for WGS sequencing, it is essential to have sufficient amounts of good-quality DNA, which can be challenging in samples from extreme environments where cellular densities are low. DNA extraction methods include mechanical lysis, the use of lysis buffers or a phenol-chloroform mixture for DNA extraction and finally the precipitation of DNA with isopropanol or ethanol or the binding of nucleic acids on the surface of silica matrices (Dairawan and Shetty, 2020). The isolated DNA is prepared for NGS by preparing a collection of similar sized DNA fragments labelled with known adapter sequences, which contain the primer annealing region and a sequence binding to the surface of the flow cell. In WGS sequencing, the entire genomic DNA is randomly broken up into short segments which are then sequenced multiple times to obtain overlapping reads. This fragmentation is performed for several reasons, including the technical limitations of sequencing platforms, which can only read relatively short DNA segments, and improved accuracy due to the production of several replicates per fragment ("sequencing depth") (Navgire et al., 2022).

Bioinformatic treatment starts with the removal of adapter and low-quality sequences as well as contamination. The further workflow strongly varies depending on data type and research question. Reads produced by WGS sequencing are usually reassembled into longer fragments of continuous sequences ("contigs" and "scaffolds") using either a reference-based or a *de novo* approach (Thomas et al., 2012). When working with data from several samples, the choice between co-assembly (assembling sequences from multiple samples into one metagenome) and independent assembly (assembling sequences from each sample into separate metagenomes) is crucial. Co-assembly is usually performed for samples from the same

or very similar environments. It can facilitate the recovery of low-abundance organisms by increasing the overall genome coverage and requires less computational resources. Independent assemblies are performed on samples from different environments. This approach accurately reflects the relative abundance of rare organisms and preserves sample-specific variation (Ghurye et al., 2016).

Contigs or scaffolds originating from the same or closely related genomes can be grouped together ("binned") into so-called bins or metagenome-assembled genomes (MAGs). The binning process relies on different metrics depending on the used algorithm, for example on conserved nucleotide patterns in subsequences of fixed length ("k-mers") and on genomic regions that are registered in reference databases (Thomas et al., 2012). Bin completeness and contamination thresholds are chosen depending on the input data; for sequences from extreme environments, one may opt for slightly more flexible thresholds to compensate for low sample biomass, microdiversity and the often poorly characterized genomic content. Binning leads to information loss, as contigs have to pass through several filters to be included into a MAG. Meanwhile, taxonomic resolution and functional inference are improved by grouping high-quality fragments from closely related organisms together (Alneberg et al., 2014; Ramos-Barbero et al., 2019).

The next step is usually to predict and annotate protein coding regions in the final MAG sequences (the same can be done for unbinned contigs or scaffolds). Annotated protein sequences can be taxonomically classified by aligning them against a reference database, which usually differs from those used for amplicon data as a set of multiple core marker genes is used (Thomas et al., 2012). This might cause comparability issues: For this thesis, relevant taxonomic information was compared by hand between the Genome Taxonomy database (GTDB) and the SILVA ribosomal RNA gene database used in chapter four (Quast et al., 2013; Parks et al., 2022). The same approach can be applied to reconstruct phylogenomic trees, for example with the bacterial (120 core genes) or archaeal (53 core genes) marker set of GTDB (Chaumeil et al., 2022).

Next to taxonomic marker genes, protein-coding sequences can be annotated regarding their function. This allows the metabolic characterization of MAGs, and the reconstruction of trophic networks (Nam et al., 2023). The most commonly used database for functional analysis is the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG organizes gene sequences into orthologous groups, allowing their comparison across different taxa. In addition, it features a catalogue of metabolic reactions. Both gene families and metabolic reactions are compiled in metabolic pathway maps, which are then organized into functional units (Kanehisa et al., 2016).

2.3.2 The metagenomic workflow

The samples assessed in the third chapter remained in storage for five and three years respectively after their collection, until genomic sequences were recovered in 2016. DNA extraction was performed by Gaël Erauso and Eleonore Frouin, using a phenol-chloroform-based approach in combination with bead beating. This mechanical treatment aids the lysis of the cell, making it less reliant on the chemical reaction (Dairawan and Shetty, 2020). Especially for low-biomass samples containing abiotic material, this approach has proven successful in

our laboratory. WGS sequencing was performed by GetGenotoul (Castanet-Tolosan, France) on an Illumina HiSeq 3000 System, which has since been replaced by newer platform types.

The WGS sequencing data was analyzed for the first time here. After read quality control, independent *de novo* assemblies were performed using metaSPAdes, which is one of the most commonly used and reliable algorithms currently available (Nurk et al., 2017). The independent assembly approach was chosen after careful consideration: While RdK, BdJ and ST07 are part of the same system and primarily influenced by serpentinization, the sites are distinct in their terrestrial, intertidal and marine environment. In addition, the samples were collected in two different years and analyzed separately for each year, adding a temporal bias. We therefore opted to preserve and compare the sample-specific variation rather than working with a single co-assembled metagenome.

After assembly, we curated a set of MAGs to optimize the taxonomic resolution of our analysis. Initial binning was performed with the three different algorithms of MetaBAT2, MaxBin2 and CONCOCT (Alneberg et al., 2014; Wu et al., 2016; Kang et al., 2019). To achieve optimal results, we subsequently performed a consensus bin refinement with metaWRAP bin_refinement in which the three bin sets are compared and only the most reliable bins retained (Uritskiy et al., 2018). For the final data set, we chose only MAGs that were at least 50% complete and featured a maximum contamination of 10%. These cutoff values are considered the minimum for publication and were chosen to preserve as much genomic information as possible (The Genome Standards Consortium et al., 2017). The final set of MAGs constitutes the base for all further analyses.

In the first part of the chapter, MAGs from the five different metagenomes were characterized and compared with each other. Protein coding regions in MAGs were predicted using Bakta (integrating Pyrodigal), a relatively recent algorithm optimized for computational efficiency, which is an important consideration when working on several metagenomes at once (Schwengers et al., 2021; Larralde, 2022). The amino acid sequences were then used to calculate the average protein oxidation state of each metagenome. To contextualize our data, we additionally calculated the average protein oxidation states of aerobic, microaerophilic and anaerobic organisms registered in the bacterial metadatabase (BacDive) (Reimer et al., 2019). The redox state of enzymes and cofactors plays an important role in the regulation of metabolic reactions in different O_2 conditions. In aerobic organisms, cellular proteins cycle between oxidized and reduced states; in anaerobes, the overall protein oxidation state is decreased because O_2 is unavailable as terminal electron acceptor, and because many enzymes are redox-sensitive. The calculation of average protein oxidation states is thus useful to determine whether the respective metagenome is influenced by O_2 or not, which is fundamental for determining its metabolic make-up (Dick and Shock, 2011; Dick, 2014). Details on the calculation can be found in the Materials and Methods section of chapter three.

Subsequently, we explored the phylogenomic relationships between MAGs from different samples and their relationship to sequences from other serpentinite-hosted ecosystems. For this, we used the GTDB-tk algorithm which constructs a phylogenomic tree based on GTDB's marker gene sets (Chaumeil et al., 2022). We also calculated the relative abundance of MAGs in their respective samples, as ecological significance is usually correlated to the abundance of an organism in the system. For this, we used the CoverM algorithm which normalizes by genome length and coverage, mapping reads to MAG sequences (Aroney et al., 2024).

In the second part of the chapter, we explore the MAGs' metabolic capacity to use bicarbonate, formate, acetate and glycine as carbon source. The basis of this analysis is a comprehensive literature review identifying the metabolic reactions and pathways involved in the uptake of each putative carbon source. Details on the proposed functional units can be found in the introduction of chapter three and in Figure 3-6. Briefly, the uptake of all four carbon compounds relies on transporter proteins which facilitate their passage through the cellular membrane. Once in the intracellular milieu, the metabolic route of bicarbonate and formate diverges from acetate and glycine. Bicarbonate and formate are transformed into CO_2 via the carbonic anhydrase and formate dehydrogenase respectively. The yielded CO_2 can then pass by one of the seven known carbon fixation pathways to produce acetyl coenzyme A (acetyl-CoA) or one of the other central metabolic precursors (pyruvate, oxaloacetate, succinate, α-ketoglutarate). Bicarbonate and formate are thus discussed as autotrophic carbon sources, and organisms capable to grow on them are discussed as potential autotrophic foundation species. Acetate and glycine are not transformed into CO_2, but directly assimilated into acetyl-CoA via the acetyl-coA synthetase (alternatively acetate kinase and phosphate acetyltransferase), or the glycine reductase respectively. Acetate and glycine are thus discussed as heterotrophic carbon sources and organisms featuring the respective enzymes are discussed as potential heterotrophic foundation species. Our MAGs were scanned for genes encoding corresponding transporters, and variants of the carbonic anhydrase, formate dehydrogenase, acetyl-coA synthetase, acetate kinase, phosphate acetyltransferase and glycine reductase. This was done with the kofam_scan algorithm, which scans input protein sequences for KEGG ortholog (KO) identifiers based on hidden Markov model (HMM) profiles (Aramaki et al., 2020).

To identify carbon fixation pathways in MAGs with bicarbonate and formate uptake genes, we initially based our analysis on the respective KEGG modules. When verifying certain reactions on the MetaCyc database (Caspi et al., 2018), however, we were surprised to find that proposed pathway structures varied strongly between the two databases. The enzyme commission (EC) numbers constituting the pathways were not coherent, and for some pathways different versions were specified in one database but not documented in the other. In addition, we noticed that the KEGG modules, which are based on KO identifiers grouped by EC number, do not take all enzyme variants or gene families into account that are able to perform the respective reaction. This poses an important bias when calculating pathway completeness, which is based on the percentage of pathway reactions that can potentially be executed by a MAG. Based on these observations we decided to merge the KEGG and MetaCyc databases and to curate a custom collection of KO identifiers for each pathway version. To do this, EC numbers and corresponding KO identifiers listed in the KEGG module were extracted. Furthermore, the list of EC numbers for the same pathway was extracted from MetaCyc and matched with KO identifiers. This provided us with an exhaustive collection of KOs for each pathway EC number. In the following, we manually reviewed each EC number in the pathway collection to determine the KO combinations allowing the reaction to operate. KOs were classified as subdomains of the same protein (all of them must be present to render the reaction operational), as synonyms or alternatives to each other (only one of them must be present to render the reaction operational). The final pathway structure was encoded into a bioinformatic script scanning input sequences for respective KO identifiers via kofam_scan and calculating a pathway completion score for each MAG.

Next to carbon uptake and pathway genes, we scanned our MAGs for gene families playing a key role in H_2, CO, CH_4, nitrogen, sulfur and O_2 metabolism using kofam_scan. This was done because carbon uptake cannot be taken out of its metabolic context: For example, a MAG contained all genes for the uptake and fixation of bicarbonate but was unable to use a lithotrophic energy source.

Finally, all of the metabolic information was synthesized to identify MAGs with a complete set of genes allowing the fixation or assimilation of one of the four putative carbon sources, attempting to identify potential autotrophic and heterotrophic foundation species in the different samples.

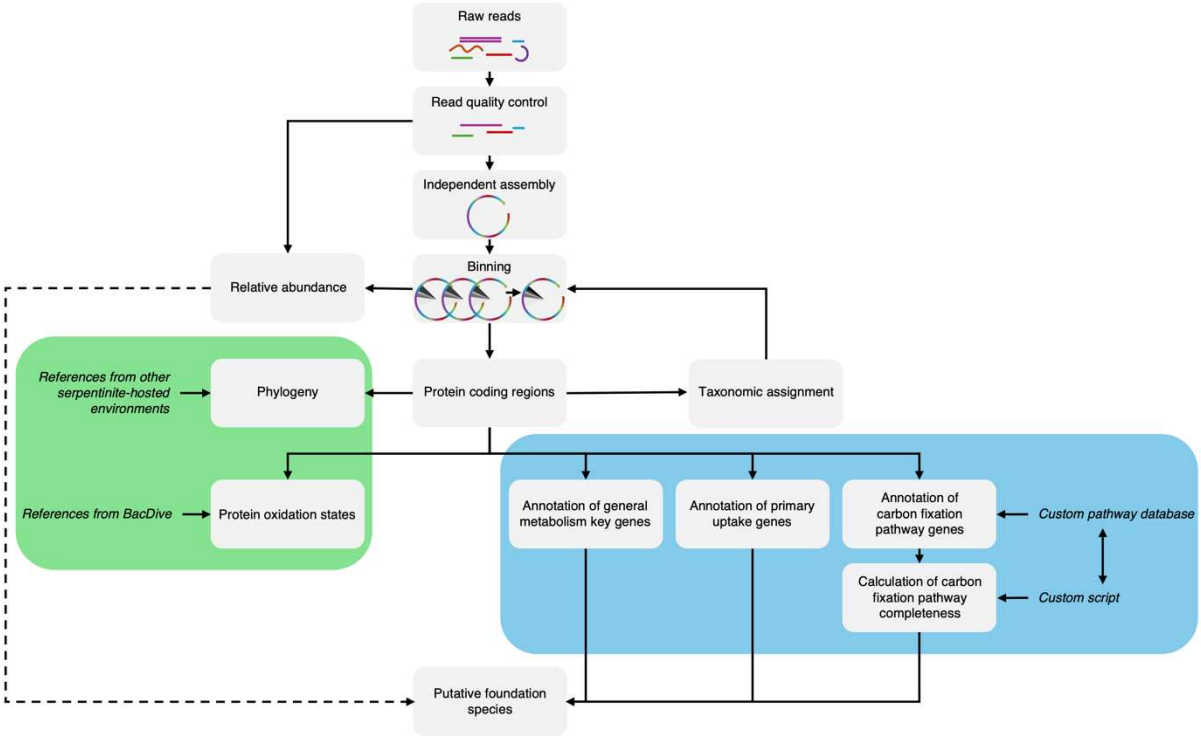


Figure 2-2. Metagenomic workflow. Sample characterization steps are highlighted in green, the functional analysis in blue

2.3.3 Benefits and drawbacks of the metagenomic approach

Metagenomics present a powerful and non-invasive way to describe not only the taxonomic, but also the functional diversity of an ecosystem. This approach can be considered relatively accurate in representing the natural community, as it facilitates the study of organisms that cannot be cultivated and is independent from the polymerase chain reaction (PCR) which can introduce bias when assessing abundance (further developed under 2.4).

However, the variation in carbon fixation pathway structures between different databases illustrates one of the fundamental drawbacks of metagenomics. This approach is heavily dependent on the scope of reference databases and well-maintained collections are often behind a paywall. When dealing with MAGs, it is important to note that they are not equivalent to

genomes. MAGs are mostly incomplete, and the absence of a gene does not necessarily reflect a biological reality: The gene could simply be encoded in a region that is missing from the MAG sequence. The absence of single enzymes, as well as barely incomplete pathways thus need to be interpreted with caution. Most metagenomes contain a very high percentage of unidentified protein sequences, which further limits the prediction of metabolic functions in a metagenome or MAG (Thomas et al., 2012).

The most relevant issue with regard to this thesis is enzyme multifunctionality. The carbonic anhydrase, formate dehydrogenase, acetyl-coA synthetase, acetate kinase, phosphate acetyltransferase and glycine reductase are all bidirectional (Kanehisa et al., 2016). Even if those enzymes are present in an input sequence, it is thus unclear whether they perform the reaction we are interested in (e.g. bicarbonate to CO_2 for the carbonic anhydrase) or the opposite one (CO_2 to bicarbonate). This illustrates the next drawback: Many organisms encode genes that are obsolete but still present in the genome. From metagenomic data alone, it is impossible to predict whether a gene is transcribed and actively used.

Metabolic activity can for example be verified by observing a relevant microbial consortium in culture. This is the approach we chose for the fourth chapter of this thesis, i.e. the monitoring of long-term cultures in specialized bioreactors.

2.4 The cultivation approach

The fourth chapter of this thesis describes the cultivation of microbial consortia from the submarine ST07 on each of the four putative carbon sources. This analysis is based on samples that were collected during a field campaign in 2022. Cultivation experiments were conducted under anaerobic conditions and with H_2 as an energy source to enrich the hydrogenotrophic subsurface communities, which live in close contact with the hydrothermal fluid and are most influenced by serpentinization.

2.4.1 Introduction to the cultivation of anaerobic extremophiles

Metagenomic studies have revealed that less than 1% of the overall prokaryotic diversity has been brought into culture and that only a minuscule proportion of these isolates are from extreme environments (Alain and Querellou, 2009; Schultz et al., 2023). Cultivating extremophiles presents a variety of challenges that go beyond the usual problems when attempting to develop cultures from an environmental sample. Firstly, it can be challenging to collect (enough) material for the inoculation of culture experiments. Extreme environments are often remote and difficult to access: For example, Prony Bay is only accessible by off-road vehicle or by boat, and some of its terrestrial springs can only be reached by experienced hikers or by helicopter. This delays inoculation, which has been shown to significantly affect both the abundance and diversity of cultures (Schultz et al., 2023). Furthermore, extreme environments are often characterized by a low cellular abundance, which does not only pose difficulties for DNA extraction as described under 2.3.1 but also for the inoculation of cultures (Rampelotto, 2024).

Secondly, extremophiles have very specific growth requirements that can be difficult to identify and fulfil in a laboratory setting. Standard cultivation approaches usually provide nutrient-rich media containing several sources of organic carbon, in hopes that one of them can be used by the community. However, the growth of most extremophiles is inhibited by such media as their systems of origin are oligotrophic. Unfortunately, the design of low-nutrient media requires relatively detailed knowledge of the metabolism of targeted organisms (Schultz et al., 2023). This can be further complicated by the fact that some metabolic precursors are not easy to supply in a laboratory setting. For example, the cultivation of hydrogenotrophic organisms is not possible in every laboratory as H_2 is highly flammable and its use subject to strict safety regulations. Lastly, extreme ecosystems are often characterized by complex syntrophic interactions, which present an important survival strategy by lowering energetic cost and "sharing" a pool of useful adaptions. An example is syntrophic acetate oxidation (SAO), which provides H_2 and CO_2 for methanogens and falls precisely into the scope of this thesis (Rampelotto, 2024). If the cultivation of one symbiotic organism fails, this might concern a whole subnetwork of the trophic chain. The cultivation of extremophiles thus requires detailed knowledge of the targeted ecosystem and the technology to collect samples and recreate the

conditions of the natural system in the laboratory. In recent years, progress has been made due to the development of omics techniques and the implementation of advanced culture platforms as illustrated in this thesis (Boileau et al., 2016; Rampelotto, 2024).

When targeting anaerobic extremophiles, it is furthermore necessary to follow special protocols as contamination with O_2 must be avoided at all times. This requires culture vessels that can be hermetically sealed, such as Hungate and Balch tubes, Penicillin flasks as well as serum vials and the above-mentioned Schott bottles closed with butyl rubber stoppers, or anaerobic bioreactors (Yang et al., 2023). When preparing anaerobic cultures, the first difficulty is the removal of O_2 from the growth medium. This can be achieved by heating the medium and then flushing it with O_2-free gas such as N_2 prior to sterilization. The heating process decreases the solubility of O_2 and provokes its diffusion from the liquid phase into the gas phase, where it is subsequently replaced (Wagner et al., 2019). The removal of O_2 from the growth medium requires addition of alternative reducing agents to control the oxidation-reduction potential (ORP), which is most commonly achieved with sodium sulfide (Hanišáková et al., 2022). Due to practical reasons, anaerobes are mostly cultivated in liquid growth media. Nevertheless, it is possible to set up cultures on solid medium (solidified with agar or phytagel for extremophiles) by placing petri dishes in an anaerobic chamber or jar, or by performing Hungate's roll tube technique where a thin layer of inoculated solid medium is distributed on the inside of a Hungate tube (Hungate and Macy, 1973; Hanišáková et al., 2022). The inoculation procedure depends on the nature of the sample. In case of a liquid preculture, inoculation is best performed with a syringe that is flushed with O_2-free gas before sample transfer. If the sample cannot be transferred with a syringe, it is possible to work in an anaerobic chamber; however, it is important to note that this does not provide a sterile environment. Otherwise, O_2 exposure can be minimized by working quickly under a strong flow of non-flammable O_2-free gas next to a flame to ensure sterility. During the incubation period, the culture vessel is usually flushed with O_2-free gas to maintain an anoxic atmosphere. More sophisticated platforms such as fed-batch bioreactors permit the establishment of a continuous gas flow in the reactor. Gas solubility is usually ensured by stirring the growth medium, as continuous gassing makes it hard to keep the bioreactor pressurized. This might prevent the growth of organisms sensitive to agitation, such as some methanogens. Hungate tubes or serum bottles might need to be manually repressurized multiple times, as cultivated organisms can consume the gases in the headspace over time (Hanišáková et al., 2022).

2.4.2 The cultivation workflow

The samples from ST07 were crushed to a slurry within one month after their collection and used for inoculation within one and five months after their collection. This was the minimum delay considering transport between New Caledonia and France, as well as the availability of the culture platform. Four different culture conditions were established, each with a single carbon source: either bicarbonate, formate, acetate or glycine. Each culture condition was tested twice, resulting in a total of eight cultures which were each maintained for 25 – 26 days. The grown microbial consortia were compared with each other regarding cellular growth, consumption of carbon source and electron acceptors, community composition and diversity.

2.4.2.1 The bioreactor platform

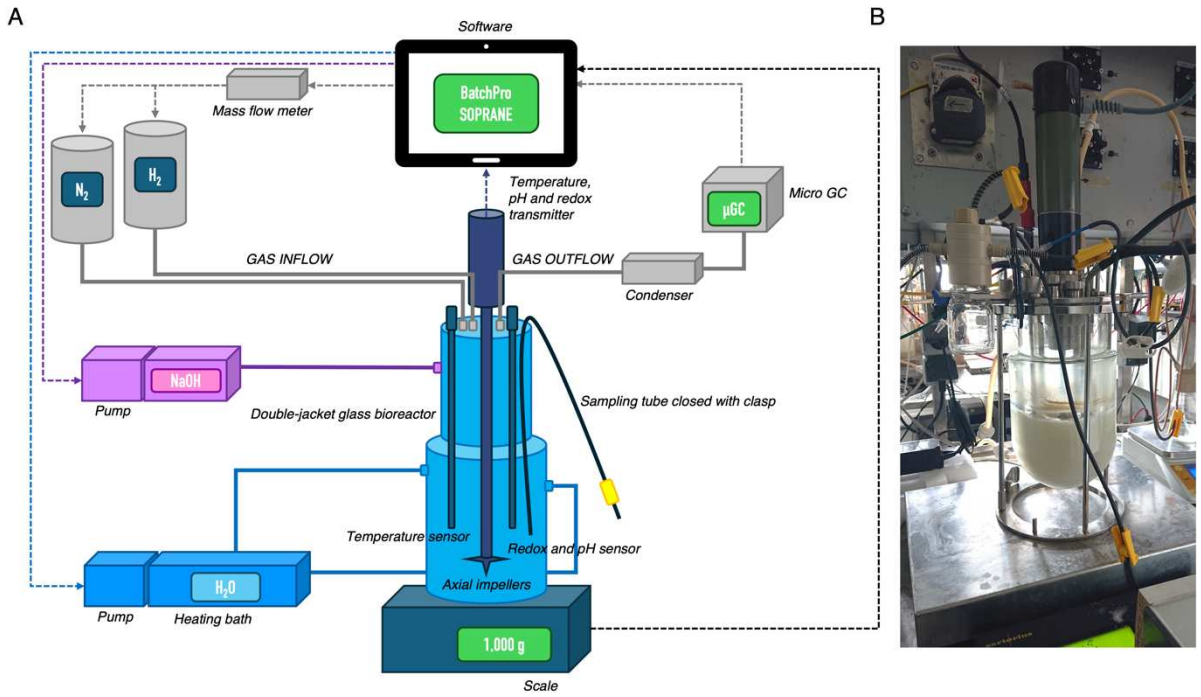


Figure 2-3. Anaerobic fed-batch bioreactor used in cultivation experiments. (A) Scheme detailing technical set-up (B) Photo of a bioreactor in use

Cultivation experiments were performed in anaerobic fed-batch bioreactors as described by Boileau et al. (2016). The platform consisted of two double-jacket glass bioreactors with an effective volume of 1.5 L (FairMenTec, Switzerland and Pierre Guerrin, France), which were each placed on a scale to measure the volume of contained liquids (Sartorius Combics 1 and BP 4100, France). The growth medium was stirred with axial impellers at 450 or 500 rpm, respectively, allowing an equivalent gas mass transfer for both bioreactor (K_La = 45 h^{-1}). Several key parameters characterizing the natural serpentinite-hosted environment could be controlled. The temperature was measured with a sensor (Prosensor pt 100, France) and automatically regulated at 35 ± 2 °C with water heated in a bath and subsequently pumped through the double-jacket (Julabo F25, France). Likewise, redox potential and pH were measured with a sensor (Mettler Toledo InPro 3253, Switzerland) and the pH regulated at 10.5 ± 0.5 by automatic or manual sodium hydroxide addition. Both bioreactors were alimented with a continuous inflow of N_2 and H_2 gas at 5 ml min^{-1} each to maintain anaerobic conditions and supply a metabolic energy source for the targeted hydrogenotrophic community. The rate of the gas inflow was controlled by two mass flow meters (Bronkhorst El-Flow, Netherlands). The gas outflow was condensed in a glass water cooler at 4°C to prevent the loss of liquid volume from the bioreactor (Julabo F25, France). The composition of the gas outflow was measured on a downstream micro gas chromatography system with a catharometric detector (MS5A, SRA Instrument, France) and a CO_2 probe (Vaisala GMT 221, Finland). The gas outflow streamline was closed with a hydraulic seal to prevent contamination with air. Samples

were taken via a tube immersed into the growth medium and closed with a clasp on the outside of the bioreactor.

Agitation, pH, temperature and gas input were automatically regulated via a local feedback loop (Wago, France). The system was monitored and controlled using the BatchPro (Decobecq Automatismes, France) and the SOPRANE (SRA Instruments, France) software packages.

2.4.2.2 The growth medium

The growth medium was based on the mineral composition of the hydrothermal fluid measured by Monnin et al. (2014). We added Balch's trace element solution, an additional trace metal solution containing nickel, iron, tungstate and selenite, and Balch's vitamin solution. These solutions provide important enzymatic cofactors that are particularly important for the growth of anaerobic chemolithotrophs such as methanogens (Balch et al., 1979). Furthermore, 5 mM each of thiosulfate (which can also serve as metabolic energy source) and sulfate were added as electron acceptors to isolate the anaerobic microbial community. This decision was also influenced by the results of previous cultivation experiments on BdJ, which tested various electron acceptors and showed that sulfate and especially thiosulfate facilitated the development of diverse microbial consortia (unpublished data). Lastly, 5mM of either sodium acetate, sodium formate, glycine or sodium bicarbonate were added as the sole carbon source depending on the culture condition. Details on the composition of the growth media can be found in the materials and methods section of chapter four.

Sterilization, degassing and pH adjustment of a total of 1L of growth medium were performed directly on the culture platform. The medium was sterilized by filtration: it was pumped through a sterile tube into the autoclaved bioreactor (Julabo F25, France), passing through a Sterivex unit (0.2 µm). Subsequently, the medium was stirred at high speed under a strong flow of N_2 gas for several hours until O_2 was fully removed from the bioreactor (as indicated by the redox sensor). Then, temperature and pH were adjusted as described above.

2.4.2.3 Inoculation

The inoculation process proved to be challenging, as the slurry could not be transferred with a syringe and the bioreactor could not be placed into an anaerobic chamber. Finally, we decided to slightly open the lid of the bioreactor under a very high flow rate of N_2 gas and very slow agitation to minimize air diffusion into the growth medium. This was done next to a flame to ensure sterility. Quickly, the slurry was transferred to the bioreactor using a sterile spoon. The glass bottle containing the inoculum was rinsed with a subsample of sterile, degassed and pH adjusted growth medium.

2.4.2.4 The sampling procedure

A sample of approximately 20 ml was collected from each bioreactor every 2-3 days, the first one directly after inoculation and the last one directly before the end of the experiment. To avoid contamination with O_2, a sterile needle was attached to the end of the sampling tube and inserted into a hermetically sealed, anoxic and sterile Hungate tube before opening the clasp. Subsequently, a syringe flushed with N_2 gas was inserted into the Hungate tube and the plunger was pulled up to create negative pressure, provoking the transfer of culture medium from the

bioreactor into the Hungate tube. Subsamples were immediately processed for different types of analyses.
A subsample of 4 ml was fixed with 2% of formaldehyde for fluorescence microscopy and stored at 4°C. A subsample of 14 ml was centrifuged at 7000 rpm and 4°C for 20 min to precipitate a cell pellet from the culture sample. The pellet was stored at -80°C for DNA extraction. The supernatant was stored at -20°C for high-performance liquid chromatography (HPLC) and ion chromatography (IC). The remaining 2 ml of the original sample were kept in the sealed Hungate tube and stored at 4°C as a backup.

2.4.2.5 Chromatography

The supernatant recovered by centrifugation was analyzed via column-based HPLC and anion-exchange IC to establish the consumption of formate and acetate, as well as sulfate and thiosulfate. Column-based HPLC is a technique used to analyze the chemical composition of liquid samples, identifying and quantifying their components. High pressure pumps transport the sample via a mobile phase into columns, the so-called stationary phase. These columns are specific for certain classes of compounds (for example, organics acids such as formate and acetate are usually analyzed with non-polar silica columns) and filled with solid particles interacting in different migration rates with the sample compounds. Transversing the stationary phase at different speeds, the compounds reach the downstream detector at different points in time. This allows their identification and quantification and is illustrated in a chromatogram, which depicts the signal intensity versus time or volume. Each compound is depicted with a curve starting at the compound-specific timepoint, its surface representing the compound's quantity in the sample (Harde et al., 2023). Here, we used a mobile phase of ultrapure water with 5 mM sulfuric acid eluted at a rate of 0.6 ml min^{-1}. The column (Biorad HPX 87H, Biorad, USA) was maintained at 45°C. Peak detection was conducted with a UV detector (Dionex ultimate 3000, Thermofisher Scientific, USA) and a refractometer (Shimadzu RID6A, Shimadzu, Japan).

Contrary to HPLC, the columns in anion-exchange IC (Thermofisher Scientific, Dionex AS-DV, USA) consist of positively charged groups that interact with anions in the sample (Ngere et al., 2023). We used a mobile phase of pure water with 9 mM of sodium carbonate eluted at 1 ml min^{-1}. The column (Dionex AS9HC, Thermofisher Scientific, USA) was maintained at 45°C. An amperometric suppressor (Dionex AERS 500, 45nA, Thermofisher Scientific, USA) was placed before the conductivity detector in order to improve peak detection. Machine supervision and signal analysis were performed with the Chromeleon software (Thermofisher, USA).

2.4.2.6 Cellular growth

The subsample fixed with formaldehyde was diluted with sterile growth medium and filtered onto white polycarbonate filters (0.2 µm porosity and 25 mm diameter from Nucleopore Whatman, USA) using a vacuum pump. For each sample, at least two filters were prepared. The filers were stained with a 3.15 µM solution of 4′,6-Diamidin-2-phenylindol (DAPI), which is a strongly fluorescent stain that allows to visually distinguish cells from inorganic material by binding to adenine–thymine-rich regions in DNA. Subsequently, the filters were analyzed under an OLYMPUS BX-61 microscope with a filter at 372 nm (Evident, Japan). Cells were

counted in 15 random fields and these counts were averaged between replicates to establish a growth curve for each culture.

2.4.2.7 Excursion to the metagenomic approach: Targeted gene analysis

To analyze composition and diversity of the eight cultivated microbial consortia, we performed amplicon-based analysis of the 16S rRNA gene. This presents the second branch of metagenomics briefly mentioned under 2.2.

DNA was extracted from the pellets recovered in the centrifugation step, using the commercially available DNAeasy PowerSoil Pro kit (Qiagen, USA). Contrary to the DNA extractions performed on the environmental samples from chapter three, this kit does not use a phenol-chloroform based approach but relies on proprietary lysis buffers in combination with bead beating.

In amplicon sequencing, a PCR is conducted to amplify the region containing the marker gene. Here, the V3-V4 region of the 16S rRNA gene was amplified with both a bacterial and an archaeal primer pair and then sequenced on an Illumina MiSeq System by MRDNA (Shallowater, Texas, USA). After read quality control in DADA2, which is one of the most commonly used algorithms for amplicon sequences (Callahan et al., 2016), amplicon-sequence variants (ASVs) were reconstructed and taxonomically assigned using the SILVA ribosomal RNA gene database (Quast et al., 2013). ASVs are groups of variants of a DNA sequence belonging to a specific organism. Contrary to the commonly used operational taxonomic units (OTUs), ASVs are distinguished at single-nucleotide resolution instead of an overall similarity threshold. Due to the improved resolution and comparability, it has been argued that ASVs are superior to OTUs (Callahan et al., 2017). The data set was normalized at a carefully selected number of reads ("rarefaction") and then further analyzed regarding its diversity. Several alpha diversity indexed were calculated and compared between the culture conditions (Lahti and Shetty, 2017; Xu et al., 2023), and a distance matrix was constructed to assess the influence of the carbon source on the community structure of the different microbial consortia (Palarea-Albaladejo and Martín-Fernández, 2015; Oksanen et al., 2024). Finally, the relative abundance of ASVs at three sample timepoints per culture was calculated (McMurdie and Holmes, 2013) to illustrate the evolution of the community structure and the establishment of dominant taxonomic groups (putative autotrophic and heterotrophic foundation species) in each culture condition.

2.4.3 Benefits and drawbacks of the cultivation approach

The cultivation approach allows to go beyond the community's genetic potential and to observe its metabolic activity: An organism with acetate uptake genes can potentially grow on acetate, an organism enriched in a bioreactor alimented with acetate is most likely able to actually do so. Furthermore, cultivated microbial consortia can be a starting point for many subsequent analyses such as the isolation of interesting strains, which is further developed in chapter five.

On the flipside, the cultivated microbial consortia only reflect a small part of the natural community. It is safe to assume that the majority of Prony Bay's serpentinite-hosted organisms were not brought into culture (see the reasons discussed under 2.4.1), which likely includes community members with high ecological significance. It is thus unclear whether identified putative foundation species play a key role in the natural community or only dominate its cultivable fraction. Combining the cultivation approach with metagenomics allows an estimation of this discrepancy.

2.5 References

Alain, K., and Querellou, J. (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* 13, 583–594. doi: 10.1007/s00792-009-0261-3

Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., et al. (2014). Binning metagenomic contigs by coverage and composition. *Nat. Methods* 11, 1144–1146. doi: 10.1038/nmeth.3103

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., et al. (2020). KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics* 36, 2251–2252. doi: 10.1093/bioinformatics/btz859

Aroney, S. T. N., Newell, R. J. P., Nissen, J., Camargo, A. P., Tyson, G. W., and Woodcroft, B. J. (2024). CoverM: Read coverage calculator for metagenomics. doi: 10.5281/ZENODO.10531253

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* 43, 260–296.

Boileau, C., Auria, R., Davidson, S., Casalot, L., Christen, P., Liebgott, P.-P., et al. (2016). Hydrogen production by the hyperthermophilic bacterium Thermotoga maritima part I: effects of sulfured nutriments, with thiosulfate as model, on hydrogen production and growth. *Biotechnol. Biofuels* 9. doi: 10.1186/s13068-016-0678-8

Callahan, B. J., McMurdie, P. J., and Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11, 2639–2643. doi: 10.1038/ismej.2017.119

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caspi, R., Billington, R., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., et al. (2018). The MetaCyc database of metabolic pathways and enzymes. *Nucleic Acids Res.* 46, D633–D639. doi: 10.1093/nar/gkx935

Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P., and Parks, D. H. (2022). GTDB-Tk v2: memory friendly classification with the Genome Taxonomy Database. Bioinformatics. doi: 10.1101/2022.07.11.499641

Dairawan, M., and Shetty, P. J. (2020). The Evolution of DNA Extraction Methods. *Am. J. Biomed. Sci. Res.* 8, 39.

Dick, J. M. (2014). Average oxidation state of carbon in proteins. *J. R. Soc. Interface* 11, 20131095. doi: 10.1098/rsif.2013.1095

Dick, J. M., and Shock, E. L. (2011). Calculation of the Relative Chemical Stabilities of Proteins as a Function of Temperature and Redox Chemistry in a Hot Spring. *PLOS ONE* 6, e22782. doi: 10.1371/journal.pone.0022782

Ghurye, J. S., Cepeda-Espinoza, V., and Pop, M. (2016). Metagenomic Assembly: Overview, Challenges and Applications. *Yale J. Biol. Med.* 89, 353–362.

Hanišáková, N., Vítězová, M., and Rittmann, S. K.-M. R. (2022). The Historical Development of Cultivation Techniques for Methanogens and Other Strict Anaerobes and Their Application in Modern Microbiology. *Microorganisms* 10, 412. doi: 10.3390/microorganisms10020412

Harde, C. D., Khedkar, D. A. N., and Sake, V. S. (2023). A Review on High Performance Liquid Chromatography. 8.

Hungate, R. E., and Macy, J. (1973). The Roll-Tube Method for Cultivation of Strict Anaerobes. *Bull. Ecol. Res. Comm.*, 123–126.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 44, D457–D462. doi: 10.1093/nar/gkv1070

Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., et al. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7, e7359. doi: 10.7717/peerj.7359

Lahti, L., and Shetty, S. (2017). microbiome. doi: 10.18129/B9.BIOC.MICROBIOME

Larralde, M. (2022). Pyrodigal: Python bindings and interface to Prodigal,an efficient method for gene prediction in prokaryotes. *J. Open Source Softw.* 7, 4296. doi: 10.21105/joss.04296

Launay, J., and Fontes, J.-Ch. (1985). Les sources thermales de Prony (Nouvelle Calédonie) et leurs précipités chimiques, exemple de formation de brucite primaire. *Geol. Fr.* 1, 83–100.

Mante, J., Groover, K. E., and Pullen, R. M. (2024). Environmental community transcriptomics: strategies and struggles. *Brief. Funct. Genomics*, elae033. doi: 10.1093/bfgp/elae033

McMurdie, P. J., and Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* 8, e61217. doi: 10.1371/journal.pone.0061217

Monnin, C., Chavagnac, V., Boulart, C., Ménez, B., Gérard, M., Gérard, E., et al. (2014). Fluid chemistry of the low temperature hyperalkaline hydrothermal system of Prony Bay (New Caledonia). *Biogeosciences* 11, 5687–5706. doi: 10.5194/bg-11-5687-2014

Monnin, C., Quéméneur, M., Price, R., Jeanpert, J., Maurizot, P., Boulart, C., et al. (2021). The Chemistry of Hyperalkaline Springs in Serpentinizing Environments: 1. The Composition of Free Gases in New Caledonia Compared to Other Springs Worldwide. *J. Geophys. Res. Biogeosciences* 126, e2021JG006243. doi: 10.1029/2021JG006243

Nam, N. N., Do, H. D. K., Loan Trinh, K. T., and Lee, N. Y. (2023). Metagenomics: An Effective Approach for Exploring Microbial Diversity and Functions. *Foods* 12, 2140. doi: 10.3390/foods12112140

Navgire, G. S., Goel, N., Sawhney, G., Sharma, M., Kaushik, P., Mohanta, Y. K., et al. (2022). Analysis and Interpretation of metagenomics data: an approach. *Biol. Proced. Online* 24, 18. doi: 10.1186/s12575-022-00179-7

Ngere, J. B., Ebrahimi, K. H., Williams, R., Pires, E., Walsby-Tickle, J., and McCullagh, J. S. O. (2023). Ion-Exchange Chromatography Coupled to Mass Spectrometry in Life Science, Environmental, and Medical Research. *Anal. Chem.* 95, 152–166. doi: 10.1021/acs.analchem.2c04298

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P. A. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome Res.* 27, 824–834. doi: 10.1101/gr.213959.116

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., et al. (2024). *vegan: Community Ecology Package*. Available at: https://CRAN.R-project.org/package=vegan

Palarea-Albaladejo, J., and Martín-Fernández, J. A. (2015). zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. *Chemom. Intell. Lab. Syst.* 143, 85–96. doi: 10.1016/j.chemolab.2015.02.019

Parks, D. H., Chuvochina, M., Rinke, C., Mussig, A. J., Chaumeil, P.-A., and Hugenholtz, P. (2022). GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Res.* 50, D785–D794. doi: 10.1093/nar/gkab776

Pelletier, B. (2005). 2005-NC-PL cruise,Alis R/V. doi: 10.17600/18002441

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ramos-Barbero, M. D., Martin-Cuadrado, A.-B., Viver, T., Santos, F., Martinez-Garcia, M., and Antón, J. (2019). Recovering microbial genomes from metagenomes in hypersaline environments: The Good, the Bad and the Ugly. *Syst. Appl. Microbiol.* 42, 30–40. doi: 10.1016/j.syapm.2018.11.001

Rampelotto, P. H. (2024). Extremophiles and Extreme Environments: A Decade of Progress and Challenges. *Life* 14, 382. doi: 10.3390/life14030382

Reimer, L. C., Vetcininova, A., Carbasse, J. S., Söhngen, C., Gleim, D., Ebeling, C., et al. (2019). Bac *Dive* in 2019: bacterial phenotypic data for High-throughput biodiversity analysis. *Nucleic Acids Res.* 47, D631–D636. doi: 10.1093/nar/gky879

Schultz, J., Modolon, F., Peixoto, R. S., and Rosado, A. S. (2023). Shedding light on the composition of extreme microbial dark matter: alternative approaches for culturing extremophiles. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1167718

Schwengers, O., Jelonek, L., Dieckmann, M. A., Beyvers, S., Blom, J., and Goesmann, A. (2021). Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification: Find out more about Bakta, the motivation, challenges and applications, here. *Microb. Genomics* 7. doi: 10.1099/mgen.0.000685

Shakya, M., Lo, C.-C., and Chain, P. S. G. (2019). Advances and Challenges in Metatranscriptomic Analysis. *Front. Genet.* 10, 904. doi: 10.3389/fgene.2019.00904

The Genome Standards Consortium, Bowers, R. M., Kyrpides, N. C., Stepanauskas, R., Harmon-Smith, M., Doud, D., et al. (2017). Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nat. Biotechnol.* 35, 725–731. doi: 10.1038/nbt.3893

Thomas, T., Gilbert, J., and Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis. *Microb. Inform. Exp.* 2, 3. doi: 10.1186/2042-5783-2-3

Uritskiy, G. V., DiRuggiero, J., and Taylor, J. (2018). MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome* 6, 158. doi: 10.1186/s40168-018-0541-1

Wagner, A. O., Markt, R., Mutschlechner, M., Lackner, N., Prem, E. M., Praeg, N., et al. (2019). Medium Preparation for the Cultivation of Microorganisms under Strictly Anaerobic/Anoxic Conditions. *J. Vis. Exp. JoVE*, 10.3791/60155. doi: 10.3791/60155

Wu, Y.-W., Simmons, B. A., and Singer, S. W. (2016). MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* 32, 605–607. doi: 10.1093/bioinformatics/btv638

Xu, S., Zhan, L., Tang, W., Wang, Q., Dai, Z., Zhou, L., et al. (2023). MicrobiotaProcess: A comprehensive R package for deep mining microbiome. *The Innovation* 4, 100388. doi: 10.1016/j.xinn.2023.100388

Yang, Z.-W., Lian, Z.-H., Liu, L., Fang, B.-Z., Li, W.-J., and Jiao, J.-Y. (2023). Cultivation strategies for prokaryotes from extreme environments. *iMeta* 2, e123. doi: 10.1002/imt2.123



CHAPTER 3
The metagenomic approach

3.1 Preface

In the third chapter, we explore the genetic potential of the natural microbial communities from RdK, BdJ and ST07. In the first part, the metagenomes from the different samples were characterized by calculating their average protein oxidation states and by comparing their phylogenomic diversity to other serpentinite-hosted systems. Subsequently, the most abundant MAGs (which we assume are ecologically most relevant) were screened for bicarbonate, formate, acetate and glycine transporter proteins, acetate and glycine assimilation genes and eleven versions of the seven known carbon fixation pathways. Carbon fixation pathway completeness was estimated based on a custom database. In addition, the MAGs were screened for key genes of general metabolism. The aim was to identify primary carbon sources in the different microbial communities, as well as putative autotrophic and heterotrophic foundation taxa.

The contributions made in the frame of this thesis include the design of the study, the construction of the pathway database, the performance of data analysis and the writing of the manuscript. The bioinformatic analysis was conducted in collaboration with Mélanie Hennart.

This chapter is presented in the form of a manuscript. It is currently revised by the authors and planned to be submitted before the PhD defense.

3.2 Manuscript

Carbon fixation and assimilation by autotrophic and heterotrophic foundation species in the serpentinite-hosted Prony Bay

Rabja Maria Popall[1], Mélanie Hennart[1], Sophie Marre[2], Pierre Peyret[2], Anne Postec[1] and Gaël Erauso[1]

[1]Aix-Marseille Univ, Univ Toulon, CNRS, IRD, MIO UM 110, Marseille, France
[2]CaptGEN, Université Clermont Auvergne, UMR MEDIS, CBRV, Clermont-Ferrand, France

*** Correspondence:**
Gaël Erauso
gael.erauso@mio.osupytheas.fr

Keywords: serpentinization, alkaline hydrothermal system, alkaliphile, carbon fixation, carbon assimilation, primary production.

Abstract

In serpentinite-hosted environments, alkaliphile microbial communities grow in the absence of CO_2. Their primary carbon sources remain elusive but might include bicarbonate, abiotically produced formate, acetate and glycine. While bicarbonate and formate facilitate autotrophic growth, heterotrophic assimilation of acetate and glycine may play an equally important role in the base of the trophic network by producing both biomass and CO_2 from an abiotic source. Here, we identify primary carbon sources and putative foundation species from different sites of the shallow Prony Bay hydrothermal field. Sample protein oxidation states and diversity suggest that the assessed metagenomes represent a gradient from aerobic and microaerophilic to anaerobic serpentinite-hosted communities that are dominated by corresponding marker taxa. Bicarbonate and acetate uptake genes are abundant across all environments, while formate and glycine metabolizing organisms are poorly represented. We identify five aerobic and microaerophilic MAGs that feature a full set of bicarbonate or acetate uptake genes and likely play a key role in surface environments. These putative surface foundation species include a cyanobacterial acetate-assimilating MAG, two mixotrophic organisms classified as *Rhodobacterales* and *Burkholderiales*, as well as two bicarbonate-fixing MAGs assigned to the *Xanthomonadales* and UBA7966 orders. Autotrophic foundation species use H_2, sulfide or thiosulfate as energy source and feature a modified version of the CBB cycle missing the RuBisCo gene. The subsurface trophic network remains elusive but might include bicarbonate-

fixing *Methanocellales* as well as CO_2 producing fermenters such as the deep-branching *Bipolaricaulales*.

Introduction

The autotrophic fixation of carbon dioxide (CO_2) into organic carbon compounds is the basis of all known ecosystems. In serpentinite-hosted environments, however, microbial communities grow in the absence of CO_2. Serpentinization occurs in geological subduction zones, where the Earth's mantle is uplifted and exposed to water. The hydration of ultramafic rock produces hydrothermal fluids enriched in hydrogen (H_2) gas and hydroxide ions, increasing the pH to at least 11. The ultrabasic milieu of those fluids drives the precipitation of CO_2 into solid calcium carbonate, forming chimney-like deposits over time (McCollom and Seewald, 2013; Schrenk et al., 2013). Little is known on how microbial ecosystems can develop in these conditions, which limit not only the availability of dissolved inorganic carbon (DIC) but also of other essential nutrients such as phosphorous (Frouin et al., 2022).

While CO_2 is not available as a primary carbon source, the serpentinite-hosted milieu promotes the abiotic production of small organic molecules. This is not only relevant with regard to the origin of life, but also a potential solution to the primary production paradox. Formate and acetate are yielded in Fischer-Tropsch reactions, and it has been shown that serpentinization can facilitate the abiotic synthesis of glycine (Brazelton et al., 2011; McCollom and Seewald, 2013; Ménez et al., 2018; Fones et al., 2021; Nobu et al., 2022). In addition, some serpentinite-hosted organisms have been observed to grow on bicarbonate, which they probably redissolve from the carbonaceous hydrothermal chimneys (Suzuki et al., 2014).

While bicarbonate, formate, acetate and glycine do not qualify as primary carbon sources by definition, they could provide carbon from an abiotic source for the trophic network in two different ways. Bicarbonate and formate may serve as autotrophic carbon sources, while acetate and glycine might facilitate the development of a heterotrophic foundation community as proposed by Lang et al. (2018).

The uptake of all four compounds into the cell is mediated by membrane-bound transporter or channel proteins: Bicarbonate uptake mostly depends on the sodium-dependent channels sbtA and mpsAB (Fan et al., 2021; Suzuki et al., 2024); formate uptake is based on the ion-channels focA, focB or the fdhC transporter (White and Ferry, 1992; Trchounian and Trchounian, 2014); acetate uptake can be mediated by the proton-dependent symporter actP (Gimenez et al., 2003), and the uptake of glycine by the proton-dependent cycA transporter (Ghrist and Stauffer, 1995). Once in the cytoplasm, however, the compounds are metabolized differently. Bicarbonate and formate are assimilated into CO_2 via the carbonic anhydrase or formate dehydrogenase respectively (Smith and Ferry, 2000; McGonigle et al., 2020). Their uptake is thus dependent on a carbon fixation pathway, which transforms the yielded CO_2 into acetyl-coA and other precursors for respiration, fermentation and heterotrophic metabolism. To date, at least seven autotrophic carbon fixation pathways have been described. The most common one is the Calvin-Benson-Bassham (CBB) cycle, which is employed by all photoautotrophic organisms and some chemotrophic bacteria and archaea, including common serpentinite-hosted taxa such as *Methanomicrobiales* and *Burkholderiales* (Asplund-

Samuelsson and Hudson, 2021). Next to the CBB cycle, the only oxygen-tolerant carbon fixation pathways are the 3-hydroxypropionate (3HP) and the hydroxypropionate-hydroxybutyrate (HP/HB) cycle. The dicarboxylate-hydroxybutyrate cycle (DC/HB), the reductive tricarboxylic acid (rTCA) cycle, the Wood-Ljungdahl pathway and most likely the recently proposed reductive glycine pathway rely on oxygen-sensitive enzymes and are restricted to microaerophilic or anaerobic conditions (Ward and Shih, 2019). Limited research on carbon fixation in serpentinite-hosted environments has highlighted the CBB cycle, the rTCA cycle and the Wood-Ljungdahl pathway so far (Seyler et al., 2020). However, a recent study suggests that most carbon fixation pathways are associated with a greater phylogenetic diversity that previously assumed, which might also be the case for serpentinite-hosted ecosystems (Garritano et al., 2022).

Contrary to bicarbonate and formate, acetate and glycine are directly transformed into acetyl-coA. Acetate is either assimilated by the acetyl-CoA synthetase or the acetate kinase and phosphate acetyltransferase (Brazelton et al., 2017). Glycine is assimilated via the glycine reductase complex, the central enzyme of the glycine reductase pathway where glycine is normally produced as an intermediate (Sánchez-Andrea et al., 2020). Speaking of autotrophic carbon uptake would be inappropriate here, as no carbon fixation pathway is involved. However, it has been suggested that heterotrophs play an equally fundamental role in serpentinite-hosted ecosystems (Lang et al., 2018). Small abiotically produced carbon compounds such as acetate and glycine could fuel a community of heterotrophs that is independent from biological production. These heterotrophs would produce CO_2 via respiration or fermentation, which may be accumulated in microenvironments and drive autotrophic activity there. This would include autotrophs without formate and bicarbonate transporters and thus lead to a diversification of the base of the trophic network.

While the calcium carbonate chimneys constitute a vast carbon source for organisms capable of redissolving bicarbonate, the concentrations and thus probably the influence of formate, acetate and glycine vary between serpentinite-hosted systems. It is likely that those variations are especially prominent between terrestrial ophiolitic systems such as The Cedars, and deep marine hydrothermal fields such as Lost City (Kohl et al., 2016b; Lang et al., 2018). A unique transition site between these extremes is located on a serpentinized peridotite nappe in the south of New Caledonia. The coastal Prony Bay hydrothermal field comprises different sites ranging from terrestrial to intertidal to submarine at a maximum depth of 50 m (Monnin et al., 2014). Depending on their location in the bay, these sites feature characteristics of both terrestrial and marine serpentinite-hosted ecosystems (Quéméneur et al., 2014; Postec et al., 2015; Frouin et al., 2018). Comparison of the different microbial communities may provide insight into primary carbon uptake in various serpentinite-hosted settings.

Here, we analyzed the carbon fixation and assimilation potential of five different metagenomes from Prony Bay's terrestrial "Rivière des Kaoris" (RdK), intertidal "Bain des Japonais" (BdJ) and submarine "Aiguille de Prony" (ST07). BdJ and ST07 were sampled in two different years, adding a temporal dimension for these sites. Initially, the metagenomes were characterized by calculating their average protein oxidation states and by comparing their phylogenomic diversity to other serpentinite-hosted systems. Subsequently, the most abundant (thus supposedly most ecologically relevant) metagenome-assembled genomes (MAGs) were screened for formate, bicarbonate, acetate and glycine transporter proteins, acetate and glycine

assimilation genes and eleven versions of the seven carbon fixation pathways described above. In order to estimate carbon fixation pathway completeness, we curated a custom database and developed a score system. In addition, MAGs were screened for key genes of general metabolism. The aim was to identify primary carbon sources in the different microbial communities, as well as putative autotrophic and heterotrophic foundation taxa.

Material and methods

Sampling and sample processing

Hydrothermal chimney samples were collected during two field campaigns to Prony Bay in 2011 and 2014. The terrestrial RdK site was sampled in 2014, while the intertidal BdJ as well as the submarine ST07 site were sampled in both years. The outside layer of the chimney fragments was removed to eliminate environmental contaminants and isolate the serpentinite-hosted microbial community. Subsamples of the chimney cores were stored at -80°C until further use. In 2016, DNA extractions were performed using a phenol-chloroform based approach in combination with bead beating. Whole genome shotgun sequencing was performed by GetGenotoul (Castanet-Tolosan, France) on an Illumina HiSeq 3000 System.

Reconstruction of metagenome-assembled genomes

Raw reads were preprocessed with AlienTrimmer v2.0 (Criscuolo and Brisse, 2013). The 5' and 3' regions were trimmed up to a maximum of 9 consecutive non-problematic bases. Adapter sequences and phiX contamination were removed at a k-mer length of 10. In addition, reads shorter than 50 bp and reads containing more than 50% of low-quality bases with a phred score below 13 were removed. The quality of trimmed reads was evaluated with fastqc v0.12.1 and fastqscreen v0.15.3 (Andrews, 2010; Wingett and Bottoms, 2023). High-quality reads were assembled into contigs using k-mer lengths of 21, 33 and 55 with metaSPAdes v3.15.5 (Nurk et al., 2017). In order to preserve site- and year-specific variation, this was performed for each sample individually. The quality of final assemblies was assessed with Quast v5.0.2 at a minimum contig length of 500 bp (Mikheenko et al., 2018). Subsequently, scaffolds were binned using the metabat2 v2.15, maxbin2 v2.2.7 and concoct v1.1.0 algorithms with default parameters (Alneberg et al., 2014; Wu et al., 2016; Kang et al., 2019). Obtained bins were refined with the metaWRAP v1.2 bin_refinement module, allowing no less than 50% completeness and no more than 10% contamination (Uritskiy et al., 2018). The quality of final MAGs was evaluated with CheckM v1.2.2 (Parks et al., 2015) (Supplementary Table 1).

Sample characterization

Calculation of protein oxidation states

MAG protein sequences were annotated with Bakta v1.9.1 in metagenome mode, using Pyrodigal v1.3.0 for the prediction of protein-coding regions (Schwengers et al., 2021; Larralde, 2022). In order to compare the cellular redox milieu between samples, average protein oxidation states of MAG amino acid sequences were calculated using a script from

Poudel et al. (2018) based on a previously described approach (Dick and Shock, 2011). Briefly, the average protein oxidation state Z_C is calculated as

$$Z_C = \frac{Z - n_H + 2(n_O + n_S) + 3n_N}{n_C}$$

where Z is the total charge of the protein, n_H, n_O, n_S and n_N are the numbers of hydrogen, oxygen, sulfur and nitrogen atoms to which formal charge values are attributed, and n_C is the number of carbon atoms in the protein. Obtained Z_C values are normalized with the total number of protein sequences in the corresponding MAG. Based on the MAG Z_C values, average cellular protein oxidation states were calculated for each sample. In addition, average protein oxidation states were calculated for the "aerobe", "microaerophile" and "anaerobe" categories of the Bacterial Diversity Metadatabase (BacDive) to use as a point of reference (Reimer et al., 2019). BacDive species assigned to multiple categories or absent from the Genome Taxonomy Database (GTDB) R214 (Chaumeil et al., 2022; Parks et al., 2022) were not taken into account. References were downloaded from The National Center for Biotechnology Information (NCBI) with ncbi-datasets-cli v16.18.0 (Sayers et al., 2022).

Phylogeny and diversity

Taxonomic annotation of MAGs was performed with GTDB-Tk v2.3.2 referencing the GTDB R214. To identify organisms with putatively high ecological significance, coverage and relative abundance of MAGs in their respective samples were calculated using CoverM v0.7.0 in genome mode (Aroney et al., 2024). To verify the taxonomic composition of the samples, 16S rRNA sequences were reconstructed from each metagenome using the RiboTaxa v1.5 pipeline (Chakoory et al., 2022). Reads were trimmed at a k-mer length of 21 before discarding reads shorter than 60 bp and with a quality score lower than 20. Subsequently, 16S genes were filtered from high-quality reads using the indexed SortMeRNA v2.0 database (Kopylova et al., 2012). Corresponding sequences were reconstructed at a maximum length of 151 bp, a mean insert size of 300 bp, the default number of iterations and the default minimum coverage of reads mapped to candidate reference sequences at a standard deviation of 100. Finally, 16S sequences were classified with the plugin sklearn_classifier of QIIME2 v2022.11.1 according to the SILVA138 database at the default threshold (Yilmaz et al., 2014; Bolyen et al., 2019).

A phylogenomic tree was constructed to explore the relationships between MAGs from different samples, as well as their relationships to organisms from other serpentinite-hosted systems. Publicly available MAGs from both terrestrial and marine ultramafic systems were downloaded from corresponding NCBI bioprojects with ncbi-datasets-cli v16.18.0 (Supplementary Table 2). The tree was based on the bacterial marker gene set from GTDB R214. Marker gene calling and alignment, as well as *de novo* taxonomic classification were performed with GTDB-tk, using IQ-TREE for tree inference. The tree was rooted at midpoint and subsequently edited in iTOL v6.9.1 (Letunic and Bork, 2024).

Prediction of carbon fixation potential and general metabolism

Presence and absence of transporter and assimilation genes for the primary uptake of bicarbonate, formate, acetate, and glycine as putative carbon sources were predicted in MAG sequences via kofam_scan v1.3.0 using default parameters (Aramaki et al., 2020). Only hits with a significant hmmsearch score were considered. In addition to primary carbon uptake

genes, we assessed enzymes playing a key role in hydrogen, carbon monoxide (CO), methane, nitrogen, sulfur and oxygen (O_2) metabolism.

In order to evaluate completeness of carbon fixation pathways in the reconstructed MAGs, we built a custom database based on modules from the Kyoto Encyclopedia of Genes and Genomes (KEGG) R109 and pathway entries from the MetaCyc database R27.1 (Kanehisa et al., 2016; Caspi et al., 2018). The decision for this approach was based on a review of both public databases, which showed that the proposed pathway structures were not coherent. For all carbon fixation pathways, the list of genes in one database included entries that were not present in the other. Furthermore, multiple versions of some pathways were recognized in one database but not available in the other. Notably, this was the case for the Wood-Ljungdahl pathway (acetogenic vs. methanogenic on MetaCyc), the rTCA cycle (version I vs. II on MetaCyc) and the 3HP cycle (s-malate production vs. glyoxylate production vs. pyruvate production on KEGG). To construct the custom database, the Enzyme Commission (EC) numbers and corresponding KEGG Orthology (KO) identifiers listed in the pathway KEGG module were extracted for each carbon fixation pathway. In addition, the EC numbers of the same pathway were extracted from the MetaCyc database. Subsequently, the MetaCyc EC numbers were matched with KO identifiers, creating a collection of KOs reflecting both the KEGG and MetaCyc database for each reaction of each pathway. This collection was then manually filtered and curated. For each EC number, the KO combinations rendering the reaction functional were determined. KOs were classified as subdomains of the same protein (hence all of them must be present to render the reaction functional), as synonyms or alternatives to each other depending on the organism (only one of them must be present to render the reaction functional). The final pathway structure was then embedded into a custom script calculating a pathway completion score based on the percentage of functional pathway reactions present in a MAG. The presence of corresponding KOs was predicted with kofam_scan as described above.

Results

A total of 346 MAGs with a completeness higher than 50% and a contamination lower than 10% could be reconstructed. This included 339 bacterial and 7 archaeal MAGs. The number of MAGs decreased along the transect from land to ocean and was higher for samples from the year 2014 than from 2011: 114 MAGs were assembled from the RdK14 metagenome, 78 MAGs from the BdJ14 metagenome, 69 MAGs from the BdJ11 metagenome, 65 MAGs from the ST0714 metagenome and 20 MAGs from the ST0711 metagenome.

Sample characterization

Sample oxidation states

The mean sample oxidation state was highest in ST0714, followed by RdK14 and BdJ14. All three samples corresponded most to the aerobic category of the BacDive database. Between BdJ11 and BdJ14 the mean sample oxidation state decreased considerably, approaching the microaerophilic BacDive category. The mean sample oxidation state of ST0711 was lowest and had highest similarity with anaerobic BacDive sequences (Figure 1).

Phylogeny and diversity

Prony Bay MAGs were evenly distributed across the phylogenomic tree (Supplementary Figure 1). In closely related phylogenomic clades, Prony Bay MAGs from all sample types generally clustered together. Larger clades with Prony Bay MAGs mostly included sequences from the serpentinite-hosted Costa Rica Margin. This especially concerned *Burkholderiales, Desulfovibrionales* and *Bacteroidales* groups. Furthermore, the SZUA-161 clade contained sequences from The Cedars, Lost City and the Costa Rica Margin, and the UBA994 cluster several genomes from The Cedars and Hakuba Happo ophiolites. The remaining clades mostly contained single and sometimes no sequences from other serpentinite-hosted systems.

Out of 346 MAGs, only 42 had a relative abundance greater than 1% in their respective samples ("high-abundance MAGs") (Supplementary Table 1). This included 41 bacterial and 1 archaeal MAG. The percentage of these high-abundance MAGs was greatest in ST0711 (30%) and lowest in ST0714 (5%) and mostly included taxonomic groups previously associated with serpentinite-hosted ecosystems (Figure 2). This was especially the case for the submarine ST0711 metagenome, which included significant proportions of the order UBA148 (also called *Methanocellales*) associated with the *Methanomicrobia*, two orders related to the *Bipolaricaulia*, and the *Desulfovibrionales*. The latter were additionally present in both terrestrial and intertidal samples. Furthermore, a considerable amount of *Gammaproteobacteria* and especially *Burkholderiales* (including *Serpentinimonas*) was present in terrestrial and intertidal samples. Next to these marker taxa, five orders of *Cyanobacteriia* were distributed across all samples except for ST0711. Other abundant taxa were the *Rhodobacterales* and the order UBA994 associated with the *Bacillota* in both intertidal samples, two orders of *Paceibacteriia* and the *Desulfobulbales* in BdJ14, the *Aggregatilineales,* DTKZ01 associated with the *Spirochaetia* and RFP12 related to the *Kiritimatiellia* in RdK14, the *Bacteroidales* as well as two orders of *Chlorobiales* in in BdJ14 and RdK14, and two orders associated with the *Deinococci* in RdK14 and ST0711. In addition, the order SZUA-161 belonging to the *Dehalococcoida* accounted for a significant proportion of the ST0711 community. The taxonomy of 16S rRNA sequences with a relative abundance greater than 1% confirmed the presence of most marker taxa, as well as phototrophic orders. Instead of the order UBA148, the 16S rRNA data suggested a major occurrence of the closely related *Methanosarcinales* not only in ST0711 but in all samples except for ST0714. Furthermore, the candidate division MSBL5 was relatively abundant in ST0711 while SZUA-161 was absent (Supplementary Figure 2).

Primary carbon uptake

In high-abundance MAGs, only acetate and bicarbonate uptake genes were present (Figure 3). The bicarbonate transporter sbtA and the bicarbonate assimilating gamma carbonic anhydrase co-occurred in all MAGs where they were present, except for a *Desulfobulbales* MAG from BdJ14 which exclusively featured sbtA. A *Rhodobacterales* MAG from BdJ11, two *Xanthomonadales* MAGs from BdJ11 and ST0714 and a *Burkholderiales* as well as an UBA7966 MAG from RdK14 - which additionally featured the mpsAB transporter - had both genes. Furthermore, a *Leptolyngbales* MAG from RdK14 featured a beta carbonic anhydrase.

The acetate transporter actP was present in three high-abundance MAGs, including a *Burkholderiales* MAG from RdK14 and two MAGs from BdJ11 assigned to the *Rhodobacterales* and *Cyanobacterales*. Acetate assimilation genes were widespread. The acetyl-coA synthetase was found in all MAGs with an actP transporter, and it was overall present in 63% of high-abundance MAGs. The acetate kinase was found in even more MAGs, but acetate assimilation via this enzyme is dependent on the phosphate acetyltransferase. The two enzymes only co-occurred in six MAGs, including three *Desulfovibrionales* MAGs from BdJ11, BdJ14 and ST0711, a *Phormidesmiales* MAG from BdJ11, a *Burkholderiales* MAG from RdK14 – which also featured the actP transporter - and a *Deinococcales* MAG from ST0711.

Formate and glycine uptake genes were absent from high-abundance MAGs (Figure 3). Concerning the glycine transporter cycA, this was also the case for all MAGs with a relative abundance lower than 1% ("low-abundance MAGs"). The glycine reductase was present in four low-abundance MAGs across all samples except for BdJ11. The formate transporter fdhC was present in eleven low-abundance MAGs, which included nine *Methylococcales* MAGs from BdJ11, BdJ14, RdK14 and ST0714. It never co-occurred with a formate dehydrogenase, which was found in a total of four MAGs from RdK14 and ST0714 that featured either the NAD-reducing or the coenzyme F420 reducing version of the enzyme (Supplementary Figure 3).

Carbon fixation

The CBB cycle was the only pathway reaching 100% completeness across both high- and low-abundance MAGs (Figure 4, Supplementary Figure 4). High-abundance MAGs with a complete CBB cycle were present in terrestrial and intertidal samples and exclusively belonged to the *Cyanobacteriia* or *Gammaproteobacteria*. More precisely, this included a *Cyanobacteriales* MAG (BdJ11), a *Cardiobacteriales* MAG (BdJ11), two *Pseudanabaenales* MAGs (BdJ14, RdK14), a *Burkholderiales* MAG (RdK14) and a *Leptolyngbyales* MAG (RdK14). In BdJ11 and BdJ14, also the second-highest scoring pathway was oxygen-tolerant: The 3-HP cycle reached 62% for the pyruvate-yielding version in both samples and 57% for the S-malate-yielding version in BdJ11. Both versions of the pathway were found in MAGs assigned to the *Rhodobacterales*. The highest-scoring anoxic pathway in both samples was the DC/HB cycle with a maximum completeness of 57% in two *Xanthomonadales* and *Burkholderiales* MAG. In the terrestrial RdK14, the anoxic rTCA I and II cycles came second to the CBB cycle with a maximum score of 60% in an *Aggregatilineales* MAG.

In submarine samples, none of the high-abundance MAGs featured a complete CBB cycle. In ST0714, The CBB cycle still scored higher than the other pathways with a maximum completeness of 80% in a PCC-6307 MAG, followed by the DC/HB cycle reaching 64% in a *Xanthomonadales* MAG. Contrary to the other samples, the highest-scoring pathway in ST0711 was anoxic: The DC/HB cycle scored a maximum completeness of 71% in a *Deinococcales* MAG, while the CBB cycle reached 70% an UBA7950 and in a *Desulfovibrionales* MAG.

The remaining pathways scored high in single, mostly low-abundance MAGs but were globally less relevant. Especially the acetogenic and methanogenic Wood-Ljungdahl pathways featured overall negligible scores, with only one high-abundance UBA148 MAG from ST0711

reaching barely above 50% completeness for the methanogenic version (Figure 4, Supplementary Figure 4).

General metabolism

Key genes for H_2, CO, nitrogen, sulfur and O_2 metabolism were present in high-abundance MAGs. Methanogenesis and aerobic methanotrophy enzymes were absent from both high-abundance and low-abundance MAGs (Figure 5, Supplementary Figure 5).

Hydrogenases were present in high-abundance bins from all samples except for ST0714. This included the O_2-labile iron-sulfur reducing hydrogenase hydA and all tested O_2-tolerant hydrogenases except for the coenzyme F420-reducing hydrogenase mvhA, which was also absent from low-abundance MAGs. The key gene for aerobic carboxydotrophy was present in three high-abundant MAGs from ST0711 and RdK14, and the key gene for anaerobic carboxydotrophy in a *Desulfobulbales* MAG from BdJ14. Ammonia-oxidizing, nitrification and denitrification genes were absent from high-abundance MAGs. One high-abundance *Burkholderiales* MAG from RdK14 featured the nitrate-reducing napA gene and several high-abundance MAGs the key genes for both periplasmic and cytoplasmic nitrite reduction as well as for nitrogen fixation. Likewise, sulfate reduction, sulfite reduction, sulfide oxidation and thiosulfate oxidation genes were relatively widespread amongst high-abundance MAGs from all samples except for ST0714. The cytochromes aa3 and cbb3 were present in most high-abundance MAGs from all samples except for ST0711.

Characterization of MAGs with a complete set of carbon uptake genes

In total, six high-abundance MAGs featured both transporter and primary assimilation enzymes for the same carbon source (Table 1). A *Cyanobacteriales* MAG (classified to the genus level as RDYZ01) featured both the actP transporter and the acetyl-coA synthetase for the assimilation of acetate. Two *Xanthomonadales* MAGs from BdJ11 (classified to the genus level as *Silanimonas*) and ST0714 (classified to the family level as *Wenzhouxiangellaceae*), as well as an UBA7966 MAG (classified to the species level as UBA7966 sp003485715) from RdK14 featured a bicarbonate transporter (all had sbtA and the UBA7966 MAG additionally mpsAB) and a gamma carbonic anhydrase for the assimilation of bicarbonate into CO_2. Furthermore, these MAGs had a 70% or 90% complete CBB cycle. Two MAGs featured the genetic makeup for both acetate and bicarbonate uptake: A *Burkoholderiales* MAG (classified to the genus level as JALHMQ01) from RdK14 as well as a *Rhodobacterales* MAG (classified to the genus level as *Roseinatronobacter*) from BdJ11. The CBB cycle in these MAGs scored 70% and 80% respectively. Four out of the five MAGs with bicarbonate uptake genes were missing both subunits of the ribulose-bisphosphate carboxylase (RuBisCo); the only exception was the *Burkholderiales* MAG which featured the small chain and a non-significant hit for the large chain of the protein.

Regarding energy metabolism, the *Cyanobacteriales*, UBA7966 and *Burkoholderiales* MAGs featured at least one of the O_2-tolerant NiFe hydrogenases hyaB and hoxH. The *Rhodobacterales, Xanthomonadales,* UBA7966 and *Burkoholderiales* MAGs had the key genes for sulfide and/or thiosulfate oxidation. The *Burkoholderiales* MAG additionally

featured the key gene for aerobic carboxydotrophy. In terms of respiration, all six MAGs featured at least one cytochrome. The *Burkoholderiales* MAG additionally had the key genes for nitrate and nitrite reduction, as well as for sulfite reduction.

Discussion

Prony Bay constitutes an ecological niche in phylogenomic clades

The average sample oxidation state decreased from light- and air-influenced terrestrial to more anoxic submarine sites, with the exception of ST0714 which featured the largest percentage of aerobic sequences (Figure 1). This sample was also an outlier concerning its high taxonomic diversification, featuring only three high-abundance MAGs out of which two were cyanobacterial (Figure 2). It is likely that ST0714 was contaminated with seawater during the sampling process, which is challenging to avoid when collecting material from the hydrothermal chimneys. This sample can thus be interpreted as representative of the mixing zone between hydrothermal fluid and ambient seawater. All other samples featured a classic serpentinite-hosted community, which shifted from aerobic and microaerophilic *Gammaproteobacteria* such as *Burkholderiales* to anaerobes such as *Desulfovibrionales*, *Methanocellales* and *Bipolaricaulia* in ST0711. The distribution of MAGs across the phylogenomic tree was neither site- nor system-specific, suggesting that Prony Bay does not constitute an isolated ecological niche (Supplementary Figure 1). In phylogenomic clades, however, MAGs from all Prony Bay sites formed microclusters. When associated to foreign genomes, Prony Bay sequences mostly co-occurred with genomes from the Costa Rica Margin, and in some cases with organisms from Lost City and The Cedars. While single taxa are thus closely linked to sequences from both marine and continental serpentinite-hosted systems, caution is required when drawing generalized conclusions from specific Prony Bay sites for other ultramafic environments.

Prony Bay communities are alimented by bicarbonate and acetate

Microbial communities from all sites rely on bicarbonate and acetate as primary carbon sources (Figure 6). It is unlikely that glycine assimilation plays a role at Prony Bay, as only few low-abundance MAGs featured a glycine reductase and none of them a glycine transporter (Supplementary Figure 3). The use of glycine in serpentinite-hosted conditions was suggested based on the occurrence of the glycine reductase in metagenome-assembled genomes from the Japanese Hakuba Happo and the Californian The Cedars springs (Nobu et al., 2022). This enzyme is incorporated in the reductive glycine pathway, and while it can assimilate exogenous glycine, it may also process endogenous glycine yielded from bicarbonate or formate as an intermediate in carbon fixation (Sánchez-Andrea et al., 2020).

Likewise, formate fixation does probably not contribute significantly to the carbon budget of the sampled Prony Bay communities. Few low-abundant MAGs featured a formate transporter or a formate dehydrogenase, and none of them had both proteins (Supplementary Figure 3). The reported utilization of formate as a carbon source is inconsistent. Especially in Lost City, where mantle-derived formate reaches concentration of up to 158 µmol/L in the hydrothermal fluid, it has been proposed to fuel autotrophic methanogenesis in biofilms

dominated by the Lost City *Methanosarcinales* (LCMS). This probably involves symbiotic relationships with sulfate-reducers and *Chloroflexi*, as the LCMS themselves do not feature formate transporters or formate dehydrogenase genes (Lang et al., 2018; McGonigle et al., 2020). Formate uptake genes have also been observed in the continental Voltri, Samail and Santa Elena ophiolites, and a microcosm study provided experimental evidence for autotrophic CH_4 production from formate in The Cedars (Kohl et al., 2016a; Brazelton et al., 2017; Crespo-Medina et al., 2017; Fones et al., 2019). On the other hand, formate uptake genes were found to be absent from The Coast Range ophiolitic communities, and unexpectedly also from a recently published Lost City metagenome which only contained a formate-dehydrogenase-like sequence with unknown function (Seyler et al., 2020; Brazelton et al., 2022). Furthermore, two other microcosm experiments on The Coast Range and Tableland ophiolites did not confirm significant CH_4 production from formate (Crespo-Medina et al., 2014; Morrill et al., 2014). Interestingly, almost all low-abundance Prony Bay MAGs featuring a formate transporter belonged to the *Methylococcales*. This order in normally methanotrophic but the methanol dehydrogenase was absent from all MAGs. Instead, the respective organisms were able to oxidize H_2, ammonia and reduced sulfur species, and respire nitrite as well as O_2 (Supplementary Figure 5). It is possible that these MAGs heterotrophically assimilate formate via the pyruvate-formate lyase, occupying a low-abundance and low-diversity ecological niche across Prony Bay sites (Bar-Even, 2016).

Contrary to glycine and formate, the acetate transporter actP and especially the acetyl-coA synthetase were present in a considerable number of MAGs (Supplementary Figure 3). Acetate assimilation genes have been documented in metagenomes from Voltri, Santa Elena and Lost City, where syntrophic acetate oxidizers could provide H_2 and CO_2 for autotrophic methanogens (Brazelton et al., 2011, 2017; Crespo-Medina et al., 2017; McGonigle et al., 2020). In addition, CH_4 was yielded from the methyl group of acetate in microcosm experiments on The Cedars and The Coast Range ophiolites; however, the same experiment failed with communities from The Tablelands ophiolite (Crespo-Medina et al., 2014; Morrill et al., 2014; Kohl et al., 2016a). While acetate can be produced abiotically in serpentinite-hosted systems, it is probably also yielded via acetogenesis and fermentation. The true contribution of acetate-assimilating organisms to the base of the trophic network is thus difficult to establish and would require more detailed analyses of Prony Bay's hydrothermal fluid. In Lost City, DIC stable isotope measurements suggest that, unlike formate, the majority of acetate is yielded biologically (Lang et al., 2010). Conversely, a study on serpentinite-hosted mud volcanos along the Mariana Forearc leans towards an abiotic origin of measured acetate pools (Eickenbusch et al., 2019). While the ratio of abiotic and biotic acetate in Prony Bay thus remains unclear, it is likely that the base of the trophic network is constituted at least to some extend by organisms assimilating acetate from an abiotic source.

Next to acetate, a relatively large proportion of high-abundance MAGs featured the sbtA bicarbonate transporter and a gamma carbonic anhydrase (Supplementary Figure 3). Bicarbonate is not only inorganic but also vastly abundant in serpentinite-hosted systems, rendering it an ideal primary carbon source for organisms capable of redissolving it from the carbonaceous hydrothermal chimneys. *Serpentinimonas,* which is associated with the *Burkholderiales* order, was observed to form aggregates on calcium carbonate particles in culture. It has been hypothesized that these aggregates decrease the pH in microenvironments

via extracellular carbonic anhydrases interconverting CO_2 and bicarbonate, which would permit to locally concentrate the latter (Suzuki et al., 2014). It is likely that other taxonomic groups have evolved the same carbon fixation strategy to adapt to DIC limitation in serpentinite-hosted environments. For example, *Thiomicrospira* genomes from Lost City feature partial carboxysome operons including a carbonic anhydrase (Brazelton and Baross, 2010).

Autotrophic and heterotrophic foundation species of surface communities

Five potential autotrophic and heterotrophic foundation taxa with a complete set of bicarbonate or acetate uptake genes could be identified (Figure 6). Across both high- and low-abundance MAGs, this only concerned aerobes and facultative anaerobes, with the exception a low-abundance *Desulfobulbales* MAG from ST0714 (Table 1, Supplementary Figure 3, Supplementary Figure 5). We therefore only sampled foundation species from the surface environment.

The first putative surface foundation taxon is an aerobic acetate-assimilating organism associated with the *Cyanobacteriia* (RDYZ01 genus) (Table 1). This MAG featured a NiFe hydrogenase, which is used for the splitting of water in photosynthesis. It has been shown that acetate can enhance the growth of genetically engineered cyanobacteria as an additional carbon source in low-light conditions, suppressing photosynthetic activity and enhancing glycolytic metabolism (Thiel et al., 2017). Related taxa such as RDYZ01 could have naturally acquired the actP gene in DIC-limited conditions. Two other putative surface foundation species had a mixotrophic genetic make-up, featuring both acetate and bicarbonate uptake genes (Table 1). An aerobic *Rhodobacterales* MAG from BdJ11 was able to oxidize sulfide and thiosulfate. It has been suggested that thiosulfate may provide a supplementary "energy boost" for ATP production and CO_2 assimilation in hyperalkaline conditions. This was the case for a chemoheterotrophic *Rhodobacterales* isolate from the continental Ney Springs, which grew on acetate and thiosulfate at a pH of up to 12.4 (Trutschel et al., 2022). In addition, our MAG can likely switch to a chemoautotrophic lifestyle by fixing bicarbonate via sulfide or thiosulfate oxidation. The use of reduced sulfur species as energy source has been reported multiple times in serpentinite-hosted environments, and probably constitutes a valuable ecological niche (Schrenk et al., 2004; Brazelton et al., 2006; Sabuda et al., 2020; Frouin et al., 2022). Next to the *Rhodobacterales* MAG, a *Burkholderiales* MAG from RdK14 featured a complete set of both acetate and bicarbonate uptake genes. The metabolic make-up of this organism was strikingly diverse, and it was the only facultatively anaerobic foundation taxon as it presented not only cytochromes but also genes for nitrate, nitrite and sulfite respiration. It is possible that also the *Burkholderiales* MAGs aliments heterotrophic growth on acetate via sulfide oxidation. For autotrophic growth, the MAG may additionally perform aerobic oxidation of CO, which is a byproduct of serpentinization and likely serves as a source of energy for some serpentinite-hosted organisms (Morrill et al., 2014; Twing et al., 2017). In line with previous reports on serpentinite-hosted *Burkholderiales*, which generally seem to be hydrogenotrophs, the MAG also featured a H_2 oxidizing NiFe hydrogenase (Brazelton et al., 2012; Suzuki et al., 2014; Twing et al., 2017; Bird et al., 2021). Besides these mixotrophic surface foundation taxa, three

more MAGs featured bicarbonate uptake genes (Table 1). This included two organisms associated with the *Xanthomonadales* from BdJ11 (*Silanimonas* genus) and ST0711 (*Wenzhouxiangellaceae* family). The former was able to oxidize sulfide, while the latter did not present any genes to use a lithotrophic energy source. The *Wenzhouxiangellaceae* are usually heterotrophic, and our MAG might employ a bicarbonate transporter in combination with a carbonic anhydrase to regulate intracellular pH without having evolved the ability to fix yielded CO_2 (Wang et al., 2015). It is thus unlikely that the *Wenzhouxiangellaceae* taxon contributes to primary carbon fixation in Prony Bay. The last putative autotrophic foundation species was associated with the gammaproteobacterial UBA7966 (UBA7966 sp003485715) and able to utilize both thiosulfate and hydrogen as an energy source. Intriguingly, all proposed bicarbonate fixers scored high for the CBB cycle but were missing the RuBisCo enzyme. The only exception might be the *Burkholderiales* MAG, which featured the small chain of the protein: since RuBisCo's small and large subunits are located directly next to each other, it is plausible that the absence of the latter is due to fragmentation of the corresponding contig. RuBisCo catalyzes the fixation of CO_2 into 3-phospho-D-glycerate and is normally essential for the CBB cycle to operate, especially since no other known protein performs the same chemical reaction (Santos Correa et al., 2023). Our MAGs may employ a modified version of the CBB cycle with a to date unidentified carboxylase. RuBisCo is characterized by slow catalysis rates and low substrate specificity, rendering it one of the least efficient enzymes (Bar-Even et al., 2010). Especially in DIC-limited environments, this is likely a disadvantage and may favor the evolution of new gene variants. Presently, three different *bona fide* RuBisCo forms and at least six additional clades of diverse RuBisCO-like proteins have been identified. However, none of the latter can fix CO_2 and RuBisCo remains one of the evolutionary most successful proteins, with evidence pointing out that it is highly optimized (Tcherkez et al., 2006; Tabita et al., 2007). Alternatively, MAGs with bicarbonate uptake genes might rely on symbiotic partners performing CO_2 fixation. Three cyanobacterial MAGs, a *Cardiobacteriales* MAG and a *Burkholderiales* MAG (*Calidifontimicrobium* genus) feature a complete CBB cycle and might enter symbioses with organisms providing CO_2 from bicarbonate (Figure 4).

Hypotheses on foundation species of subsurface communities

As all MAGs with a (nearly) complete acetate or bicarbonate uptake mechanism were aerobes or facultative anaerobes, the trophic base of Prony Bay's subsurface environment remains elusive. The fact that we did not recover anaerobic foundation species might be partially due to a methodological bias: The number of MAGs reconstructed from the anoxic ST0711 was much lower than for other samples. The more anoxic and extreme its environment, the less biomass a sample usually contains. This complicates DNA extraction and renders the reconstruction of high-quality sequences overall more challenging.

It is possible that the archaeal community contributes to carbon uptake in the subsurface, similar to the consortia of LCMS and symbiotic bacteria fixing formate in Lost City. Prony Bay may feature an archaeal community capable of fixing bicarbonate, as has recently been suggested for an archaeal *Methanocellales* acetogen from The Cedars (Suzuki et al., 2024). The only high-abundance archaeal MAG we were able to reconstruct was assigned to the *Methanocellales* and it is closely related to the organism described by Suzuki et al. (Supplementary Figure 6). Our MAG lacks bicarbonate uptake genes, but in this case, this

might be due to the relatively low completeness (64%) of the genomic sequence (Supplementary Table 1). While archaea such as *Methanocellales* might contribute to the development of higher trophic levels, archaeal abundance is generally low in Prony Bay (Postec et al., 2015; Frouin et al., 2018). Therefore, the base of the trophic network is likely constituted by a majority of bacterial foundation species. The sampled ST0711 community is dominated by two orders of *Bipolaricaulia*, an ancient lineage which has been found in several serpentinite-hosted systems (Brazelton et al., 2022; Colman et al., 2022). While this taxon is usually described as an autotrophic acetogen, it has been shown that some genotypes are heterotrophic and lack a Wood-Ljungdahl pathway like our MAG (Coskun et al., 2023) (Figure 4). Related strains are able to perform fermentation of peptides, amino acids, and simple sugars to acetate, formate and H_2 (Hao et al., 2018). Abiotic synthesis of these compound classes has long been debated and was partially demonstrated in laboratory experiments, including the Miller-Urey experiment, Strecker synthesis and formose reaction (Strecker, 1850; Butlerow, 1861; Miller and Urey, 1959; Müller et al., 2022). On condition that such abiotic reactions occur in the hydrothermal fluid of Prony Bay's active sites, CO_2 producing fermenters such as *Bipolaricaulia* might offer a new perspective into primary carbon uptake in the subsurface.

Conclusion

We successfully reconstructed MAGs from five Prony Bay samples representing a gradient from aerobic and microaerophilic to anaerobic serpentinite-hosted communities. Except for ST0714, which illustrates the mixing zone of hydrothermal fluid and seawater, the microbial consortia were dominated by corresponding key taxa such as aerobic and microaerophilic *Burkholderiales* and anaerobic *Desulfovibrionales*, *Methanocellales* and *Bipolaricaulia*. MAGs were phylogenomically evenly distributed but formed microclusters in clades that were only sporadically associated with foreign serpentinite-hosted sequences. There are thus limitations to the adaptability of Prony Bay results to other ultramafic environments. All sampled communities featured bicarbonate and acetate uptake genes, while formate and glycine seemed to play a minor role. MAGs featuring a full set of genes for bicarbonate or acetate uptake were aerobic or facultatively anaerobic, suggesting that only foundation species from the surface environment were sampled. Proposed surface foundation taxa include a cyanobacterial acetate-assimilating MAG, two mixotrophic organisms classified as *Rhodobacterales* and *Burkholderiales*, as well as two bicarbonate-fixing MAGs assigned to the *Xanthomonadales* and UBA7966 orders. The autotrophs were able to oxidize H_2, sulfide or thiosulfate and featured a nearly complete CBB cycle missing the RuBisCo gene. It is possible that they rely on a to date unidentified carboxylase or on symbionts. Future studies should attempt to target subsurface foundation species, for example by further adapting DNA extraction protocols to low biomass samples. Other than bicarbonate, formate, acetate and glycine, CO_2-yielding pathways involved in the fermentation of other amino acids, peptides and simple sugars might be interesting to assess in organisms such as acetogenic *Methanocellales* and fermenting *Bipolaricaulia*.

Acknowledgements

We are grateful to Philippe Ortet, Marine Bergot and Caroline Monteil of the CEA BIC bioinformatics platform for providing help with the functional analysis. Furthermore, we would like to thank all partners of the MICROPRONY project, including everyone who was involved in the 2011 and 2014 field campaigns. Many thanks to Eléonore Frouin for performing the DNA extractions with GE. RMP was awarded a PhD Fellowship from the French Ministry of Education and Scientific Research.

Author contributions

GE and AP supervised the study. RMP and MH designed the study, with revisions from GE. RMP built the carbon fixation pathway database. MH developed the carbon fixation pathway score and performed the bioinformatic analysis. SM and PP performed SSU rRNA reconstruction. RMP performed the data analysis and wrote the manuscript. All authors read and accepted the final version.

References

Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., et al. (2014). Binning metagenomic contigs by coverage and composition. *Nat Methods* 11, 1144–1146. doi: 10.1038/nmeth.3103

Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., et al. (2020). KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics* 36, 2251–2252. doi: 10.1093/bioinformatics/btz859

Aroney, S. T. N., Newell, R. J. P., Nissen, J., Camargo, A. P., Tyson, G. W., and Woodcroft, B. J. (2024). CoverM: Read coverage calculator for metagenomics. doi: 10.5281/ZENODO.10531253

Asplund-Samuelsson, J., and Hudson, E. P. (2021). Wide range of metabolic adaptations to the acquisition of the Calvin cycle revealed by comparison of microbial genomes. *PLOS Computational Biology* 17, e1008742. doi: 10.1371/journal.pcbi.1008742

Bar-Even, A. (2016). Formate Assimilation: The Metabolic Architecture of Natural and Synthetic Pathways. *Biochemistry* 55, 3851–3863. doi: 10.1021/acs.biochem.6b00495

Bar-Even, A., Noor, E., Lewis, N. E., and Milo, R. (2010). Design and analysis of synthetic carbon fixation pathways. *Proceedings of the National Academy of Sciences* 107, 8889–8894. doi: 10.1073/pnas.0907176107

Bird, L. J., Kuenen, J. G., Osburn, M. R., Tomioka, N., Ishii, S., Barr, C., et al. (2021). Serpentinimonas gen. nov., Serpentinimonas raichei sp. nov., Serpentinimonas barnesii

sp. nov. and Serpentinimonas maccroryi sp. nov., hyperalkaliphilic and facultative autotrophic bacteria isolated from terrestrial serpentinizing springs. *International Journal of Systematic and Evolutionary Microbiology* 71. doi: 10.1099/ijsem.0.004945

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brazelton, W. J., and Baross, J. A. (2010). Metagenomic Comparison of Two Thiomicrospira Lineages Inhabiting Contrasting Deep-Sea Hydrothermal Environments. *PLOS ONE* 5, e13530. doi: 10.1371/journal.pone.0013530

Brazelton, W. J., McGonigle, J. M., Motamedi, S., Pendleton, H. L., Twing, K. I., Miller, B. C., et al. (2022). Metabolic Strategies Shared by Basement Residents of the Lost City Hydrothermal Field. *Appl Environ Microbiol* 88, e00929-22. doi: 10.1128/aem.00929-22

Brazelton, W. J., Mehta, M. P., Kelley, D. S., and Baross, J. A. (2011). Physiological Differentiation within a Single-Species Biofilm Fueled by Serpentinization. *mBio* 2, e00127-11. doi: 10.1128/mBio.00127-11

Brazelton, W. J., Nelson, B., and Schrenk, M. O. (2012). Metagenomic Evidence for H2 Oxidation and H2 Production by Serpentinite-Hosted Subsurface Microbial Communities. *Front. Microbio.* 2. doi: 10.3389/fmicb.2011.00268

Brazelton, W. J., Schrenk, M. O., Kelley, D. S., and Baross, J. A. (2006). Methane- and Sulfur-Metabolizing Microbial Communities Dominate the Lost City Hydrothermal Field Ecosystem. *Appl Environ Microbiol* 72, 6257–6270. doi: 10.1128/AEM.00574-06

Brazelton, W. J., Thornton, C. N., Hyer, A., Twing, K. I., Longino, A. A., Lang, S. Q., et al. (2017). Metagenomic identification of active methanogens and methanotrophs in serpentinite springs of the Voltri Massif, Italy. *PeerJ* 5, e2945. doi: 10.7717/peerj.2945

Butlerow, A. M. (1861). Bildung einer zuckerartigen Substanz durch Synthese. *Justus Liebigs Annalen der Chemie* 120, 295–298.

Caspi, R., Billington, R., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., et al. (2018). The MetaCyc database of metabolic pathways and enzymes. *Nucleic Acids Research* 46, D633–D639. doi: 10.1093/nar/gkx935

Chakoory, O., Comtet-Marre, S., and Peyret, P. (2022). RiboTaxa: combined approaches for rRNA genes taxonomic resolution down to the species level from metagenomics data revealing novelties. *NAR Genomics and Bioinformatics* 4, lqac070. doi: 10.1093/nargab/lqac070

Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P., and Parks, D. H. (2022). GTDB-Tk v2: memory friendly classification with the Genome Taxonomy Database. Bioinformatics. doi: 10.1101/2022.07.11.499641

Colman, D. R., Kraus, E. A., Thieringer, P. H., Rempfert, K., Templeton, A. S., Spear, J. R., et al. (2022). Deep-branching acetogens in serpentinized subsurface fluids of Oman. *Proc. Natl. Acad. Sci. U.S.A.* 119, e2206845119. doi: 10.1073/pnas.2206845119

Coskun, Ö. K., Gomez-Saez, G. V., Beren, M., Ozcan, D., Hosgormez, H., Einsiedl, F., et al. (2023). Carbon metabolism and biogeography of candidate phylum "Candidatus Bipolaricaulota" in geothermal environments of Biga Peninsula, Turkey. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1063139

Crespo-Medina, M., Twing, K. I., Kubo, M. D. Y., Hoehler, T. M., Cardace, D., McCollom, T., et al. (2014). Insights into environmental controls on microbial communities in a continental serpentinite aquifer using a microcosm-based approach. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00604

Crespo-Medina, M., Twing, K. I., Sánchez-Murillo, R., Brazelton, W. J., McCollom, T. M., and Schrenk, M. O. (2017). Methane Dynamics in a Tropical Serpentinizing Environment: The Santa Elena Ophiolite, Costa Rica. *Front. Microbiol.* 8, 916. doi: 10.3389/fmicb.2017.00916

Criscuolo, A., and Brisse, S. (2013). AlienTrimmer: A tool to quickly and accurately trim off multiple short contaminant sequences from high-throughput sequencing reads. *Genomics* 102, 500–506. doi: 10.1016/j.ygeno.2013.07.011

Dick, J. M., and Shock, E. L. (2011). Calculation of the Relative Chemical Stabilities of Proteins as a Function of Temperature and Redox Chemistry in a Hot Spring. *PLOS ONE* 6, e22782. doi: 10.1371/journal.pone.0022782

Eickenbusch, P., Takai, K., Sissman, O., Suzuki, S., Menzies, C., Sakai, S., et al. (2019). Origin of Short-Chain Organic Acids in Serpentinite Mud Volcanoes of the Mariana Convergent Margin. *Front. Microbiol.* 10, 1729. doi: 10.3389/fmicb.2019.01729

Fan, S.-H., Matsuo, M., Huang, L., Tribelli, P. M., and Götz, F. (2021). The MpsAB Bicarbonate Transporter Is Superior to Carbonic Anhydrase in Biofilm-Forming Bacteria with Limited CO2 Diffusion. *Microbiology Spectrum* 9, 10.1128/spectrum.00305-21. doi: 10.1128/spectrum.00305-21

Fones, E. M., Colman, D. R., Kraus, E. A., Nothaft, D. B., Poudel, S., Rempfert, K. R., et al. (2019). Physiological adaptations to serpentinization in the Samail Ophiolite, Oman. *ISME J* 13, 1750–1762. doi: 10.1038/s41396-019-0391-2

Fones, E. M., Colman, D. R., Kraus, E. A., Stepanauskas, R., Templeton, A. S., Spear, J. R., et al. (2021). Diversification of methanogens into hyperalkaline serpentinizing

environments through adaptations to minimize oxidant limitation. *ISME J* 15, 1121–1135. doi: 10.1038/s41396-020-00838-1

Frouin, E., Bes, M., Ollivier, B., Quéméneur, M., Postec, A., Debroas, D., et al. (2018). Diversity of Rare and Abundant Prokaryotic Phylotypes in the Prony Hydrothermal Field and Comparison with Other Serpentinite-Hosted Ecosystems. *Front. Microbiol.* 9, 102. doi: 10.3389/fmicb.2018.00102

Frouin, E., Lecoeuvre, A., Armougom, F., Schrenk, M. O., and Erauso, G. (2022). Comparative Metagenomics Highlight a Widespread Pathway Involved in Catabolism of Phosphonates in Marine and Terrestrial Serpentinizing Ecosystems. *mSystems*, e00328-22. doi: 10.1128/msystems.00328-22

Garritano, A. N., Song, W., and Thomas, T. (2022). Carbon fixation pathways across the bacterial and archaeal tree of life. *PNAS Nexus* 1, pgac226. doi: 10.1093/pnasnexus/pgac226

Ghrist, A. C., and Stauffer, G. V. (1995). The Escherichia coli glycine transport system and its role in the regulation of the glycine cleavage enzyme system. *Microbiology* 141, 133–140. doi: 10.1099/00221287-141-1-133

Gimenez, R., Nuñez, M. F., Badia, J., Aguilar, J., and Baldoma, L. (2003). The Gene *yjcG* , Cotranscribed with the Gene *acs* , Encodes an Acetate Permease in *Escherichia coli*. *J Bacteriol* 185, 6448–6455. doi: 10.1128/JB.185.21.6448-6455.2003

Hao, L., McIlroy, S. J., Kirkegaard, R. H., Karst, S. M., Fernando, W. E. Y., Aslan, H., et al. (2018). Novel prosthecate bacteria from the candidate phylum Acetothermia. *The ISME Journal* 12, 2225–2237. doi: 10.1038/s41396-018-0187-9

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 44, D457–D462. doi: 10.1093/nar/gkv1070

Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., et al. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7, e7359. doi: 10.7717/peerj.7359

Kohl, L., Cumming, E., Cox, A., Rietze, A., Morrissey, L., Lang, S. Q., et al. (2016a). Exploring the metabolic potential of microbial communities in ultra-basic, reducing springs at The Cedars, CA, USA: Experimental evidence of microbial methanogenesis and heterotrophic acetogenesis. *Journal of Geophysical Research: Biogeosciences* 121, 1203–1220. doi: 10.1002/2015JG003233

Kohl, L., Cumming, E., Cox, A., Rietze, A., Morrissey, L., Lang, S. Q., et al. (2016b). Exploring the metabolic potential of microbial communities in ultra-basic, reducing springs at The Cedars, CA, USA: Experimental evidence of microbial methanogenesis

and heterotrophic acetogenesis: Methanogenesis at The Cedars. *J. Geophys. Res. Biogeosci.* 121, 1203–1220. doi: 10.1002/2015JG003233

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611

Lang, S. Q., Butterfield, D. A., Schulte, M., Kelley, D. S., and Lilley, M. D. (2010). Elevated concentrations of formate, acetate and dissolved organic carbon found at the Lost City hydrothermal field. *Geochimica et Cosmochimica Acta* 74, 941–952. doi: 10.1016/j.gca.2009.10.045

Lang, S. Q., Früh-Green, G. L., Bernasconi, S. M., Brazelton, W. J., Schrenk, M. O., and McGonigle, J. M. (2018). Deeply-sourced formate fuels sulfate reducers but not methanogens at Lost City hydrothermal field. *Sci Rep* 8, 755. doi: 10.1038/s41598-017-19002-5

Larralde, M. (2022). Pyrodigal: Python bindings and interface to Prodigal,an efficient method for gene prediction in prokaryotes. *JOSS* 7, 4296. doi: 10.21105/joss.04296

Letunic, I., and Bork, P. (2024). Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Research* 52, W78–W82. doi: 10.1093/nar/gkae268

McCollom, T. M., and Seewald, J. S. (2013). Serpentinites, Hydrogen, and Life. *Elements* 9, 129–134. doi: 10.2113/gselements.9.2.129

McGonigle, J. M., Lang, S. Q., and Brazelton, W. J. (2020). Genomic Evidence for Formate Metabolism by *Chloroflexi* as the Key to Unlocking Deep Carbon in Lost City Microbial Ecosystems. *Appl Environ Microbiol* 86, e02583-19. doi: 10.1128/AEM.02583-19

Ménez, B., Pisapia, C., Andreani, M., Jamme, F., Vanbellingen, Q. P., Brunelle, A., et al. (2018). Abiotic synthesis of amino acids in the recesses of the oceanic lithosphere. *Nature* 564, 59–63. doi: 10.1038/s41586-018-0684-z

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., and Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics* 34, i142–i150. doi: 10.1093/bioinformatics/bty266

Miller, S. L., and Urey, H. C. (1959). Organic Compound Synthesis on the Primitive Earth. *Science* 130, 245–251. doi: 10.1126/science.130.3370.245

Monnin, C., Chavagnac, V., Boulart, C., Ménez, B., Gérard, M., Gérard, E., et al. (2014). Fluid chemistry of the low temperature hyperalkaline hydrothermal system of Prony Bay (New Caledonia). *Biogeosciences* 11, 5687–5706. doi: 10.5194/bg-11-5687-2014

Morrill, P. L., Brazelton, W. J., Kohl, L., Rietze, A., Miles, S. M., Kavanagh, H., et al. (2014). Investigations of potential microbial methanogenic and carbon monoxide utilization pathways in ultra-basic reducing springs associated with present-day continental serpentinization: the Tablelands, NL, CAN. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00613

Müller, F., Escobar, L., Xu, F., Węgrzyn, E., Nainytė, M., Amatov, T., et al. (2022). A prebiotically plausible scenario of an RNA–peptide world. *Nature* 605, 279–284. doi: 10.1038/s41586-022-04676-3

Nobu, M. K., Nakai, R., Tamazawa, S., Mori, H., Toyoda, A., Ijiri, A., et al. (2022). Unique H2-utilizing lithotrophy in serpentinite-hosted systems. *ISME J*. doi: 10.1038/s41396-022-01197-9

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P. A. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome Res* 27, 824–834. doi: 10.1101/gr.213959.116

Parks, D. H., Chuvochina, M., Rinke, C., Mussig, A. J., Chaumeil, P.-A., and Hugenholtz, P. (2022). GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Research* 50, D785–D794. doi: 10.1093/nar/gkab776

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* 25, 1043–1055. doi: 10.1101/gr.186072.114

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00857

Poudel, S., Colman, D. R., Fixen, K. R., Ledbetter, R. N., Zheng, Y., Pence, N., et al. (2018). Electron Transfer to Nitrogenase in Different Genomic and Metabolic Backgrounds. *Journal of Bacteriology* 200, 10.1128/jb.00757-17. doi: 10.1128/jb.00757-17

Quéméneur, M., Bes, M., Postec, A., Mei, N., Hamelin, J., Monnin, C., et al. (2014). Spatial distribution of microbial communities in the shallow submarine alkaline hydrothermal field of the Prony Bay, New Caledonia: Microbial communities of Prony Hydrothermal Field. *Environmental Microbiology Reports* 6, 665–674. doi: 10.1111/1758-2229.12184

Reimer, L. C., Vetcininova, A., Carbasse, J. S., Söhngen, C., Gleim, D., Ebeling, C., et al. (2019). Bac *Dive* in 2019: bacterial phenotypic data for High-throughput biodiversity analysis. *Nucleic Acids Research* 47, D631–D636. doi: 10.1093/nar/gky879

Sabuda, M. C., Brazelton, W. J., Putman, L. I., McCollom, T. M., Hoehler, T. M., Kubo, M. D. Y., et al. (2020). A dynamic microbial sulfur cycle in a serpentinizing continental ophiolite. *Environ Microbiol* 22, 2329–2345. doi: 10.1111/1462-2920.15006

Sánchez-Andrea, I., Guedes, I. A., Hornung, B., Boeren, S., Lawson, C. E., Sousa, D. Z., et al. (2020). The reductive glycine pathway allows autotrophic growth of Desulfovibrio desulfuricans. *Nat Commun* 11, 5090. doi: 10.1038/s41467-020-18906-7

Santos Correa, S., Schultz, J., Lauersen, K. J., and Soares Rosado, A. (2023). Natural carbon fixation and advances in synthetic engineering for redesigning and creating new fixation pathways. *Journal of Advanced Research* 47, 75–92. doi: 10.1016/j.jare.2022.07.011

Sayers, E. W., Bolton, E. E., Brister, J. R., Canese, K., Chan, J., Comeau, D. C., et al. (2022). Database resources of the national center for biotechnology information. *Nucleic Acids Research* 50, D20–D26. doi: 10.1093/nar/gkab1112

Schrenk, M. O., Brazelton, W. J., and Lang, S. Q. (2013). Serpentinization, Carbon, and Deep Life. *Reviews in Mineralogy and Geochemistry* 75, 575–606. doi: 10.2138/rmg.2013.75.18

Schrenk, M. O., Kelley, D. S., Bolton, S. A., and Baross, J. A. (2004). Low archaeal diversity linked to subseafloor geochemical processes at the Lost City Hydrothermal Field, Mid-Atlantic Ridge. *Environ Microbiol* 6, 1086–1095. doi: 10.1111/j.1462-2920.2004.00650.x

Schwengers, O., Jelonek, L., Dieckmann, M. A., Beyvers, S., Blom, J., and Goesmann, A. (2021). Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification: Find out more about Bakta, the motivation, challenges and applications, here. *Microbial Genomics* 7. doi: 10.1099/mgen.0.000685

Seyler, L. M., Brazelton, W. J., McLean, C., Putman, L. I., Hyer, A., Kubo, M. D. Y., et al. (2020). Carbon Assimilation Strategies in Ultrabasic Groundwater: Clues from the Integrated Study of a Serpentinization-Influenced Aquifer. *mSystems* 5, e00607-19. doi: 10.1128/mSystems.00607-19

Smith, K. S., and Ferry, J. G. (2000). Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev* 24, 335–366. doi: 10.1111/j.1574-6976.2000.tb00546.x

Strecker, A. (1850). Ueber die künstliche Bildung der Milchsäure und einen neuen, dem Glycocoll homologen Körper. *Justus Liebigs Ann. Chem.* 75, 27–45. doi: 10.1002/jlac.18500750103

Suzuki, S., Ishii, S., Chadwick, G. L., Tanaka, Y., Kouzuma, A., Watanabe, K., et al. (2024). A non-methanogenic archaeon within the order Methanocellales. *Nat Commun* 15, 4858. doi: 10.1038/s41467-024-48185-5

Suzuki, S., Kuenen, J. G., Schipper, K., van der Velde, S., Ishii, S., Wu, A., et al. (2014). Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. *Nat Commun* 5, 3900. doi: 10.1038/ncomms4900

Tabita, F. R., Hanson, T. E., Li, H., Satagopan, S., Singh, J., and Chan, S. (2007). Function, Structure, and Evolution of the RubisCO-Like Proteins and Their RubisCO Homologs. *Microbiol Mol Biol Rev* 71, 576–599. doi: 10.1128/MMBR.00015-07

Tcherkez, G. G. B., Farquhar, G. D., and Andrews, T. J. (2006). Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc Natl Acad Sci U S A* 103, 7246–7251. doi: 10.1073/pnas.0600605103

Thiel, K., Vuorio, E., Aro, E.-M., and Kallio, P. T. (2017). The effect of enhanced acetate influx on Synechocystis sp. PCC 6803 metabolism. *Microb Cell Fact* 16, 21. doi: 10.1186/s12934-017-0640-x

Trchounian, K., and Trchounian, A. (2014). Different role of *focA* and *focB* encoding formate channels for hydrogen production by *Escherichia coli* during glucose or glycerol fermentation. *International Journal of Hydrogen Energy* 39, 20987–20991. doi: 10.1016/j.ijhydene.2014.10.074

Trutschel, L. R., Chadwick, G. L., Kruger, B., Blank, J. G., Brazelton, W. J., Dart, E. R., et al. (2022). Investigation of microbial metabolisms in an extremely high pH marine-like terrestrial serpentinizing system: Ney Springs. *Science of The Total Environment* 836, 155492. doi: 10.1016/j.scitotenv.2022.155492

Twing, K. I., Brazelton, W. J., Kubo, M. D. Y., Hyer, A. J., Cardace, D., Hoehler, T. M., et al. (2017). Serpentinization-Influenced Groundwater Harbors Extremely Low Diversity Microbial Communities Adapted to High pH. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00308

Uritskiy, G. V., DiRuggiero, J., and Taylor, J. (2018). MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome* 6, 158. doi: 10.1186/s40168-018-0541-1

Wang, G., Tang, M., Li, T., Dai, S., Wu, H., Chen, C., et al. (2015). Wenzhouxiangella marina gen. nov, sp. nov, a marine bacterium from the culture broth of Picochlorum sp. 122, and proposal of Wenzhouxiangellaceae fam. nov. in the order Chromatiales. *Antonie van Leeuwenhoek* 107, 1625–1632. doi: 10.1007/s10482-015-0458-7

Ward, L. M., and Shih, P. M. (2019). The evolution and productivity of carbon fixation pathways in response to changes in oxygen concentration over geological time. *Free Radical Biology and Medicine* 140, 188–199. doi: 10.1016/j.freeradbiomed.2019.01.049

White, W. B., and Ferry, J. G. (1992). Identification of formate dehydrogenase-specific mRNA species and nucleotide sequence of the fdhC gene of Methanobacterium formicicum. *Journal of Bacteriology* 174, 4997–5004. doi: 10.1128/jb.174.15.4997-5004.1992

Wingett, S., and Bottoms, C. (2023). StevenWingett/FastQ-Screen: FastQ Screen v0.15.3. doi: 10.5281/ZENODO.1344583

Wu, Y.-W., Simmons, B. A., and Singer, S. W. (2016). MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* 32, 605–607. doi: 10.1093/bioinformatics/btv638

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Research* 42, D643–D648. doi: 10.1093/nar/gkt1209

3.3 Figures and tables

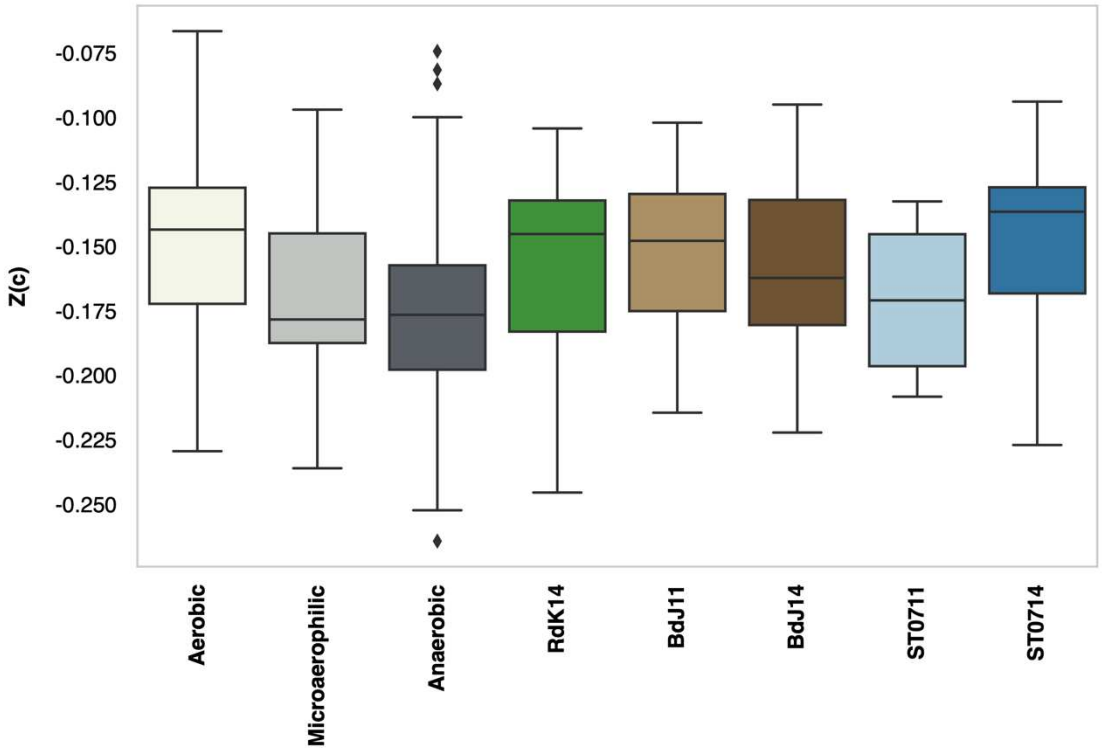


Figure 3-1. Average protein oxidation states of sample MAG sequences and reference sequences from the Bacterial Diversity Metadatabase classified as "aerobic", "microaerophilic" or "anaerobic".



Figure 3-2. Taxonomic composition per sample showing metagenome-assembled genomes (MAGs) with a relative abundance >1%. MAGs with a relative abundance <1% are grouped under "other". MAGs were assigned to orders according to GTDB. Classes shared by at least two orders are indicated with abbreviations: Pac for *Paceibacteria*; Gamma for *Gammaproteobacteria*; Dei for *Deinococci*; Chl for *Chlorobia*; Cyano for *Cyanobacteriia*; Bip for *Bipolaricaulia*. The percentages of unmapped reads are the following: BdJ11: 64%. BdJ14: 63%. RdK14: 42%. ST0711: 59%. ST0714: 82%. MAG identifiers from top to bottom are as follows (note that MAGs were reconstructed from individual assemblies, hence their numbering is not coherent between samples): BdJ11: Bin66, Bin30, Bin47, Bin41, Bin27, Bin26 and Bin46, Bin52, Bin58. BdJ-14: Bin62, Bin7, Bin57, Bin31 and Bin39, Bin33, Bin50, Bin51, Bin9, Bin65, Bin52, Bin66. RdK14: Bin98, Bin78, Bin113, Bin49, Bin2, Bin16, Bin97, Bin44, Bin12, Bin17, Bin76 and Bin92. ST0711: Bin16, Bin11, Bin15, Bin14, Bin12, Bin6. ST07-14: Bin33 and Bin46, Bin53.

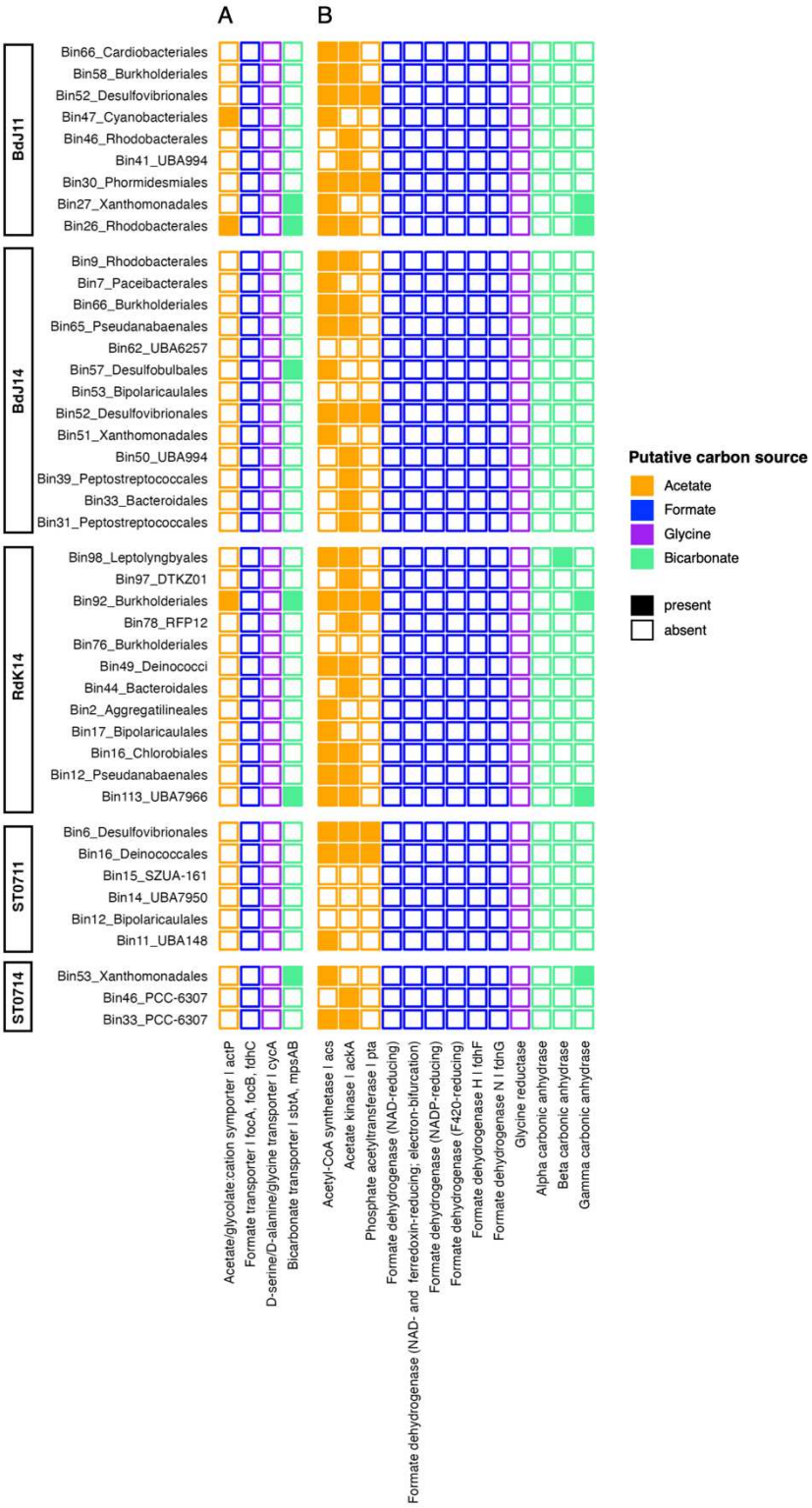


Figure 3-3. Presence and absence of A) transporters and B) primary assimilation enzymes in the most abundant metagenome-assembled genomes (relative abundance >1%) per sample. The depicted proteins facilitate the primary uptake of acetate, formate, glycine or bicarbonate as putative carbon sources. Genes were annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) R109. Where no genes are specified, all available enzyme subdomains (all must be present) or alternatives (one of them must be present) were taken into account.

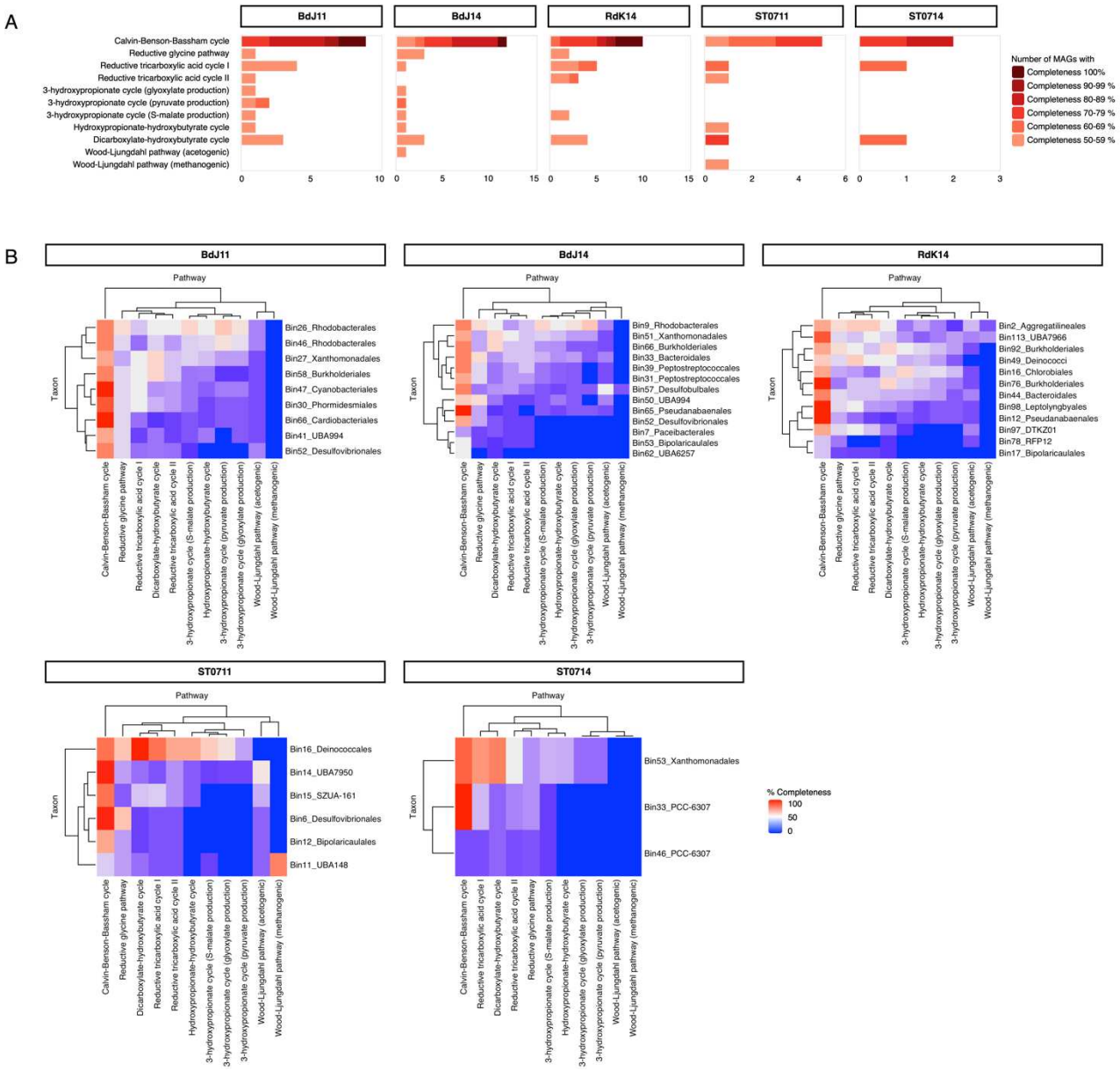


Figure 3-4. Carbon fixation pathway completeness in the most abundant metagenome-assembled genomes (relative abundance >1%) per sample. Completeness scores were calculated based on custom HMM profiles and are given in percent. A) Distribution of pathway completeness scores in each sample. B) Pathway completeness across metagenome-assembled genomes in each sample.

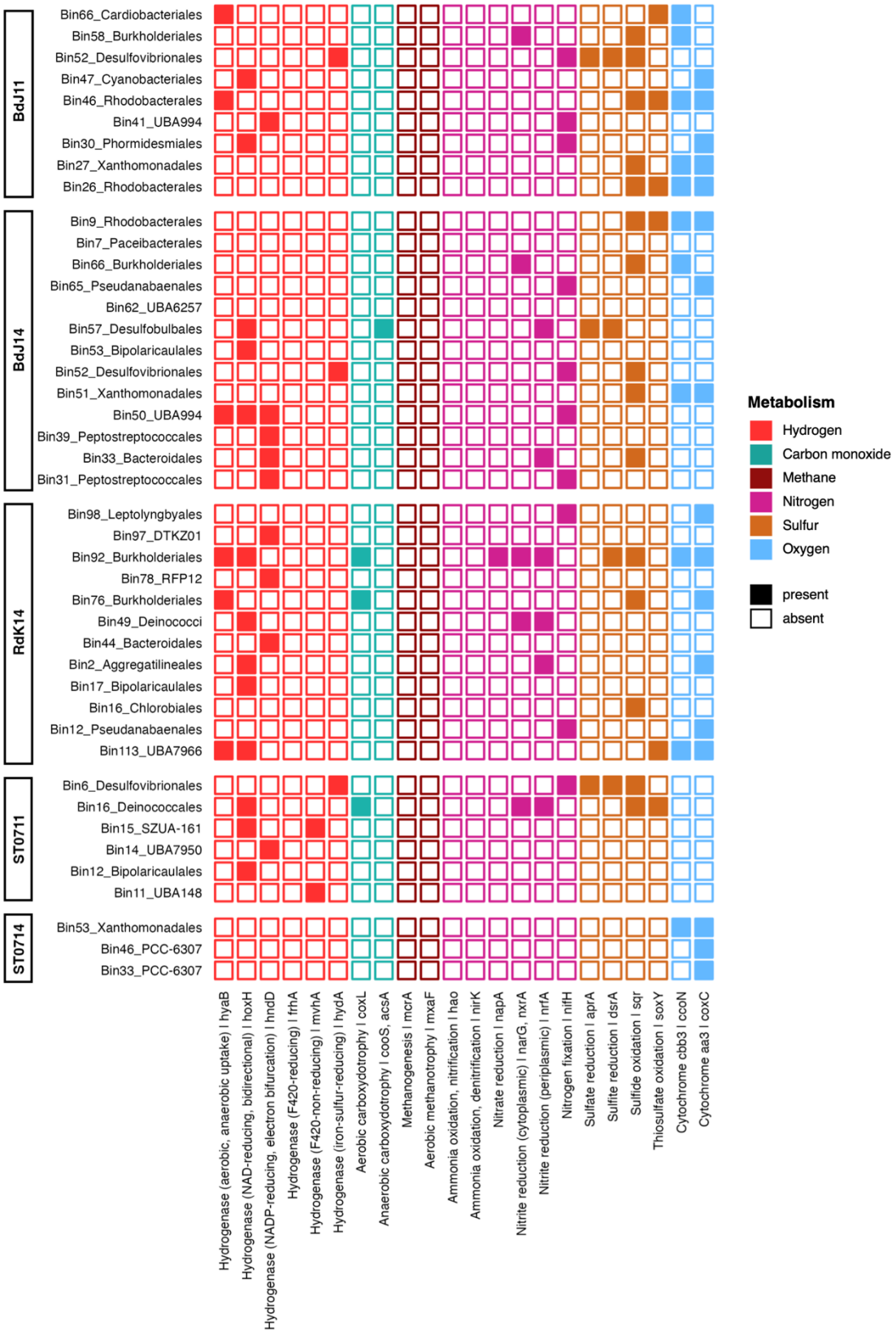


Figure 3-5. Presence and absence of key metabolic genes in the most abundant metagenome-assembled genomes (relative abundance >1%) per sample. Genes playing a crucial role in the metabolism of hydrogen, carbon monoxide, methane, nitrogen, sulfur or oxygen were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109.

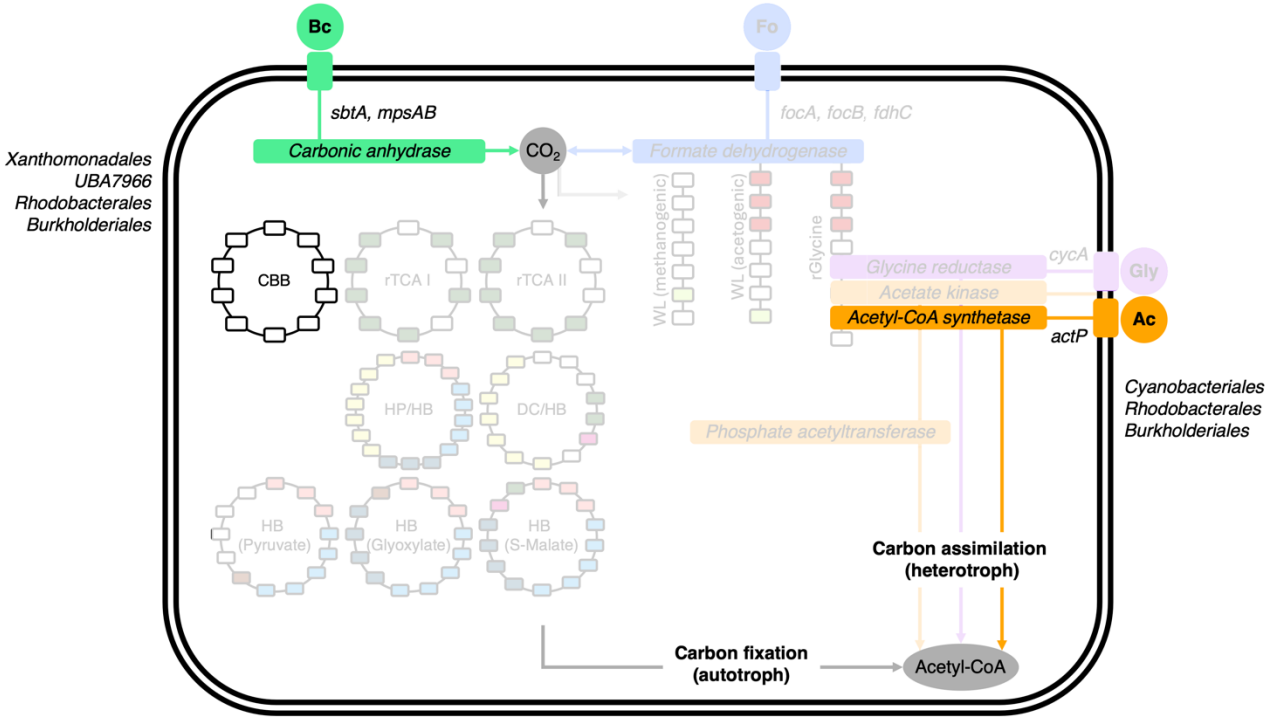


Figure 3-6. Proposed metabolism of bicarbonate-fixing autotrophic and acetate-assimilating heterotrophic foundation species in Prony Bay surface environments. The order of MAGs with a complete set of uptake genes is given next to the corresponding carbon source. Enzymes and pathways that are absent or poorly represented in the assessed metagenomes are faded out. Enzyme commission number sequences occurring in multiple carbon fixation pathways are color-coded.

Table 3-1. Characterization of MAGs that feature both a transporter and an assimilation enzyme for the same carbon source. Carbon fixation pathway completeness is given in percent.

	BdJ11			RdK14		ST0714
MAG	**Bin47_Cyanobacteriales**	**Bin26_Rhodobacterales**	**Bin27_Xanthomonadales**	**Bin113_UBA7966**	**Bin92_Burkholderiales**	**Bin53_Xanthomonadales**
Completeness	99%	97%	96%	95%	97%	97%
Contamination	0.41%	2.06%	0.24%	0%	1.37%	1.09%
Lowest tax. classification	g__RDYZ01	g__Roseinatronobacter	g__Silanimonas	s__UBA7966 sp003485715	g__JALHMQ01	f__Wenzhouxiangellaceae
Transporter + Assimilation	Acetate	Acetate, bicarbonate	Bicarbonate	Bicarbonate	Acetate, bicarbonate	Bicarbonate
Carbon fixation pathway	*CBB cycle (100%)*	CBB cycle (80%)	CBB cycle (70%)	CBB cycle (90%)	CBB cycle (70%)	CBB cycle (70%)
Hydrogen	Hydrogenase (NAD-reducing, bidirectional) | hoxH	absent	absent	Hydrogenase (aerobic, anaerobic uptake) | hyaB Hydrogenase (NAD-reducing, bidirectional) | hoxH	Hydrogenase (aerobic, anaerobic uptake) | hyaB Hydrogenase (NAD-reducing, bidirectional) | hoxH	absent
Carbon monoxide	absent	absent	absent	absent	Aerobic carboxydotrophy | coxL	
Methane	absent	absent	absent	absent	absent	absent
Nitrogen	absent	absent	absent	absent	Nitrate reduction | napA Nitrite reduction (cytoplasmic) | narG, nxrA Nitrite reduction (periplasmic) | nrfA	absent
Sulfur	absent	Sulfide oxidation | sqr Thiosulfate oxidation | soxY	Sulfide oxidation | sqr	Thiosulfate oxidation | soxY	Sulfite reduction | dsrA Sulfide oxidation | sqr	absent
Oxygen	Cytochrome aa3 | coxC	Cytochrome aa3 | coxC Cytochrome ccb3 | ccoN	Cytochrome aa3 | coxC Cytochrome ccb3 | ccoN	Cytochrome aa3 | coxC Cytochrome ccb3 | ccoN	Cytochrome aa3 | coxC Cytochrome ccb3 | ccoN	Cytochrome aa3 | coxC Cytochrome ccb3 | ccoN

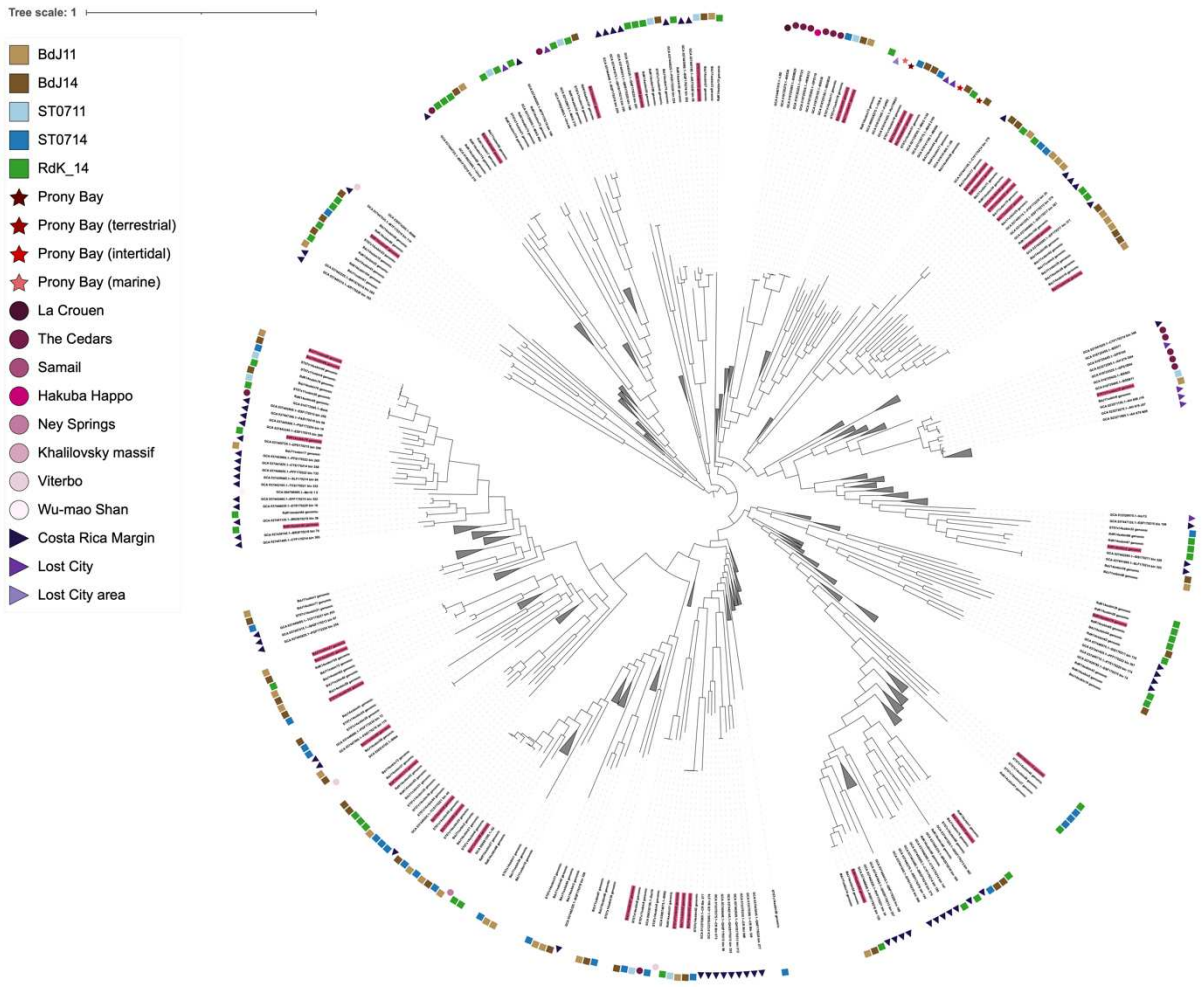
3.4 Supplementary material

Supplementary Table 3-1. Quality of metagenome-assembled genomes with a relative abundance > 1% in their respective sample

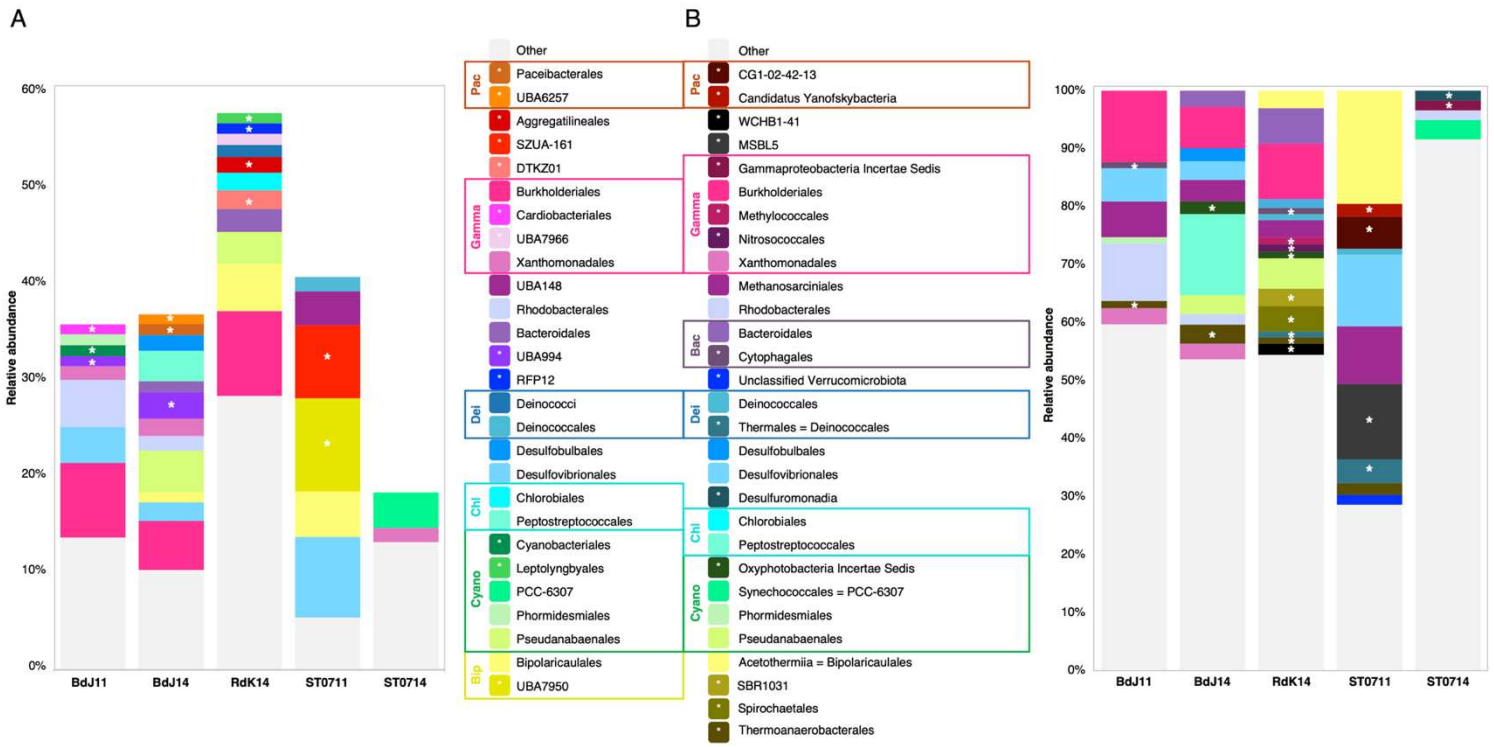
Sample	Bin	Completeness	Contamination	GC	GC std	Genome size	# scaffolds	# contigs	N50 (scaffolds)	N50 (contigs)
BdJ11	Bin26	96.76	2.06	0.63	0.02	2288419	136	137	26412	26412
BdJ11	Bin27	95.87	0.24	0.68	0.02	2180977	110	128	36277	28646
BdJ11	Bin30	93.64	1.49	0.53	0.02	4970448	529	610	15484	13756
BdJ11	Bin41	78.38	0.00	0.55	0.02	1414100	223	291	10333	6521
BdJ11	Bin46	93.59	0.76	0.63	0.01	3146158	134	139	43994	43994
BdJ11	Bin47	98.88	0.41	0.61	0.02	4302967	166	212	38136	31045
BdJ11	Bin52	94.46	0.00	0.58	0.03	1876963	212	290	12972	9401
BdJ11	Bin58	84.17	0.96	0.64	0.03	1725985	232	258	13589	10875
BdJ11	Bin66	97.51	0.00	0.46	0.01	1829966	12	45	369479	68898
BdJ14	Bin31	97.87	0.40	0.43	0.02	3143496	135	176	43192	36750
BdJ14	Bin33	97.94	0.48	0.43	0.02	3024324	33	41	253154	172844
BdJ14	Bin39	94.33	2.13	0.42	0.02	2763847	173	202	31219	28300
BdJ14	Bin50	84.82	0.00	0.55	0.04	1564664	239	276	10004	8390
BdJ14	Bin51	96.71	0.17	0.68	0.02	2181634	120	136	30908	29186
BdJ14	Bin52	77.17	0.00	0.58	0.03	1682288	347	381	5576	5167
BdJ14	Bin53	84.75	0.00	0.68	0.02	1176804	270	293	5307	4676
BdJ14	Bin57	91.47	0.00	0.54	0.02	1686203	122	167	21223	14663
BdJ14	Bin62	69.49	0.00	0.39	0.02	430703	43	57	12563	10767
BdJ14	Bin65	96.34	0.47	0.46	0.02	2608370	149	177	25347	21546
BdJ14	Bin66	90.19	0.82	0.64	0.02	1827381	206	233	15980	13476
BdJ14	Bin7	60.99	0.00	0.38	0.02	587974	54	61	19066	13993
BdJ14	Bin9	98.60	0.40	0.63	0.02	2465113	131	132	30543	30016
RdK14	Bin113	94.72	0.00	0.67	0.02	2660550	21	26	396610	290565
RdK14	Bin12	94.81	0.71	0.46	0.02	2333570	236	258	14386	13183
RdK14	Bin16	96.16	2.55	0.48	0.02	3191823	293	349	18095	14932
RdK14	Bin17	74.58	0.00	0.68	0.02	980799	207	221	5962	5240
RdK14	Bin2	95.91	0.00	0.49	0.02	3707834	191	280	33860	21365
RdK14	Bin44	95.95	1.67	0.41	0.02	2609184	177	215	23744	18148
RdK14	Bin49	98.29	0.85	0.71	0.01	2054532	65	86	55730	37474
RdK14	Bin76	82.80	4.57	0.72	0.02	3036489	668	701	5536	5073
RdK14	Bin78	85.14	1.66	0.69	0.02	2287843	425	486	7121	5727
RdK14	Bin92	96.71	1.37	0.70	0.02	3283849	238	247	21170	20286
RdK14	Bin97	98.85	1.15	0.61	0.02	2516976	108	146	41730	30388
RdK14	Bin98	97.41	0.00	0.50	0.02	4688838	341	348	21291	20606
ST0711	Bin11	64.06	0.65	0.47	0.02	682072	93	137	9692	6414
ST0711	Bin12	83.05	0.00	0.68	0.02	1063119	228	245	5939	5114
ST0711	Bin14	89.83	0.00	0.46	0.03	1111558	204	225	7763	6702
ST0711	Bin15	71.33	4.29	0.54	0.02	1143809	134	135	13473	13473
ST0711	Bin16	97.44	0.43	0.60	0.01	2884834	39	42	116943	102345
ST0711	Bin6	79.79	2.08	0.59	0.02	1584420	319	349	6629	5803
ST0714	Bin33	95.86	2.75	0.64	0.04	2099192	250	269	12012	11513
ST0714	Bin46	61.22	0.27	0.62	0.03	1688869	239	245	9589	9177
ST0714	Bin53	97.10	1.09	0.55	0.01	2581624	12	15	312953	197236

Supplementary Table 3-2. BioProject accession numbers of sequences from the National Center for Biotechnology Information database that were included in the phylogenomic tree.

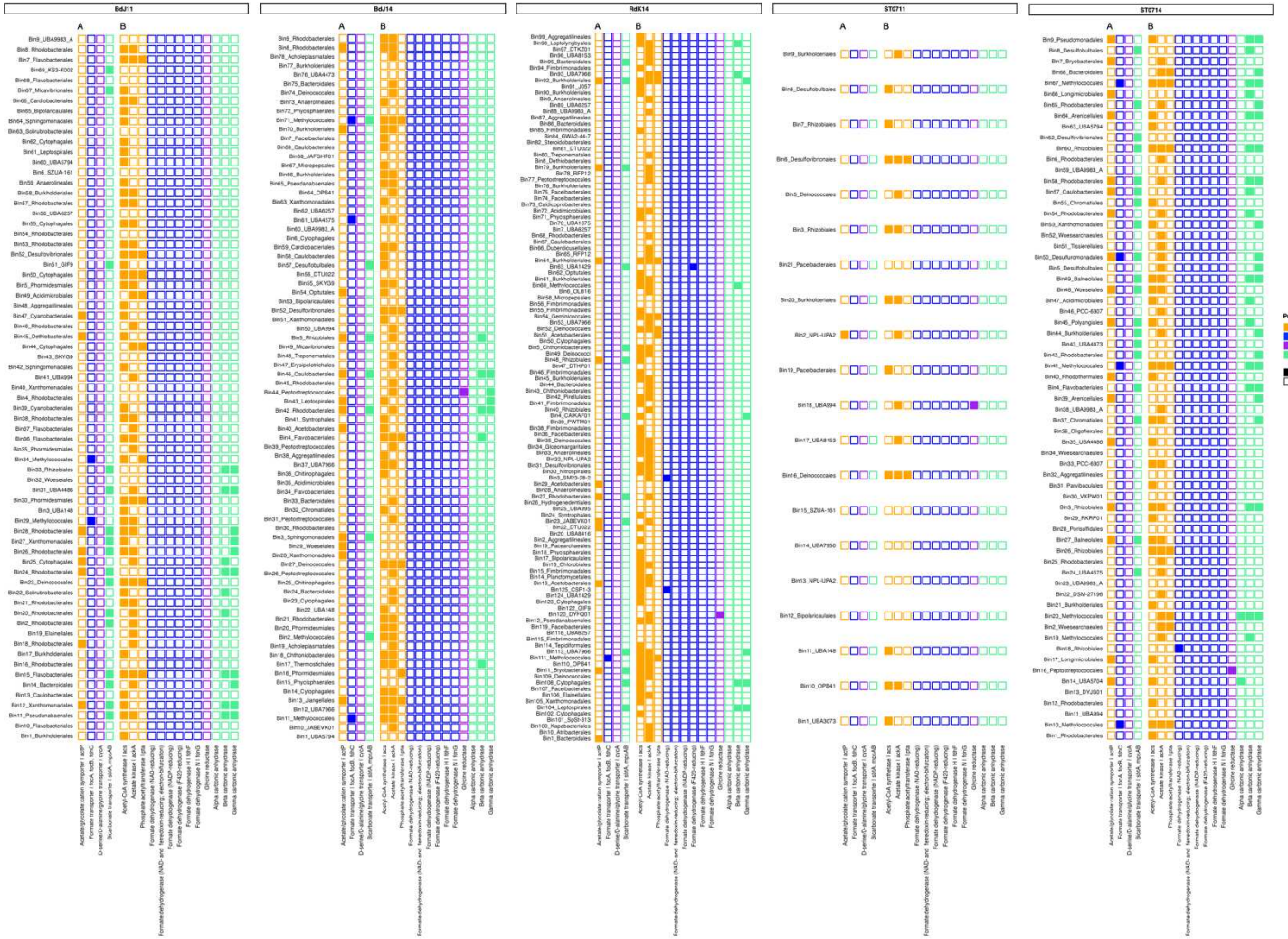
Assembly BioProject Accession	System	Number of Sequences
PRJNA599172	Costa Rica Margin	17
PRJNA627197	Costa Rica Margin	420
PRJNA453100	Hakuba Happo	6
PRJNA517583	Khalilovsky massif	2
PRJNA626556	La Crouen	4
PRJNA37895	Lost City	1
PRJNA577730	Lost City	3
PRJNA779602	Lost City	130
PRJEB28585	Lost City area	2
PRJNA945066	Ney Springs	6
PRJNA626556	Prony Bay	10
PRJNA738530	Samail	14
PRJNA930444	Samail	10
PRJDB2971	The Cedars	4
PRJNA351917	The Cedars	11
PRJNA383291	The Cedars	2
PRJNA453100	The Cedars	19
PRJNA746793	Viterbo	7
PRJNA431474	Wu-mao Shan	2



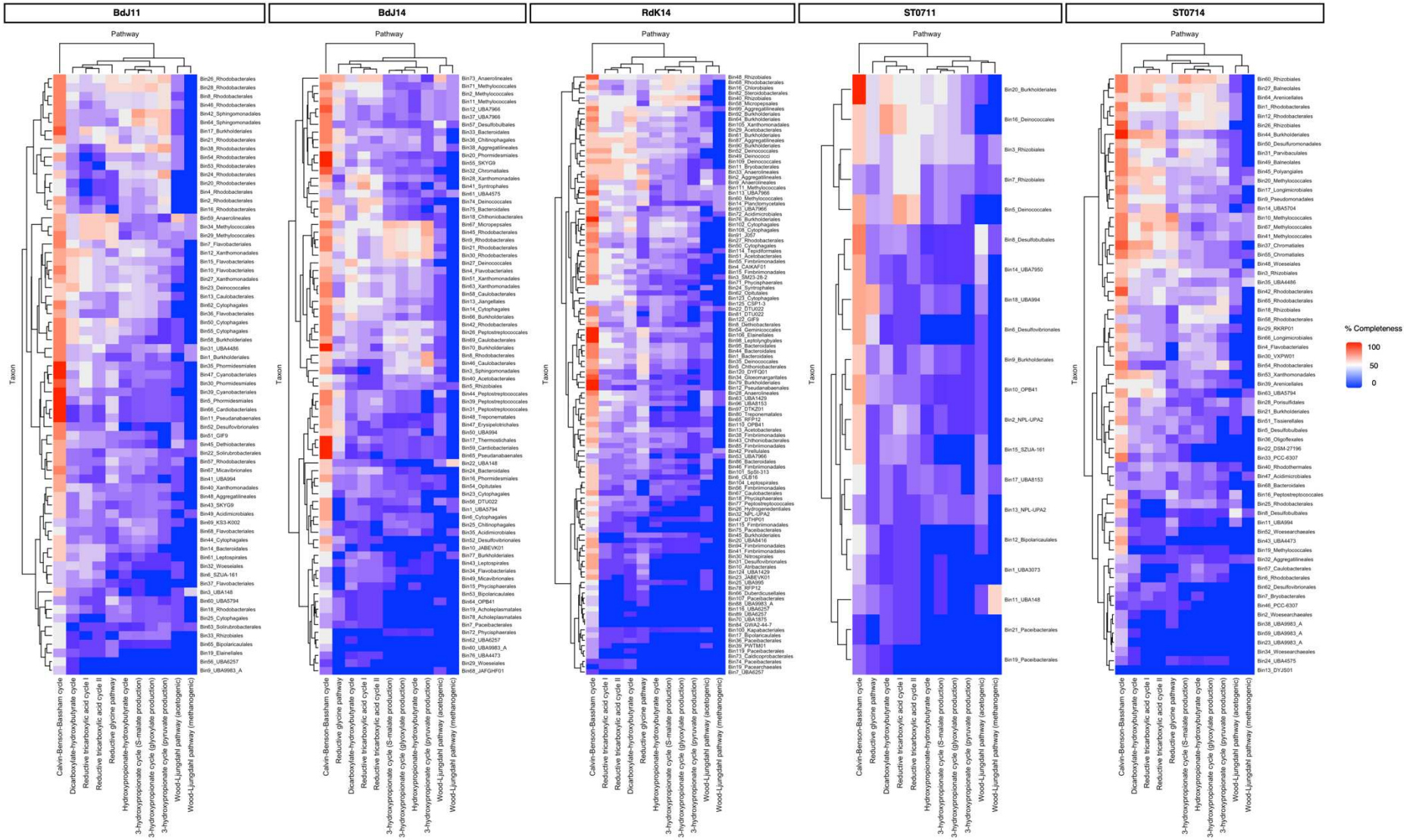
Supplementary Figure 3-1. Phylogenomic tree including all MAGs from this study and reference sequences from other Prony Bay metagenomes (stars in red shades), as well as other terrestrial (circles in pink shades) and marine (triangles in purple shades) serpentinite-hosted systems. MAGs from this study with a relative abundance >1% in their respective sample are highlighted in pink. Details of reference sequences are given in Supplementary Table 2. The tree is based on the bacterial marker gene set from GTDB R214 and rooted at midpoint. A preliminary interactive version is available here: https://itol.embl.de/tree/92184112185972617249304 63



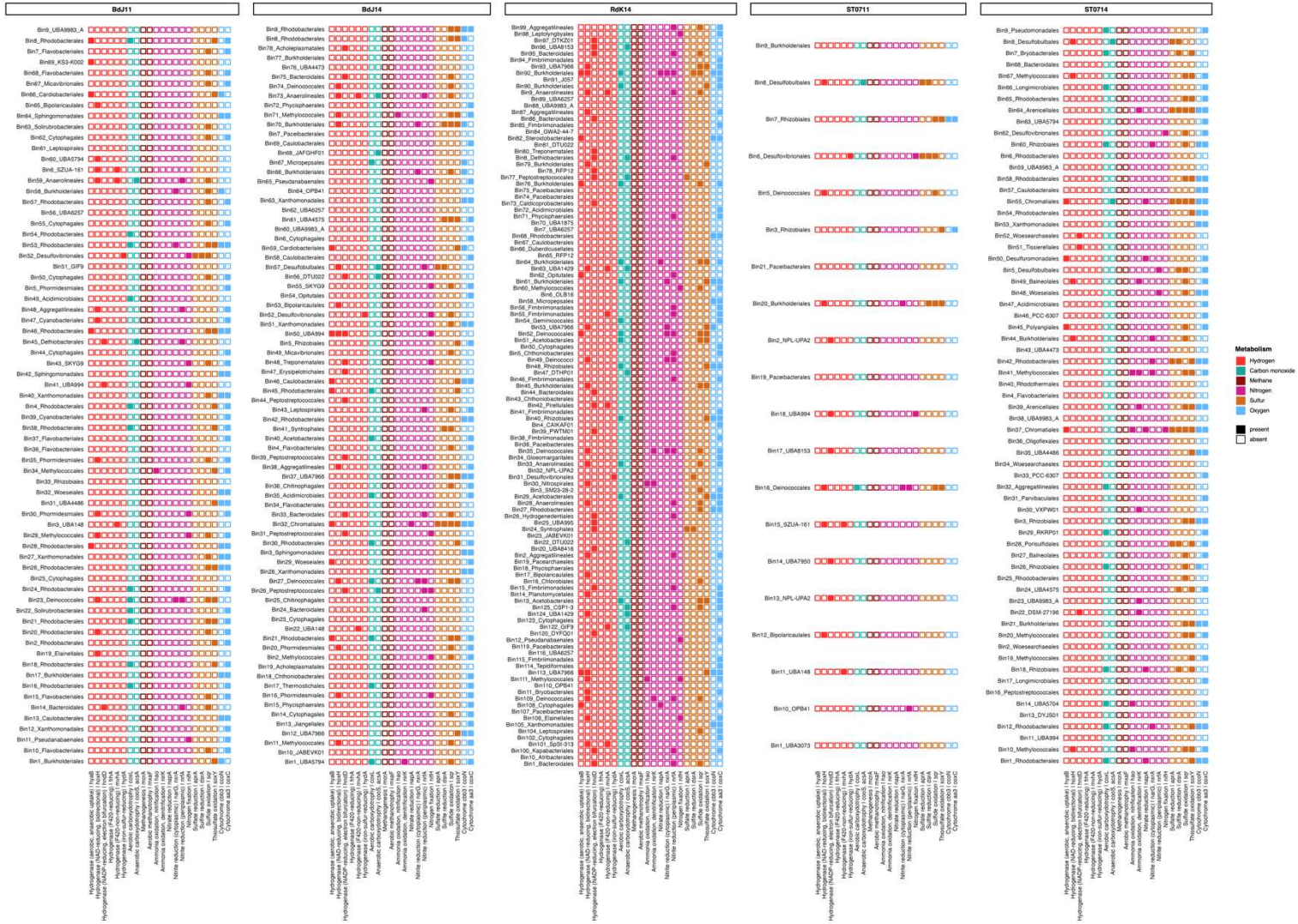
Supplementary Figure 3-2. Relative abundance of A) MAGs assigned to orders according to GTDB and B) 16S rRNA sequences assigned to orders according to SILVA-138. Orders with < 1% relative abundance are grouped under "other". Classes shared by at least two orders are indicated with abbreviations: Pac for *Paceibacteria* or *Parcubacteria*; Gamma for *Gammaproteobacteria*; Bac for *Bacteroidia*; Dei for *Deinococci*; Chl for *Chlorobia*; Cyano for *Cyanobacteriia*; Bip for *Bipolaricaulia*. Orders that occur only in the MAG or the 16S rRNA sequence data set are marked with stars. In A) note that the percentages of unmapped, thus unclassified, reads are the following: BdJ-11: 64%. BdJ-14: 63%. RdK-14: 42%. ST07-11: 59%. ST07-14: 82%. MAG identifiers from top to bottom are as follows (note that MAGs were reconstructed from individual assemblies, hence their numbering is not coherent between samples): BdJ11: Bin66, Bin30, Bin47, Bin41, Bin27, Bin26 and Bin46, Bin52, Bin58. BdJ14: Bin62, Bin7, Bin57, Bin31 and Bin39, Bin33, Bin50, Bin51, Bin9, Bin65, Bin52, Bin66. RdK14: Bin98, Bin78, Bin113, Bin49, Bin2, Bin16, Bin97, Bin44, Bin12, Bin17, Bin76 and Bin92. ST0711: Bin16, Bin11, Bin15, Bin14, Bin12, Bin6. ST0714: Bin33 and Bin46, Bin5



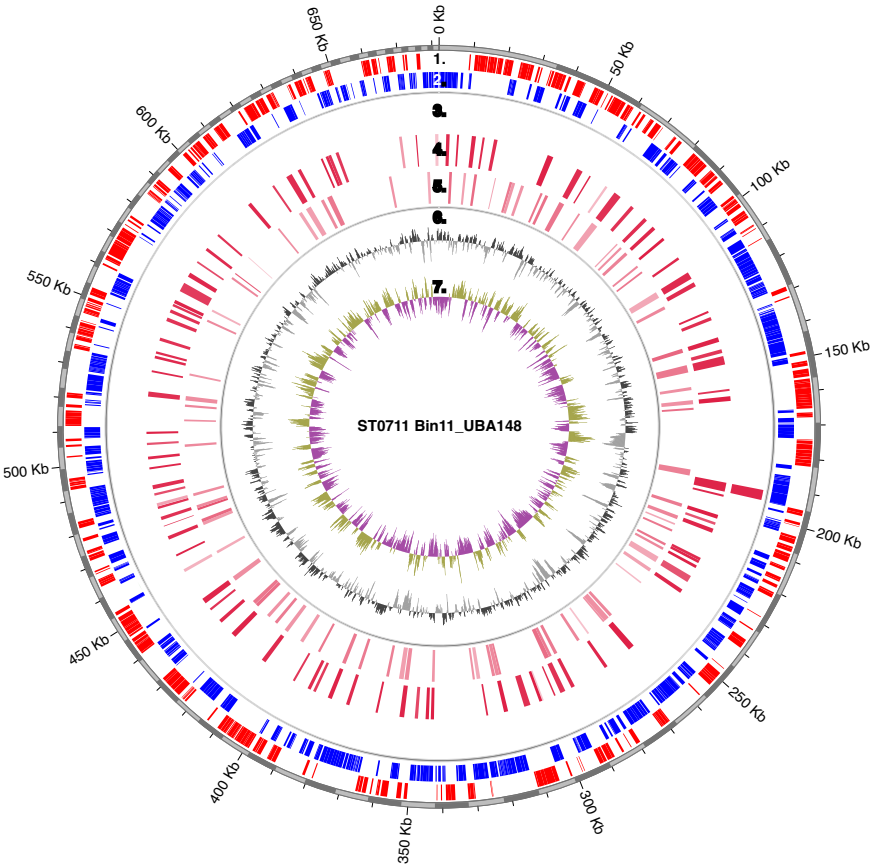
Supplementary Figure 3-3. Presence and absence of A) transporters and B) primary assimilation enzymes in all metagenome-assembled genomes per sample. The depicted proteins facilitate the primary uptake of acetate, formate, glycine or bicarbonate as putative carbon sources. Genes were annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) R109. Where no genes are specified, all available enzyme subdomains (all must be present) or alternatives (one of them must be present) were taken into account.



Supplementary Figure 3-4. Carbon fixation pathway completeness across all metagenome-assembled genomes per sample. Completeness scores were calculated based on custom HMM profiles and are given in percent.



Supplementary Figure 3-5. Presence and absence of key metabolic genes in all metagenome-assembled genomes per sample. Genes playing a crucial role in the metabolism of hydrogen, carbon monoxide, methane, nitrogen, sulfur or oxygen were annotated according to the Kyoto Encyclopedia of Genes and Genomes R109.



Supplementary Figure 3-6. Blastn results of the UBA148 (also called *Methanocellales*) MAG from ST0711 against the The Cedars *Methanocellales* MAG Met12 (GenBank accession CP017966.2), the Lost City *Methanosarcinales* MAG-838 (GenBank accession GCA_021129915.1) and The Cedars *Methanosarcinales* MAG Met13 (GenBank accession GCA_036677945.1). Outer to inner tracks show (1) Forward CDS (2) Reverse CDS (3) Met13 (4) MAG-838 (5) Met12 (6) GC content (7) GC skew.



CHAPTER 4
The cultivation approach

4.1 Preface

In chapter four, we explore the metabolic activity of microbial communities from ST07. The samples used in this chapter were collected during a one-month field campaign to Prony Bay, which was part of this thesis. Cultivation experiments were conducted on a bioreactor platform, which allowed the control of various environmental parameters such as pH, temperature, and gas supply. Four anoxic and hydrogenotrophic culture conditions were established, each alimented with either bicarbonate, formate, acetate or glycine as the sole carbon source. Carbon consumption, growth, diversity, and taxonomic composition were compared between the microbial consortia to determine which carbon sources facilitate the development of microbial communities in serpentinizing conditions and which taxonomic groups are enriched on them.

Contributions made in the frame of the thesis were sample collection, participation in experimental design, laboratory work, bioinformatical analysis, analysis of results and the writing of the manuscript. This included the supervision of a 6-month master internship, in the frame of which part of the laboratory work was conducted.

This chapter is presented in the form of a manuscript. It is currently revised by the authors and planned to be submitted before the PhD defense.

4.2 Manuscript

Cultivating hydrogenotrophic microbial communities from the serpentinite-hosted Prony Bay on different carbon sources

Rabja Maria Popall[1], Agathe Roland[1], Sylvain Davidson[1], Yannick Combet-Blanc[1], Anne Postec[1] and Gaël Erauso[1]

[1]Aix-Marseille Univ, Univ Toulon, CNRS, IRD, MIO UM 110, Marseille, France

*** Correspondence:**
Gaël Erauso
gael.erauso@mio.osupytheas.fr

Keywords: serpentinization, alkaline hydrothermal system, alkaliphile, hydrogenotroph, primary production, carbon fixation, origin of life.

Abstract

The primary source of carbon is one of the most fundamental questions regarding the development of microbial communities in serpentinite-hosted systems. The hydration of ultramafic rock to serpentinites creates hyperalkaline conditions, depleting the environment of dissolved inorganic carbon. Metagenomic studies suggest that serpentinite-hosted microbial communities rely on the local redissolution of bicarbonate instead, as well as on small organic molecules produced in abiotic reactions linked to serpentinization. To test these hypotheses, we cultivated anaerobic and hydrogenotrophic microbial consortia from the Prony Bay hydrothermal field on bicarbonate, formate, acetate or glycine as the sole carbon source. While glycine did not support growth, microbial consortia characterized by a significant enrichment of individual taxa were obtained on the remaining three carbon compounds. This included a strong enrichment of normally aerobic taxa under strictly anaerobic conditions. We propose that an intricate feedback loop between autotrophic and heterotrophic foundation species facilitates the establishment of serpentinite-hosted shallow subsurface ecosystems. Bicarbonate-fixing *Meiothermus* and *Hydrogenophaga*, as well as formate-fixing *Meiothermus*, *Thioalkalimicrobium* and potentially a novel genotype of *Roseibaca* might produce organic compounds for heterotrophs on the first trophic level. Furthermore, the base of the trophic network may include heterotrophic *Roseibaca*, *Acetoanaerobium* and *Meiothermus* species producing CO_2 from acetate for a more diverse community of autotrophs. The cultivated archaeal community is expected to cycle CH_4 and CO_2 between *Methanomicrobiales* and *Methanosarciniales* with putative *Woesearchaeales* symbionts.

Introduction

Hydrothermal vents driven by serpentinization are a window into the past, possibly all the way back to the origin of life. Serpentinization, the hydration of exposed mantle rock, creates geochemical conditions that most likely characterized early Earth (Russell et al., 2010). Most modern organisms cannot survive in these conditions, except for specialized microbial communities that grow independently of oxygen (O_2). Elucidating their metabolism may be key to the first life forms and the current limits of life (Sojo et al., 2016).

The hydration of mantle rocks to serpentinites produces hydrothermal fluids enriched in hydroxide ions and hydrogen (H_2). While the released H_2 constitutes a potent energy source for microbial metabolism, the elevated pH poses a fundamental energetic challenge to life. Firstly, it inverts the cell membrane potential, requiring particular adaptations to maintain the energetic driving force of the cell (McCollom and Seewald, 2013). It has been shown that some alkaliphiles use Na^+ instead of proton gradients or can concentrate protons at the cell surface (Suzuki et al., 2014). Secondly, the elevated pH reduces the solubility of essential nutrients such as phosphorous (Frouin et al., 2022) and dissolved inorganic carbon (DIC). The latter is usually vital for primary production, which constitutes the base of the trophic network. In hyperalkaline environments, however, carbon dioxide (CO_2) precipitates as calcium carbonate ($CaCO_3$), forming carbonaceous hydrothermal chimneys over time and removing DIC from the pool of bioavailable carbon (Schrenk et al., 2013). This phenomenon raises one of the main questions regarding the functioning of serpentinite-hosted microbial ecosystems: which carbon source can replace CO_2 in primary production?

Various small organic acids and amino acids produced abiotically by serpentinization may hold the key to this issue. Formate and acetate are yielded in Fischer-Tropsch and Sabatier-type reactions catalyzed in the hydrothermal fluid (Brazelton et al., 2011; McCollom and Seewald, 2013; Fones et al., 2021). Similarly, serpentinizing conditions have been shown to facilitate the abiotic synthesis of glycine (Ménez et al., 2018; Nobu et al., 2022). Formate, acetate and glycine concentrations vary widely between hydrothermal systems (Brazelton et al., 2011; Rempfert et al., 2017), but all three compounds are likely ubiquitous in serpentinite-hosted environments. In addition, some serpentinite-hosted microorganisms have been shown to grow on solid $CaCO_3$. The physiological mechanism they employ probably involves the local redissolution of bicarbonate ions (Suzuki et al., 2014). For organisms capable of such redissolution, the carbonaceous hydrothermal chimneys could provide a vast carbon source.

By definition, primary producers are autotrophic and use an inorganic carbon source that is converted into more complex carbohydrates via a carbon fixation pathway. For this reason, acetate and glycine in particular, but also formate, are not usually considered as primary carbon sources. Due to the lack of DIC, however, the concept of primary production in serpentinite-hosted ecosystems might need to be reconsidered. This is supported by the fact that all of the compounds mentioned above are derived from an abiotic source, which *per se* challenges the definitions of heterotrophy and autotrophy (Schönheit et al., 2016).

In this context, formate and bicarbonate can be interpreted as autotrophic carbon sources. Once accumulated in the pH-neutral cytoplasm, they are both converted into CO_2 by the formate dehydrogenase or the carbonic anhydrase, respectively (Smith and Ferry, 2000; Brazelton et al., 2022). The yielded CO_2 can then be used in any of the seven known carbon fixation

pathways to form acetyl-CoA or other precursors for respiration, fermentation, and heterotrophic metabolism. On the contrary, acetate and glycine cannot be metabolized through a canonical carbon fixation pathway and thus constitute heterotrophic carbon sources. Acetate is directly converted to acetyl-CoA by the acetyl-CoA synthetase or the acetate kinase and phosphate acetyltransferase (Brazelton et al., 2017; Sánchez-Andrea et al., 2020), while glycine is reduced to acetyl phosphate by the glycine reductase complex. The only known exception to this heterotrophic route is syntrophic acetate oxidation (SAO), where a symbiotic bacterium oxidizes acetate to CO_2 and H_2 or formate for an autotrophic methanogen or sulfate-reducer. However, SAO has only been described in a handful of taxa (Timmers et al., 2018). While heterotrophs are not typically regarded as primary producers, they might play a more fundamental role in the serpentinite-hosted trophic network than elsewhere. Lang et al. (2018) proposed that heterotrophic foundation species could produce CO_2 for a diverse community of autotrophs, including those without formate or bicarbonate uptake genes. Acetate- and glycine assimilating heterotrophs could thus be as essential for the trophic chain as bicarbonate- or formate-fixing autotrophs.

In the last decade, the metabolic potential of these hypothetical primary producers has mainly been explored through metagenomic approaches, leading to some exciting discoveries of novel routes for non-canonical carbon fixation pathways and hypotheses on ecosystem functioning (Suzuki et al., 2014; Brazelton et al., 2017, 2022; Nobu et al., 2022). However, many of the concerned enzymes are bidirectional or have other metabolic functions, rendering it difficult to evaluate such hypotheses based on metagenomic predictions alone. This requires experimental evidence, for example by observing the metabolic activity of targeted communities in culture. However, the cultivation of serpentinite-hosted communities has proven problematic, mainly because the physicochemical conditions associated with serpentinization are challenging to reproduce in the laboratory. Previous attempts have focused on microcosm experiments, but produced contradictory results: While Kohl et al. (2016a) observed methane (CH_4) production from bicarbonate, formate and acetate in microbial communities from The Cedars ophiolite, the same experiment failed with microbial communities from the Tablelands ophiolite (Morrill et al., 2014). To bridge this gap, we performed cultivation experiments on natural serpentinite-hosted communities in controlled laboratory conditions. These communities were recovered from the shallow Prony Bay hydrothermal field (Monnin et al., 2014) and enriched on a bioreactor platform, which allowed the control of various environmental parameters such as pH, temperature, and continuous gas supply. Four hydrogenotrophic culture conditions were established, each alimented with bicarbonate, formate, acetate or glycine as the sole carbon source. Carbon consumption, growth, diversity, and taxonomic composition were compared between the microbial consortia to determine which carbon sources facilitate the development of microbial communities in serpentinizing conditions and which taxonomic groups are enriched on them.

Material and methods

Study site

The shallow Prony Bay hydrothermal field is located at the southern end of New Caledonia. As a coastal system, it comprised several sites from land to sea that reflect the geomicrobiological characteristics of terrestrial and marine serpentinite-hosted systems, respectively (Quéméneur et al., 2014; Postec et al., 2015; Frouin et al., 2018, 2022; Trutschel et al., 2022). The most prominent hydrothermal mount is the "Aiguille de Prony", a large carbonate massif (38 m high from its base at a depth of around 45 m, depending on the height of the tide, to its summit). The most active zone lies at a depth of 20 meters, where numerous chimneys emit hydrothermal fluid. The concentration of DIC measured in the fluid remains below 1 mmol L^{-1}, with pH values ranging from 10 to 11.3, Eh values ranging from -540 to -680 mV, and a temperature of around 35°C measured in the endmember fluid (Monnin et al., 2014, 2021).

During a field campaign in 2022, the submittal part (~50 cm long and max 20 cm in diameter) of a mature, active chimney was sampled. The chimney was covered with a watertight bag, letting the hydrothermal fluid accumulate for a few hours until the ambient seawater was entirely removed. The chimney was then sawed off, the bag hermetically sealed and transported to the field laboratory. The chimney was then divided into slices of approximately 35 mm under sterile conditions. The outer layer was removed with a chisel to isolate the center of each slice. This part was ground with mortar and pestle in an anaerobic chamber. The resulting slurry was aliquoted into hermetically sealed Schott bottles and stored under a nitrogen (N_2) atmosphere at 4-8°C until inoculation of cultures.

Experimental set-up

Experiments were carried out in sterile, non-opaque double-jacketed glass bioreactors (Pierre Guerin, France) with a working volume of 1.5 L. The cultures were agitated at 500 rpm, the temperature was continuously montitored by a probe (Prosensor pt 100, France) and maintained at 35 ± 2 °C via hot water circulation in the double jacket via a thermostat (Julabo, SE6, Switzerland). The bioreactors were supplied with a continuous flow of H_2 and N_2 gas at 5 ml min^{-1} each by two thermal mass flow meters (Bronkhorst el flow, Netherlands) to maintain anaerobic conditions and provide a metabolic energy source. The outflow gas effluent was condensed using cold water from a cryostat (Julabo F25, switzerland) to prevent evaporation of the culture medium. The pH was continuously measured by a probe (Mettler Toledo InPro 3253, Switzerland) and maintained at 10.5 ± 0.5 by automatic addition of 1M NaOH with a peristaltic pump. All equipment was connected to a Wago PLC (France) via a serial link (RS232/RS485), 4–20 mA analog loop or a digital signal. The PLC was connected to a computer for process monitoring and data acquisition (batchPro software, Decobecq Automatismes, France).

Four culture conditions were inoculated with 35 ± 5 g of chimney slurry and supplied with 5 mM of sodium acetate, sodium formate, glycine, or sodium bicarbonate as the sole carbon source. The cultures were performed in fed-batch mode using 1L of minimal growth medium, which was inspired by the natural fluid composition of Prony Bay (Monnin et al., 2014): 3.7 mM NH_4Cl, 0.92 mM K_2HPO_4, 86 mM NaCl, 12 mM $MgCl_2(6H_2O)$, 5 mM $Na_2S_2O_3(5H_2O)$, 5

mM Na_2SO_4, 3 mM $CaCl_2(2H_2O)$, 5 mL L^{-1} Balch's trace element solution (Balch et al., 1979), 0.4 mL L^{-1} Balch's vitamin solution (Balch et al., 1979) and 1 mL L^{-1} trace metal solution, the latter containing 1.42 g L^{-1} $FeSO_4$, 1.6 g L^{-1} $NiSO_4$ $(6H_2O)$, 38 mg L^{-1} $Na_2WO_4(2H_2O)$ and 3 mg L^{-1} $Na_2SeO_3(5H_2O)$ at a concentration of 1000 fold. Sulfate and thiosulfate were incorporated in the growth media as electron acceptors for anaerobic respiration (thiosulfate can also be used as an energy source).

Each culture condition was tested twice: The first experimental run included the cultures Ac1, Fo1, G1, Bc1 and the second experimental run the cultures Ac2, Fo2, G2, Bc2. All cultures were maintained for 25 or 26 days and sampled every 2-3 days. Samples were immediately subdivided and prepared for different types of analysis.

Biochemical analysis

The consumption of acetate and formate was monitored via high-performance liquid chromatography. Samples were centrifuged at 7000 rpm for 30 min at 4°C. Subsequently, 20 µL of the recovered supernatant was injected into the column at 35°C (Dionex Ultimate 3000 from Thermofisher Scientific, USA) and eluted at 0.6 mL min^{-1} with a solution of 2.5 mM H_2SO_4. In addition, the consumption of sulfate and thiosulfate as electron acceptors was measured via ion chromatography. 10 mL of 1:10 diluted supernatant was injected into the column (Dionex AS-DV from Thermofisher Scientific, USA) and eluted at 1 mL min^{-1} with a 9 mM buffer solution of Na_2CO_3 and $NaHCO_3$.

Culture growth

The number of cells was monitored via fluorescence microscopy. Cells were fixed with formaldehyde (final concentration in sample 2%) and diluted with sterile growth media according to the cellular density. Subsequently, 2 ml of diluted sample was filtered onto white polycarbonate filters (0.2 µm porosity and 25 mm diameter from Nucleopore Whatman, USA) using a vacuum pump and sterile growth medium. After filtration, the polycarbonate filters were placed on microscope slides for staining with 10-20 µL of 3.15 µM DAPI (diluted in mounting solution from CitiFluorTM AF1 Electron Microscopy Sciences, USA). Cells were counted in 15 random fields using an OLYMPUS BX-61 microscope with a filter at 372 nm (Evident, Japan). Samples were analyzed in duplicates.

Alpha and beta diversity

The taxonomic composition at three different time points per culture (on the first day, in the middle, and on the last day), plus the inoculum of each experimental run, was assessed via 16S rRNA analysis. Samples were centrifuged at 7000 rpm and 4°C for 30 min. The pellet was recovered, and the DNA was extracted following the standard protocol of the DNAeasy PowerSoil Pro kit (Qiagen, USA). The yielded DNA quantity and quality were evaluated on a NanoDrop One (Thermofisher Scientific, USA) and a Qubit 2.0 Fluorometer (Thermofisher Scientific, USA). Amplification and Illumina MiSeq sequencing of the 16S rRNA V3-V4 region was performed on the sequencing platform of MrDNA (Texas, USA) with two primer pairs targeting bacteria (341F: CCTACGGGNBGCWSCAG and 805R:

GACTACNVGGGTATCTAATCC) and archaea (344F: AYGGGGYGCASCAGGSG and 806R: GGACTACVSGGGTMTCTAAT) respectively.

The bioinformatic analysis was performed in R and separately for each primer pair. Filtering and trimming of demultiplexed reads were performed in DADA2 (Callahan et al., 2016), and samples with a low number of reads (< 180) were removed. Denoised and dereplicated reads were merged into ASVs, allowing no mismatches in the overlapping region of forward and reverse reads. Taxonomic assignment of ASVs was performed based on the SILVA database version 138.1 (Quast et al., 2013) with a minimum bootstrap value of 60. The bacterial data set was rarefied to the minimum number of reads (11420) (Supplementary Figure 2A). The archaeal data set contained many bacterial ASVs, which we pruned to avoid bias in archaeal community analysis. Subsequently, the archaeal data set was rarefied to a depth of 12% (721 reads) (Supplementary Figure 2B), preserving 88% of samples left after the initial quality control in DADA2 (Callahan et al., 2016). Alpha diversity indices were calculated using the microbiome package (Lahti and Shetty, 2017) and the Microbiota Process package (Xu et al., 2023). Subsequently, a centered log-ratio transformation was performed on the read counts using the zCompositions package (Palarea-Albaladejo and Martín-Fernández, 2015). On the transformed data, an Aitchison distance matrix (k = 5) was constructed for both bacterial(Supplementary Figure 3A) and archaeal (Supplementary Figure 3B) sequences via non-metric multidimensional scaling (NMDS) using the vegan package (Oksanen et al., 2024). Permutational multivariate analysis of variance (PERMANOVA) tests were conducted to test the effect of carbon source, sampling timepoint, and experimental run on the observed sample variation. To ensure the validity of this analysis, a combined PERMANOVA model was calculated on the interaction of carbon source and sampling timepoint to inspect whether the data's time series structure significantly affected the observed sample variation and whether it was reasonable to assess sample variability with separate models. Furthermore, analysis of variance (ANOVA) tests was performed to verify if observed differences in distance were confounded by differences in dispersion amongst groups. To identify which ASVs were driving the individual variables' effects on sample variation the most, coefficients for the respective variable groups were extracted from the PERMANOVA model using the adonis function of the vegan package (Oksanen et al., 2024). ASV relative abundance was calculated with the phyloseq package by normalizing ASV read counts to total sample read counts (McMurdie and Holmes, 2013).

Results

Cellular net growth was highest in the microbial consortia grown on acetate (the log mean number of cells mL^{-1} was 9.31E+06 in Ac1 and 1.53E+06 in Ac2), followed by formate (3.27E+06 in Fo1 and 7.53E+05 in Fo2), bicarbonate (1.57E+06 in Bc1 and 4.59E+05 in Bc2) and lastly glycine which did not feature much growth (1.04E+06 in G1 and 1.58E+05 in G2). In the second run, net growth was reduced by an approximate factor of ten (Figure 1) and the consumption of acetate and formate was much slower (net consumption of acetate amounted to 4.65 mM in Ac1 and 1.23 mM in Ac2, net consumption of formate to 3.27 mM in Fo1 and 1.57 mM in Fo2) (Figure 1).

Overall, the number of bacterial taxa (853 ASVs before and 838 ASVs after rarefaction) was much higher than that of archaeal taxa (71 ASVs before and 66 ASVs after rarefaction).

Bacterial alpha diversity was highest in the inoculum samples (n = 2, mean number of ASVs = 315, mean Shannon index = 0.95), followed by the microbial consortia grown on glycine (n = 5, mean number of ASVs = 215.8, mean Shannon index = 0.8); it was lower in the consortia grown on formate (n = 6, mean number of ASVs = 80.5, mean Shannon index = 0.61) and bicarbonate (n = 6, mean number of ASVs = 76.17, mean Shannon index = 0.69), followed by the consortia grown on acetate (n = 6, mean number of ASVs = 72.5, mean Shannon index = 0.47). The evenness of bacterial taxa was highest in the inoculum (mean Pielou index = 4.17), followed by the microbial consortia grown on glycine (mean Pielou index = 2.93). Like the alpha diversity, it was lower in the consortia grown on bicarbonate (mean Pielou index = 2.07) and formate (mean Pielou index = 1.86), and finally acetate (mean Pielou index = 1.54) (Figure 2A). Archaeal alpha diversity was highest in the microbial consortia grown on glycine (n = 5, mean number of ASVs = 12.4, mean Shannon index = 0.79), followed by the inoculum (n = 2, mean number of ASVs = 10, mean Shannon index = 0.76); it did not differ much between the consortia grown on formate (n = 6, mean number of ASVs = 8.2, mean Shannon index = 0.75), bicarbonate (n = 6, mean number of ASVs = 8.4, mean Shannon index = 0.72), and acetate (n = 6, mean number of ASVs = 6.6, mean Shannon index = 0.75). Evenness of archaeal taxa was highest in the inoculum (mean Pielou index = 1.75) and the consortia grown on glycine (mean Pielou index = 1.74), followed by the consortia grown on formate (mean Pielou index = 1.63), acetate (mean Pielou index = 1.52) and bicarbonate (mean Pielou index = 1.5) (Figure 2B).

Bacterial beta diversity showed considerable overlap between bacteria (Figure 3A) and archaea (Figure 3B) of the different microbial consortia. Nevertheless, significant differences in bacterial variation were observed by NMDS. According to PERMANOVA tests, the carbon source explained 29.12 % of this variation ($F = 2.053$, $p = 0.009$), the time of sampling 22.3 % ($F = 2.01$, $p = 0.023$), and the batch number 14.45 % ($F = 1.859$, $p = 0.048$). The interaction between carbon source and sampling time was insignificant ($F = 0.25$, $p = 0.154$), with the individual effect of both variables remaining significant in the combined model, validating the statistical model used. Two ASVs associated with the *Rhodobacteraceae* (one classified to the order level as *Roseibaca*) were the most significant drivers of sample dissimilarity by carbon source, indicated by their relatively high PERMANOVA coefficients ($ASV_{289} = 2.459$, $ASV_{004} = 2.212$). For the sampling time, an ASV associated with *Meiothermus* was the most significant driver of sample dissimilarity ($ASV_{003} = 3.208$). For the batch number, the most critical drivers of sample dissimilarity were two ASVs associated with *Pseudahrensia* and the *Dethiobacteraceae,* respectively ($ASV_{345} = 4.325$, $ASV_{119} = 4.286$). ASVs driving sample similarity in both carbon source and sample time were associated with the cluster of uncultivated MSBL5 *(Dehalococcoidia)* (carbon source: $ASV_{042} = -2.76$, sampling time: $ASV_{006} = -3.99$), *Acetothermia* (carbon source: $ASV_{011} = -2.4$, sampling timepoint: $ASV_{005} = -3.09$ and $ASV_{011} = -3.23$), *Desulfovibrio* (carbon source: $ASV_{016} = -2.09$, sampling timepoint: $ASV_{016} = -2.78$) and ML635J-40 aquatic group *(Bacteroidales)* (carbon source: $ASV_{007} = -2.11$, sampling timepoint: $ASV_{007} = -2.95$) taxa (Figure 4A). For the archaea, the carbon source and batch number had no significant effect on the distance matrix ($F = 1.229$, $p = 0.15$ with $R^2 = 0.197$ and $F = 1.183$, $p = 0.26$ with $R^2 = 0.097$ respectively). Sampling time slightly affects community composition, explaining only 8.2 % of the variance ($F = 1.558$, $p = 0.053$). The most significant driver of sample dissimilarity according to the sampling time was an ASV identified as *Woesearchaeales* ($ASV_{037} = 1.01$). Drivers of sample similarity were ANME-3

(ASV_{007} = -2.93, ASV_{019} = -2.88, ASV_{003} = -1.4, ASV_{039} = -1.37, ASV_{045} = -1.36, ASV_{002} = -1.24) and *Syntrophoarchaeaceae* (ASV_{006} = -4.33, ASV_{034} = -1.76, ASV_{061} = -1.23) which are both associated with the *Methanosarcinales* (Figure 4B). All ANOVA tests were non-significant, confirming the reliability of the variables' effects on observed differences in bacterial and archaeal distance matrices.

The evolution of the taxonomic composition over time in the different microbial consortia was consistent with alpha and beta diversity predictions. On all carbon sources except glycine, the bacterial community changed significantly over time, and enrichment of specific taxonomic groups was observed (Figure 5A). The inoculum and samples from the first day of culture were mainly composed of the genera *Acetoanaerobium* and *Roseibaca* and a relatively smaller proportion of *Methyloceanibacter*, *Truepera*, *Desulfovibrio*, *Caldicobacter*, *Filomicrobium,* and *Ruegeria*. On acetate, a large proportion of *Roseibaca* and a small proportion of *Halomonas* were enriched after 12 (Ac2) and 14 (Ac1) days, respectively. The strong enrichment of *Roseibaca* was maintained until the end of the culture, next to a lesser enrichment of *Meiothermus* (Ac1 and Ac2). At the end of the culture on formate, a strong enrichment of *Roseibaca* (Fo1 and Fo2) and a slighter enrichment of *Meiothermus* (Fo1) were also observed, along with a small proportion of *Thioalkalimicrobium* (Fo1). On day 12 of the culture, the community was dominated by *Acetoanaerobium* (Fo2). On bicarbonate, the opposite was observed: A strong enrichment of *Meiothermus*, and a less pronounced (re-)enrichment of *Roseibaca* at the end of the culture (Bc1 and Bc2), next to a considerable enrichment of *Hydrogenophaga* (Bc1). On day 7, the culture was dominated by *Halomonas* next to a slight enrichment of *Thioalkalimicrobium* and *Reinekea* (Bc1)*,* and on day 13, *Roseibaca* next to a slight enrichment of *Halomonas* (Bc2). Little development was observed on glycine: the community structure resembled the inoculum throughout the experiment in both runs.

The archaeal community (Figure 5B) was strongly dominated by the order *Methanosarcinales* (ASV classified to the genus level as ANME-3) in the inoculum, next to a small proportion of *Nitrosopumilales* (INOC2), and in all culture samples except for the end of the culture grown on bicarbonate, where a strong enrichment of *Methanomicrobiales* was observed (Bc2). In addition, a slight enrichment of *Woesearchaeales* was observed in the middle and at the end of the culture grown on acetate (Ac1 and Ac2) and formate (Fo1), as well as on day 7 of the culture grown on bicarbonate (Bc1). They were also present at the beginning (G2), middle, and end (G1) of the culture grown on glycine, but their relative abundance decreased over time.

Discussion

The natural community

The natural bacterial community was dominated by several taxonomic groups that are commonly found in Prony Bay and other serpentinite-hosted environments. *Acetoanaerobium* is an anaerobic bacterium that can produce acetate from the fermentation of complex carbohydrates; it is one of the strains that has been isolated from Prony Bay (Bes et al., 2015). Close relatives of *Desulfovibrio*, an anaerobic sulfate-reducing bacterium, have been found in Prony Bay, at the serpentinized Costa Rica Margin and in Lost City (Postec et al., 2015;

Brazelton et al., 2022; Rogers et al., 2023). Likewise, the facultatively anaerobic chemoorganotroph *Truepera* has been documented in Prony Bay and in the Coast Range ophiolite (Frouin et al., 2018; Sabuda et al., 2021). None of these marker taxa were significantly enriched in any of the culture conditions, suggesting that they rely on lower trophic levels.

Carbon sources of cultivated microbial consortia

All carbon sources facilitated culture growth and development except for glycine. The microbial consortium maintained on glycine featured a very low growth rate (Figure 1), and diversity and evenness scores remained consistently elevated (Figure 2), suggesting that there was no significant enrichment of taxonomic groups that might be specialized on the uptake of this compound. This was confirmed by the taxonomic composition of the consortia, which did not change much over time and remained similar to the natural community (Figure 5). The use of glycine as a carbon source in a serpentinization context was proposed by Nobu et al. (2022), who showed that glycine reductase genes are abundant in metagenomes from various serpentinite-hosted systems. The glycine reductase complex is a central enzyme in the reductive glycine pathway, a recently proposed seventh carbon fixation pathway (Sánchez-Andrea et al., 2020). While it is indeed possible for exogenous glycine to enter the glycine reductase complex via a membrane-bound transporter, it might also be produced as an endogenous intermediate in the reductive glycine pathway. Similar to the other carbon fixation pathways, the reductive glycine pathway starts with one molecule of CO_2 that could be yielded from both formate (Smith and Ferry, 2000) and bicarbonate (Smith and Ferry, 2000; Brazelton et al., 2022), which would be the primary carbon source in this case. It is likely that in Prony Bay, formate and bicarbonate are thermodynamically more favorable carbon sources than glycine, whose natural concentration is expected to be low (Nobu et al., 2022). This might explain the low growth observed in this experimental condition.

Contrary to glycine, significant growth was observed in cultures grown on bicarbonate, formate and especially acetate (Figure 1). This was accompanied by a steep decrease in diversity and evenness (Figure 2), suggesting the enrichment of specific taxonomic groups on these carbon sources. This is confirmed by the evolution of the taxonomic composition of the cultures after the second and third week of incubation (Figure 5). The main driver of sample dissimilarity according to carbon source was the *Roseibacaceae* family (Figure 3, Figure 4).

Dominant taxa of cultivated microbial consortia

The consortia grown on acetate and formate were heavily dominated by the genus *Roseibaca* associated with the *Roseibacaceae*. Members of this genus are anoxygenic photoheterotrophs and rose-colored due to their bacteriochlorophyll pigments (Milford et al., 2000; Labrenz et al., 2009). They have previously been detected in Prony Bay, where their biofilms may be responsible for the dark-pink color of the chimney exteriors (Frouin et al., 2018). Intriguingly, *Roseibaca* are classified as strict aerobes but O_2 concentrations remained well below 0.5% throughout the entire incubation period in all culture conditions (Supplementary Figure 4). Since the experiments were performed in clear glass bioreactors, ambient light could have favored the growth of oxygenic phototrophs locally supplying O_2. However, no significant occurrence of cyanobacteria (the only bacterial group that performs oxygenic photosynthesis) was observed (Hanada, 2016) (Figure 5). The cultivated *Roseibaca* members may therefore be

facultative anaerobes. The carbonaceous chimney wall is very porous, and at a depth where O_2 becomes scarce, light might still penetrate. The bacteriochlorophyll pigments used in anoxygenic photosynthesis can absorb light at near-infrared wavelengths and facilitate photosynthesis in deep water or sediments (Hanada, 2016). In addition, the occurrence of normally strictly aerobic taxa in anoxic serpentinized fluids is a relatively common phenomenon and has been suggested to drive localized speciation of closely related populations along the steep O_2 and redox gradients from surface waters to subsurface fluids (Munro-Ehrlich et al., 2023). Next to *Roseibaca*, the consortia grown on acetate and formate included a significant proportion of *Meiothermus*. The cultures alimented with formate additionally featured a slight enrichment of the most likely thiosulfate-oxidizing bacteria *Thioalkalimicrobium* (see the thiosulfate consumption on formate in Supplementary Fig. S1), as well as a mid-term enrichment of *Acetoanaerobium*. The latter probably fermented organic compounds produced by the consortium during the first week and does not count amongst primary producers.

The consortia grown on bicarbonate was strongly dominated by the *Meiothermus* genus. Similar to *Roseibaca*, *Meiothermus* has been described as a strictly aerobic heterotroph; however, Munro-Ehrlich et al. (2023) provided evidence that subsurface populations can undergo parapatric speciation and develop traits of facultatively anaerobic autotrophs, such as the gain of NiFe Hydrogenases. Similar to these subsurface genotypes, it is likely that the *Meiothermus* population cultivated on bicarbonate grew chemoautotrophically by producing CO_2 in the cytoplasm with H_2 as an anaerobic electron donor. Besides *Meiothermus,* a significant enrichment of the chemoautotrophic *Hydrogenophaga,* a sister group of the serpentinite-hosted marker genus *Serpentinimonas* which features bicarbonate-uptake genes, as well as an enrichment of *Roseibaca* could be observed (Willems et al., 1989; Suzuki et al., 2014). While *Roseibaca* are usually classified as photoheterotrophs, their consistent and significant growth on bicarbonate might suggest that we enriched a novel photoautotrophic genotype. In addition, the genus *Halomonas* was dominant in the second week of the first experimental run, a thiosulfate-oxidizing bacterium that is typically observed in serpentinite-hosted environments. As this genus is heterotrophic, however, it is unlikely that it used bicarbonate as a carbon source (Ventosa et al., 2021; Alibaglouei et al., 2023; Trutschel et al., 2023) (Figure 5A).

The initial archaeal community consisted almost exclusively of the ANME-3 cluster, which belongs to the *Methanosarcinales*. This group was not enriched but maintained throughout the entirety of the cultivation experiments on all carbon sources, with a predominance so strong that the taxonomic composition did not vary significantly between carbon sources (Figure 4B, Figure 5B). The *Methanosarcinales* are one of the most typical taxa associated with serpentinization, with a very low degree of diversity to the extent that only two phylotypes have been described. The Lost City *Methanosarcinales* (LCMS) are more closely related to ANME-3 (our most abundant ANME-3 ASV shares 95.31% sequence identity with the LCMS), while The Cedars *Methanosarcinales* (TCMS) are intermediate between the ANME-2c and *Methanomicrobiales* clusters (Brazelton et al., 2006; Suzuki et al., 2013). While ANME-3 are classified as anaerobic methane oxidizers (AOM), it has been shown that they possess all the genes necessary for methanogenesis and LCMS are usually described as methanogens. It is, therefore, unclear whether LCMS are consumers or producers of methane, or both (Brazelton

et al., 2006). In addition to the maintenance of *Methanosarcinales*, a slight enrichment of *Woesearchaeales* was observed on formate, bicarbonate and particularly acetate in the first experimental run. This enrichment accounted for an almost significant influence of the sampling time on the community structure (Figure 4B). Since the abundances shown here are relative, the archaeal taxonomic composition may reflect an emerging symbiotic relationship between *Methanosarcinales* and *Woesearchaeales*. The *Woesearchaeales* are probably incapable of an independent lifestyle and have been suggested to establish symbiotic relationships with methanogens. So far, only heterotrophic genotypes are known (Liu et al., 2018). While only a handful of taxa has been shown to perform SAO, it might be possible that our *Woesearchaeales* strain is able to oxidize acetate to CO_2 and H_2 for a *Methanosarcinales* partner. In the second experimental run, a remarkable enrichment of *Methanomicrobiales* was observed on bicarbonate towards the end of the culture (81.44% ASV sequence identity with TCMS). This order is methanogenic (Rouvière et al., 1992) and probably outcompeted the symbiosis between *Methanosarcinales* and *Woesearchaeales* (Figure 5B). Interestingly, a recent study reclassified TCMS as acetogenic *Methanocellales* featuring bicarbonate uptake genes (Suzuki et al., 2024). It is thus likely that our strain is able to fix bicarbonate as an autotrophic carbon source.

The shallow subsurface foundation consortium

The predominance of phototrophic *Roseibaca* in two culture conditions suggests that our results are mostly reflective of Prony Bay's shallow subsurface community. The pronounced growth on acetate supports the foundation species theory proposed by Lang et al. (2018). Their original model was based on a high occurrence of sulfate reducers taking up formate and releasing CO_2 in the deep-sea Lost City hydrothermal field. While it is likely that sulfate reducers play an essential role in the deeper subsurface communities of Prony Bay, they were not enriched in the bioreactors despite sulfate and thiosulfate being the sole electron acceptors added to the cultures (Figure 5, Figure 6A). On the contrary, at least two of the enriched taxa (*Thioalkalimicrobium* and *Hydrogenophaga*) have previously been reported to reduce nitrate, suggesting that the respiration of nitrate plays a role in the anaerobic natural system. The electron acceptor used by the cultivated consortia remains unclear (Figure 6A). In the bioreactors alimented with acetate, the microbial consortium of putative heterotrophic foundation species was heavily dominated by the anoxygenic photoheterotroph *Roseibaca*, suggesting that this metabolic type plays a more important role in serpentinite-hosted environments than previously assumed. Besides *Roseibaca*, the putative consortium of heterotrophic foundation species included the heterotrophic genotype of *Meiothermus* (Munro-Ehrlich et al., 2023). Together, *Roseibaca* and *Meiothermus* may produce CO_2 from acetate for various autotrophs on the first trophic level in the natural ecosystem (Figure 6B).

Next to these putative acetate-assimilating foundation species, we cultivated potential autotrophic foundation consortia on bicarbonate and formate. On bicarbonate, this concerned the autotrophic *Meiothermus* genotype described by Munro-Ehrlich et al. (2023) and the hydrogenotrophic *Hydrogenophaga*. It is likely that the same *Meiothermus* genotype is part of base of the trophic network cultivated on formate, alongside the thiosulfate-oxidizing autotroph *Thioalkalimicrobium* and potentially a novel photoautotrophic genotype of *Roseibaca*. Together, these bicarbonate- and formate-fixing autotrophs could produce organic compounds

for heterotrophs on the first trophic level such as *Acetoanaerobium* and *Halomonas* (Figure 6B).

A special case of such carbon cycling between heterotrophs and autotrophs constitutes the relationship between methanogens and methanotrophs: Unidentified methanotrophs could establish a supply of CO_2 for autotrophic methanogens and vice versa. The *Methanomicrobiales* might perform methanogenesis with bicarbonate as an autotrophic carbon source, thus constituting an autotrophic foundation species. The predominant *Methanosarcinales* on the other hand are likely dependent on CO_2 that is either produced by a symbiont or locally released by other community members. While endogenous CO_2 supply might be provided by a potentially acetate-oxidizing *Woesearchaeales*, external CO_2 could rely on acetoclastic methanogenesis in addition to methanotrophy (Figure 6B).

Conclusion

Shallow subsurface microbial consortia displaying a pronounced enrichment of specific taxonomic groups were successfully cultivated from a submarine Prony Bay chimney sample in anaerobic fed-batch bioreactors simulating *in-situ* conditions. These reactors were operated with artificial hydrothermal fluid under a continuous flow of H_2 gas, supplemented with sulfate and thiosulfate as the sole electron acceptors and bicarbonate, formate, acetate or glycine as the sole carbon source. The culture on glycine exhibited only minimal growth and the taxonomic composition barely changed compared to the initial diversity observed in the inoculum. This either suggests that glycine plays a minor role in the natural microbial ecosystem, or that the experimental conditions did not accurately reproduce the natural conditions. The latter is supported by the observation of the generally very low growth rates on glycine, which might have required longer incubation periods. The pronounced growth on acetate supports a modified version of the foundation species theory proposed by Lang et al. (2018). Heterotrophic foundation taxa such as *Roseibaca* and *Meiothermus* (heterotrophic genotype) may produce CO_2 from acetate, facilitating the development of a diverse community of autotrophs, including those without bicarbonate and formate transporters or symbionts. Autotrophic foundation species might include bicarbonate-fixing *Meiothermus* (autotrophic genotype) and *Hydrogenophaga*, as well as formate-fixing *Meiothermus*, *Thioalkalimicrobium* and potentially a novel photoautotrophic genotype of *Roseibaca* producing organics for heterotrophs such as *Acetoanaerobium* and *Halomonas.* In the archaeal community, this reciprocal relationship is illustrated by CO_2 and CH_4 cycling *Methanosarcinales* with putative acetate-oxidizing *Woesearchaeales* symbionts, as well as bicarbonate-fixing *Methanomicrobiales*. We propose that such a feedback loop contributes to the diversification of the serpentinite-hosted community and explains the presence of well-established microbial communities in environments depleted of DIC.

Acknowledgments

We would like to thank Dr. Ariane Bize (INRAE Prose, Antony, France) for the training and discussions on DNA Stable Isotope Probing, which could not be included in this article but was greatly appreciated. Furthermore, we would like to thank Marc Garel and Fabrice Armougom

(MIO, Marseille) for the training on metabarcoding, which included the bioinformatic pipeline used in the analysis presented here. We are especially grateful to all participants of the 2022 field campaign to Prony Bay, including Roy Price (Stony Brook University, USA), Clarisse Majorel, Isabelle Biegala and the scientific diving team (IRD Research Center Nouméa, New Caledonia). Many thanks to all members of the ANR MICROPRONY. RMP was awarded a PhD fellowship from the French Ministry of Education and Scientific Research.

Author contributions

GE and AP designed and supervised the study. GE, AP, RMP, SD, and YCB developed the experimental design. AR, RMP, SD, and AP performed the experimental work. RMP and AR performed the initial data analysis. RMP performed the final data analysis; RMP and GE drafted the manuscript. All authors read and approved the final version.

References

Alibaglouei, M., Trutschel, L. R., Rowe, A. R., and Sackett, J. D. (2023). Complete genome sequence of Halomonas sp. strain M1, a thiosulfate-oxidizing bacterium isolated from a hyperalkaline serpentinizing system, Ney Springs. *Microbiology Resource Announcements* 12, e00508-23. doi: 10.1128/MRA.00508-23

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* 43, 260–296.

Bes, M., Merrouch, M., Joseph, M., Quéméneur, M., Payri, C., Pelletier, B., et al. (2015). Acetoanaerobium pronyense sp. nov., an anaerobic alkaliphilic bacterium isolated from a carbonate chimney of the Prony Hydrothermal Field (New Caledonia). *International Journal of Systematic and Evolutionary Microbiology* 65, 2574–2580. doi: 10.1099/ijs.0.000307

Brazelton, W. J., McGonigle, J. M., Motamedi, S., Pendleton, H. L., Twing, K. I., Miller, B. C., et al. (2022). Metabolic Strategies Shared by Basement Residents of the Lost City Hydrothermal Field. *Appl Environ Microbiol* 88, e00929-22. doi: 10.1128/aem.00929-22

Brazelton, W. J., Mehta, M. P., Kelley, D. S., and Baross, J. A. (2011). Physiological Differentiation within a Single-Species Biofilm Fueled by Serpentinization. *mBio* 2, e00127-11. doi: 10.1128/mBio.00127-11

Brazelton, W. J., Schrenk, M. O., Kelley, D. S., and Baross, J. A. (2006). Methane- and Sulfur-Metabolizing Microbial Communities Dominate the Lost City Hydrothermal Field Ecosystem. *Appl Environ Microbiol* 72, 6257–6270. doi: 10.1128/AEM.00574-06

Brazelton, W. J., Thornton, C. N., Hyer, A., Twing, K. I., Longino, A. A., Lang, S. Q., et al. (2017). Metagenomic identification of active methanogens and methanotrophs in serpentinite springs of the Voltri Massif, Italy. *PeerJ* 5, e2945. doi: 10.7717/peerj.2945

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nat Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Fones, E. M., Colman, D. R., Kraus, E. A., Stepanauskas, R., Templeton, A. S., Spear, J. R., et al. (2021). Diversification of methanogens into hyperalkaline serpentinizing environments through adaptations to minimize oxidant limitation. *ISME J* 15, 1121–1135. doi: 10.1038/s41396-020-00838-1

Frouin, E., Bes, M., Ollivier, B., Quéméneur, M., Postec, A., Debroas, D., et al. (2018). Diversity of Rare and Abundant Prokaryotic Phylotypes in the Prony Hydrothermal Field and Comparison with Other Serpentinite-Hosted Ecosystems. *Front. Microbiol.* 9, 102. doi: 10.3389/fmicb.2018.00102

Frouin, E., Lecoeuvre, A., Armougom, F., Schrenk, M. O., and Erauso, G. (2022). Comparative Metagenomics Highlight a Widespread Pathway Involved in Catabolism of Phosphonates in Marine and Terrestrial Serpentinizing Ecosystems. *mSystems*, e00328-22. doi: 10.1128/msystems.00328-22

Hanada, S. (2016). Anoxygenic Photosynthesis —A Photochemical Reaction That Does Not Contribute to Oxygen Reproduction—. *Microbes Environ* 31, 1–3. doi: 10.1264/jsme2.ME3101rh

Kohl, L., Cumming, E., Cox, A., Rietze, A., Morrissey, L., Lang, S. Q., et al. (2016). Exploring the metabolic potential of microbial communities in ultra-basic, reducing springs at The Cedars, CA, USA: Experimental evidence of microbial methanogenesis and heterotrophic acetogenesis. *Journal of Geophysical Research: Biogeosciences* 121, 1203–1220. doi: 10.1002/2015JG003233

Labrenz, M., Lawson, P. A., Tindall, B. J., and Hirsch, P. (2009). Roseibaca ekhonensis gen. nov., sp. nov., an alkalitolerant and aerobic bacteriochlorophyll a-producing alphaproteobacterium from hypersaline Ekho Lake. *International Journal of Systematic and Evolutionary Microbiology* 59, 1935–1940. doi: 10.1099/ijs.0.016717-0

Lahti, L., and Shetty, S. (2017). microbiome. doi: 10.18129/B9.BIOC.MICROBIOME

Lang, S. Q., Früh-Green, G. L., Bernasconi, S. M., Brazelton, W. J., Schrenk, M. O., and McGonigle, J. M. (2018). Deeply-sourced formate fuels sulfate reducers but not methanogens at Lost City hydrothermal field. *Sci Rep* 8, 755. doi: 10.1038/s41598-017-19002-5

Liu, X., Li, M., Castelle, C. J., Probst, A. J., Zhou, Z., Pan, J., et al. (2018). Insights into the ecology, evolution, and metabolism of the widespread Woesearchaeotal lineages. *Microbiome* 6, 102. doi: 10.1186/s40168-018-0488-2

McCollom, T. M., and Seewald, J. S. (2013). Serpentinites, Hydrogen, and Life. *Elements* 9, 129–134. doi: 10.2113/gselements.9.2.129

McMurdie, P. J., and Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* 8, e61217. doi: 10.1371/journal.pone.0061217

Ménez, B., Pisapia, C., Andreani, M., Jamme, F., Vanbellingen, Q. P., Brunelle, A., et al. (2018). Abiotic synthesis of amino acids in the recesses of the oceanic lithosphere. *Nature* 564, 59–63. doi: 10.1038/s41586-018-0684-z

Milford, A. D., Achenbach, L. A., Jung, D. O., and Madigan, M. T. (2000). Rhodobaca bogoriensis gen. nov. and sp. nov., an alkaliphilic purple nonsulfur bacterium from African Rift Valley soda lakes. *Archives of Microbiology* 174, 18–27. doi: 10.1007/s002030000166

Monnin, C., Chavagnac, V., Boulart, C., Ménez, B., Gérard, M., Gérard, E., et al. (2014). Fluid chemistry of the low temperature hyperalkaline hydrothermal system of Prony Bay (New Caledonia). *Biogeosciences* 11, 5687–5706. doi: 10.5194/bg-11-5687-2014

Monnin, C., Quéméneur, M., Price, R., Jeanpert, J., Maurizot, P., Boulart, C., et al. (2021). The Chemistry of Hyperalkaline Springs in Serpentinizing Environments: 1. The Composition of Free Gases in New Caledonia Compared to Other Springs Worldwide. *Journal of Geophysical Research: Biogeosciences* 126, e2021JG006243. doi: 10.1029/2021JG006243

Morrill, P. L., Brazelton, W. J., Kohl, L., Rietze, A., Miles, S. M., Kavanagh, H., et al. (2014). Investigations of potential microbial methanogenic and carbon monoxide utilization pathways in ultra-basic reducing springs associated with present-day continental serpentinization: the Tablelands, NL, CAN. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00613

Munro-Ehrlich, M., Nothaft, D. B., Fones, E. M., Matter, J. M., Templeton, A. S., and Boyd, E. S. (2023). Parapatric speciation of Meiothermus in serpentinite-hosted aquifers in Oman. *Front Microbiol* 14, 1138656. doi: 10.3389/fmicb.2023.1138656

Nobu, M. K., Nakai, R., Tamazawa, S., Mori, H., Toyoda, A., Ijiri, A., et al. (2022). Unique H2-utilizing lithotrophy in serpentinite-hosted systems. *ISME J*. doi: 10.1038/s41396-022-01197-9

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., et al. (2024). *vegan: Community Ecology Package*. Available at: https://CRAN.R-project.org/package=vegan

Palarea-Albaladejo, J., and Martín-Fernández, J. A. (2015). zCompositions — R package for multivariate imputation of left-censored data under a compositional approach.

Chemometrics and Intelligent Laboratory Systems 143, 85–96. doi: 10.1016/j.chemolab.2015.02.019

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00857

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41, D590–D596. doi: 10.1093/nar/gks1219

Quéméneur, M., Bes, M., Postec, A., Mei, N., Hamelin, J., Monnin, C., et al. (2014). Spatial distribution of microbial communities in the shallow submarine alkaline hydrothermal field of the Prony Bay, New Caledonia: Microbial communities of Prony Hydrothermal Field. *Environmental Microbiology Reports* 6, 665–674. doi: 10.1111/1758-2229.12184

Rempfert, K. R., Miller, H. M., Bompard, N., Nothaft, D., Matter, J. M., Kelemen, P., et al. (2017). Geological and Geochemical Controls on Subsurface Microbial Life in the Samail Ophiolite, Oman. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00056

Rogers, T. J., Buongiorno, J., Jessen, G. L., Schrenk, M. O., Fordyce, J. A., de Moor, J. M., et al. (2023). Chemolithoautotroph distributions across the subsurface of a convergent margin. *ISME J* 17, 140–150. doi: 10.1038/s41396-022-01331-7

Rouvière, P., Mandelco, L., Winker, S., and Woese, C. R. (1992). A detailed phylogeny for the Methanomicrobiales. *Syst Appl Microbiol* 15, 363–371. doi: 10.1016/S0723-2020(11)80209-2

Russell, M. J., Hall, A. J., and Martin, W. (2010). Serpentinization as a source of energy at the origin of life: Serpentinization and the emergence of life. *Geobiology* 8, 355–371. doi: 10.1111/j.1472-4669.2010.00249.x

Sabuda, M. C., Putman, L. I., Hoehler, T. M., Kubo, M. D., Brazelton, W. J., Cardace, D., et al. (2021). Biogeochemical Gradients in a Serpentinization-Influenced Aquifer: Implications for Gas Exchange Between the Subsurface and Atmosphere. *Journal of Geophysical Research: Biogeosciences* 126, e2020JG006209. doi: 10.1029/2020JG006209

Sánchez-Andrea, I., Guedes, I. A., Hornung, B., Boeren, S., Lawson, C. E., Sousa, D. Z., et al. (2020). The reductive glycine pathway allows autotrophic growth of Desulfovibrio desulfuricans. *Nat Commun* 11, 5090. doi: 10.1038/s41467-020-18906-7

Schönheit, P., Buckel, W., and Martin, W. F. (2016). On the Origin of Heterotrophy. *Trends in Microbiology* 24, 12–25. doi: 10.1016/j.tim.2015.10.003

Schrenk, M. O., Brazelton, W. J., and Lang, S. Q. (2013). Serpentinization, Carbon, and Deep Life. *Reviews in Mineralogy and Geochemistry* 75, 575–606. doi: 10.2138/rmg.2013.75.18

Smith, K. S., and Ferry, J. G. (2000). Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev* 24, 335–366. doi: 10.1111/j.1574-6976.2000.tb00546.x

Sojo, V., Herschy, B., Whicher, A., Camprubí, E., and Lane, N. (2016). The Origin of Life in Alkaline Hydrothermal Vents. *Astrobiology* 16, 181–197. doi: 10.1089/ast.2015.1406

Suzuki, S., Ishii, S., Chadwick, G. L., Tanaka, Y., Kouzuma, A., Watanabe, K., et al. (2024). A non-methanogenic archaeon within the order Methanocellales. *Nat Commun* 15, 4858. doi: 10.1038/s41467-024-48185-5

Suzuki, S., Ishii, S., Wu, A., Cheung, A., Tenney, A., Wanger, G., et al. (2013). Microbial diversity in The Cedars, an ultrabasic, ultrareducing, and low salinity serpentinizing ecosystem. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15336–15341. doi: 10.1073/pnas.1302426110

Suzuki, S., Kuenen, J. G., Schipper, K., van der Velde, S., Ishii, S., Wu, A., et al. (2014). Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. *Nat Commun* 5, 3900. doi: 10.1038/ncomms4900

Timmers, P. H. A., Vavourakis, C. D., Kleerebezem, R., Damsté, J. S. S., Muyzer, G., Stams, A. J. M., et al. (2018). Metabolism and Occurrence of Methanogenic and Sulfate-Reducing Syntrophic Acetate Oxidizing Communities in Haloalkaline Environments. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.03039

Trutschel, L. R., Chadwick, G. L., Kruger, B., Blank, J. G., Brazelton, W. J., Dart, E. R., et al. (2022). Investigation of microbial metabolisms in an extremely high pH marine-like terrestrial serpentinizing system: Ney Springs. *Science of The Total Environment* 836, 155492. doi: 10.1016/j.scitotenv.2022.155492

Trutschel, L. R., Kruger, B. R., Sackett, J. D., Chadwick, G. L., and Rowe, A. R. (2023). Determining resident microbial community members and their correlations with geochemistry in a serpentinizing spring. *Front Microbiol* 14, 1182497. doi: 10.3389/fmicb.2023.1182497

Ventosa, A., de la Haba, R. R., Arahal, D. R., and Sánchez-Porro, C. (2021). "Halomonas," in *Bergey's Manual of Systematics of Archaea and Bacteria*, (John Wiley & Sons, Ltd), 1–111. doi: 10.1002/9781118960608.gbm01190.pub2

Willems, A., Busse, J., Goor, M., Pot, B., Falsen, E., Jantzen, E., et al. (1989). Hydrogenophaga, a New Genus of Hydrogen-Oxidizing Bacteria That Includes Hydrogenophaga flava comb. nov. (Formerly Pseudomonas flava), Hydrogenophaga palleronii (Formerly

Pseudomonas palleronii), Hydrogenophaga pseudoflava (Formerly Pseudomonas pseudoflava and “Pseudomonas carboxy do flava”), and Hydrogenophaga taeniospiralis (Formerly Pseudomonas taeniospiralis). *International Journal of Systematic and Evolutionary Microbiology* 39, 319–333. doi: 10.1099/00207713-39-3-319

Xu, S., Zhan, L., Tang, W., Wang, Q., Dai, Z., Zhou, L., et al. (2023). MicrobiotaProcess: A comprehensive R package for deep mining microbiome. *The Innovation* 4, 100388. doi: 10.1016/j.xinn.2023.100388

4.3 Figures

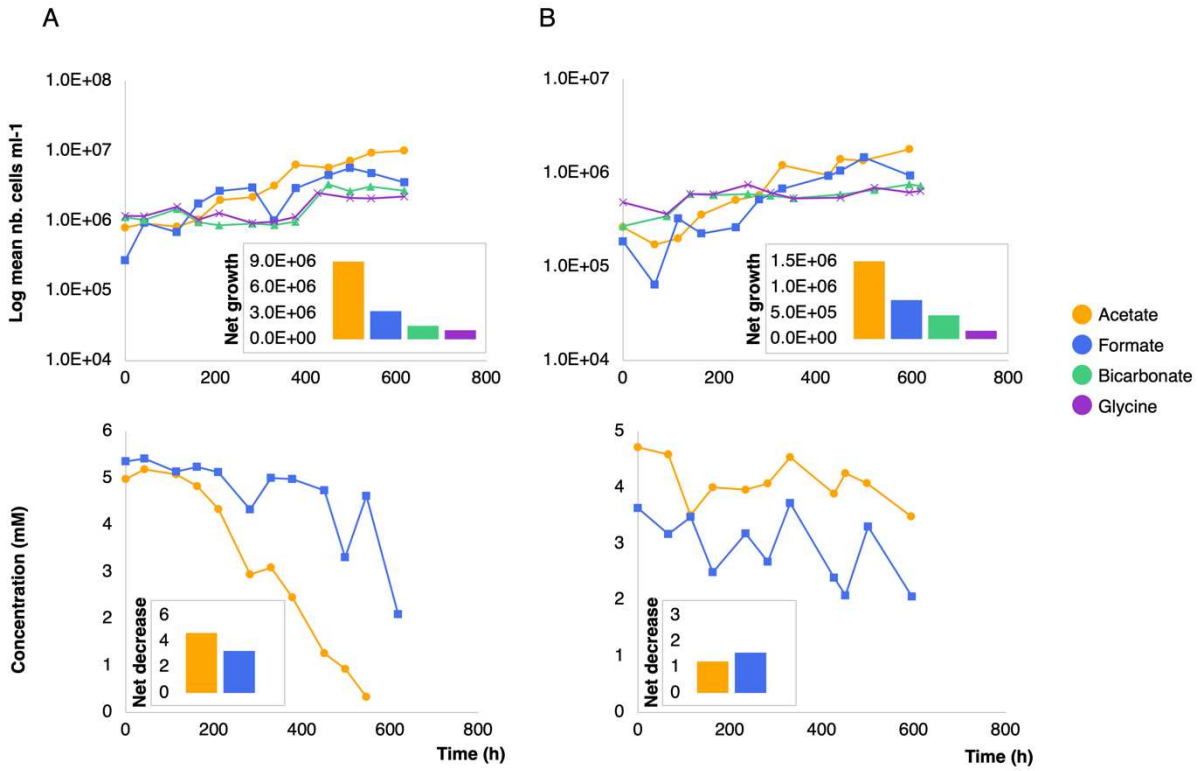


Figure 4-1. Culture development in the first experimental run (cultures Ac1, Fo1, G1, Bc1) (A) and in the second run (cultures Ac2, Fo2, G2, Bc2) (B). The number of cells was counted via DAPI-staining and fluorescence microscopy. The concentration of acetate and formate in the respective consortia was determined by high-performance liquid chromatography. The net increase of cells and the net decrease of acetate and formate (shown as inserts) were calculated as the difference between the first and last sampling timepoint

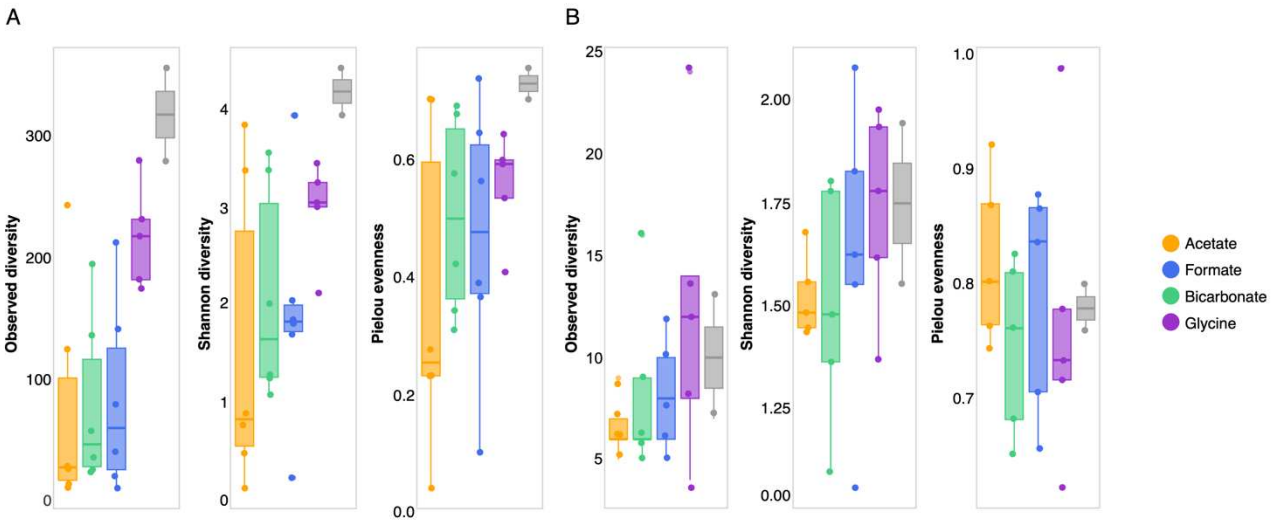


Figure 4-2. Alpha diversity and evenness calculated to the observed number of ASVs, the Shannon index and the Pielou index in bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). Samples from both experimental runs are combined

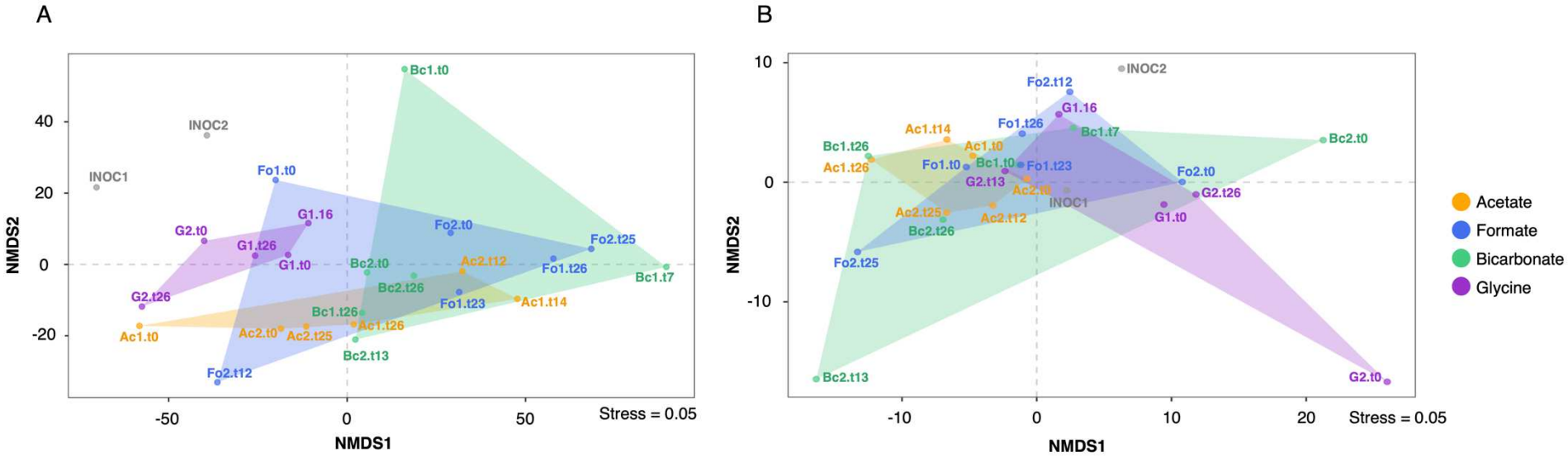


Figure 4-3. Aitchison distance matrices (k = 5) constructed on bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (blue) or glycine (purple). Distance matrices were constructed using a non-metric multidimensional scaling on log-ratio transformed data

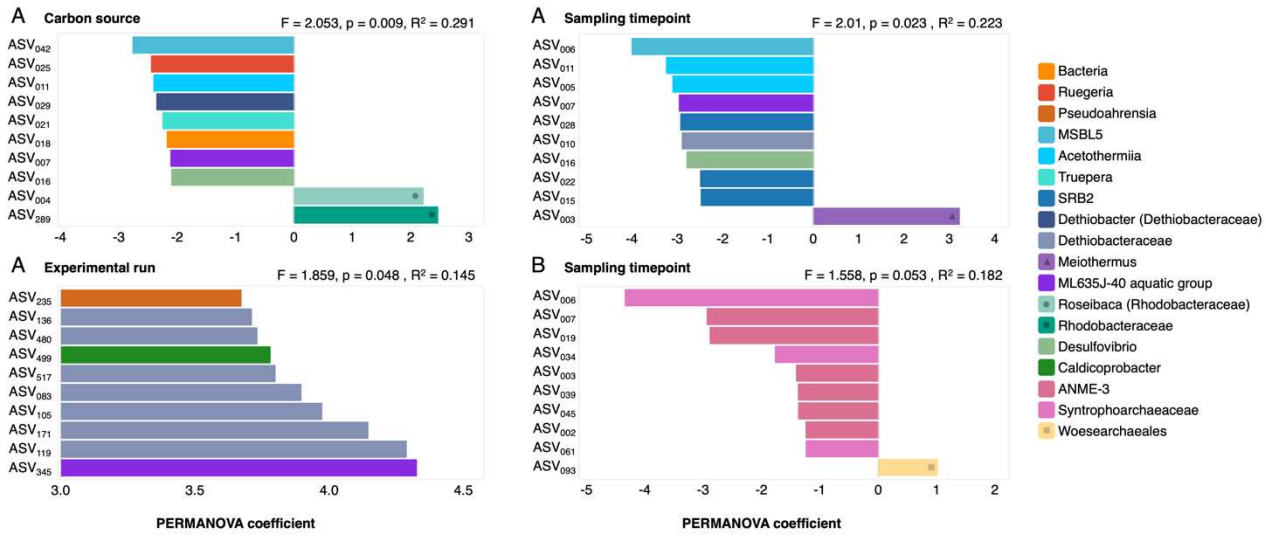


Figure 4-4. Top ASVs driving significant effects of carbon source, sampling timepoint and experimental run on sample variation in bacterial (A) and/or archaeal (B) sequences. Different ASVs are color-coded according to the lowest taxonomic level to which they were assigned. Coefficients for the respective group of variable were calculated in permutational multivariate analysis of variance tests; correspoding F values, p values and R^2 value are given on the top right of each graph. Positive coefficients indicate an effect of the respective ASV on sample dissimilarity, negative coefficients indicate an effect on sample similarity. Key taxonomic groups are highlighted with symbols to improve the readability of the graph

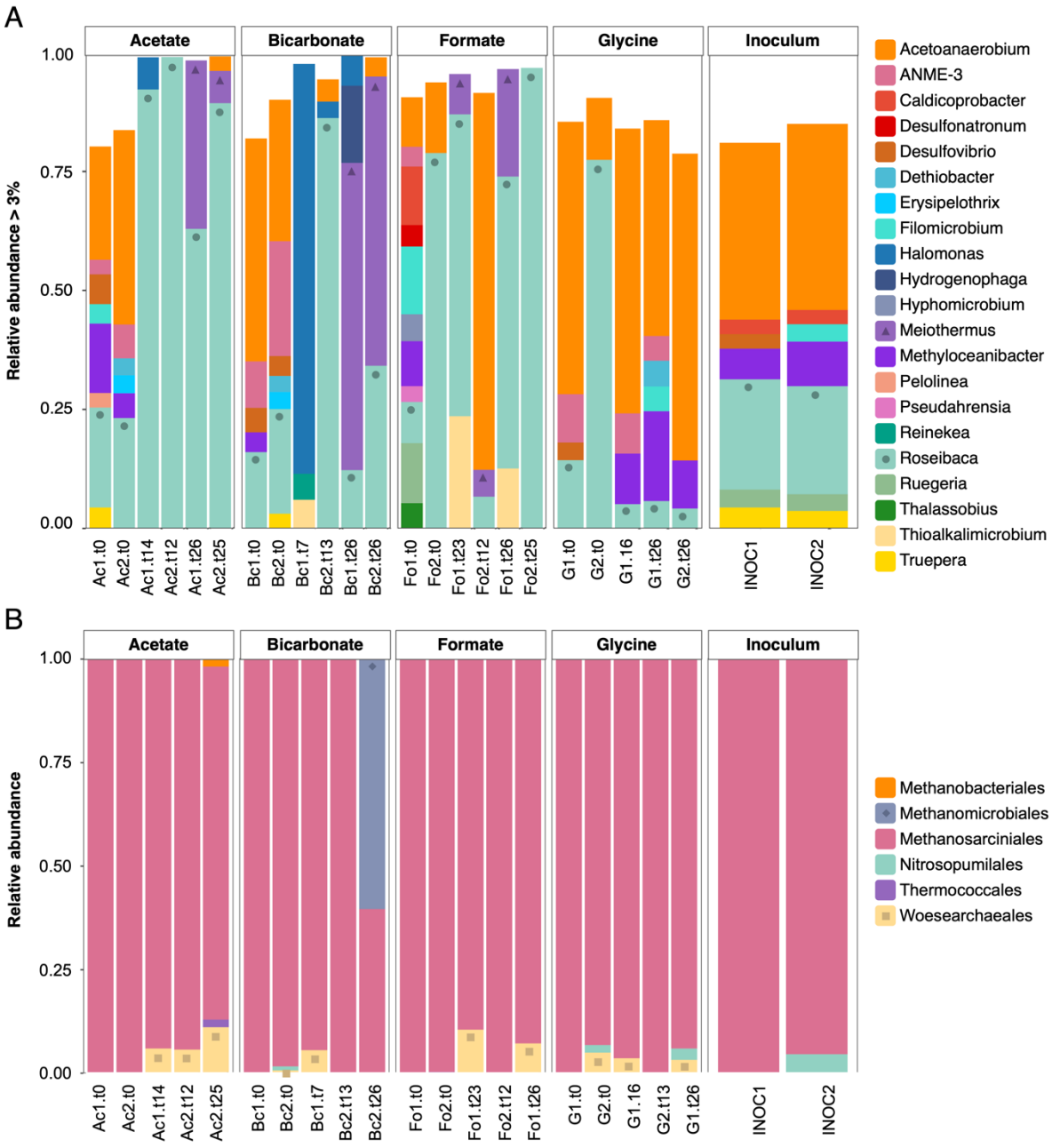


Figure 4-5. Taxonomic composition of bacterial (A) and archaeal (B) microbial consortia grown on acetate (Ac), bicarbonate (Bc), formate (Fo) or glycine (G) in the first (1) and second (2) experimental run over time. For each carbon source and experimental run, the taxonomic composition of up to three different timepoints is depicted, including the first day (t0), an intermediate day and the last day (t25 or t26) of the culture. In addition, the taxonomic composition of the inoculum used in the respective experimental run (INOC) is shown. For bacterial sequences, only genera with a relative abundance > 3% are depicted. For archaeal sequences, all orders are shown. Note that ANME-3 is a genus of the archaeal order *Methanosarcinales* (both depicted in the same color) which was also amplified by the bacterial primers. Key taxonomic groups are emphasized with symbols to improve readability of the graph

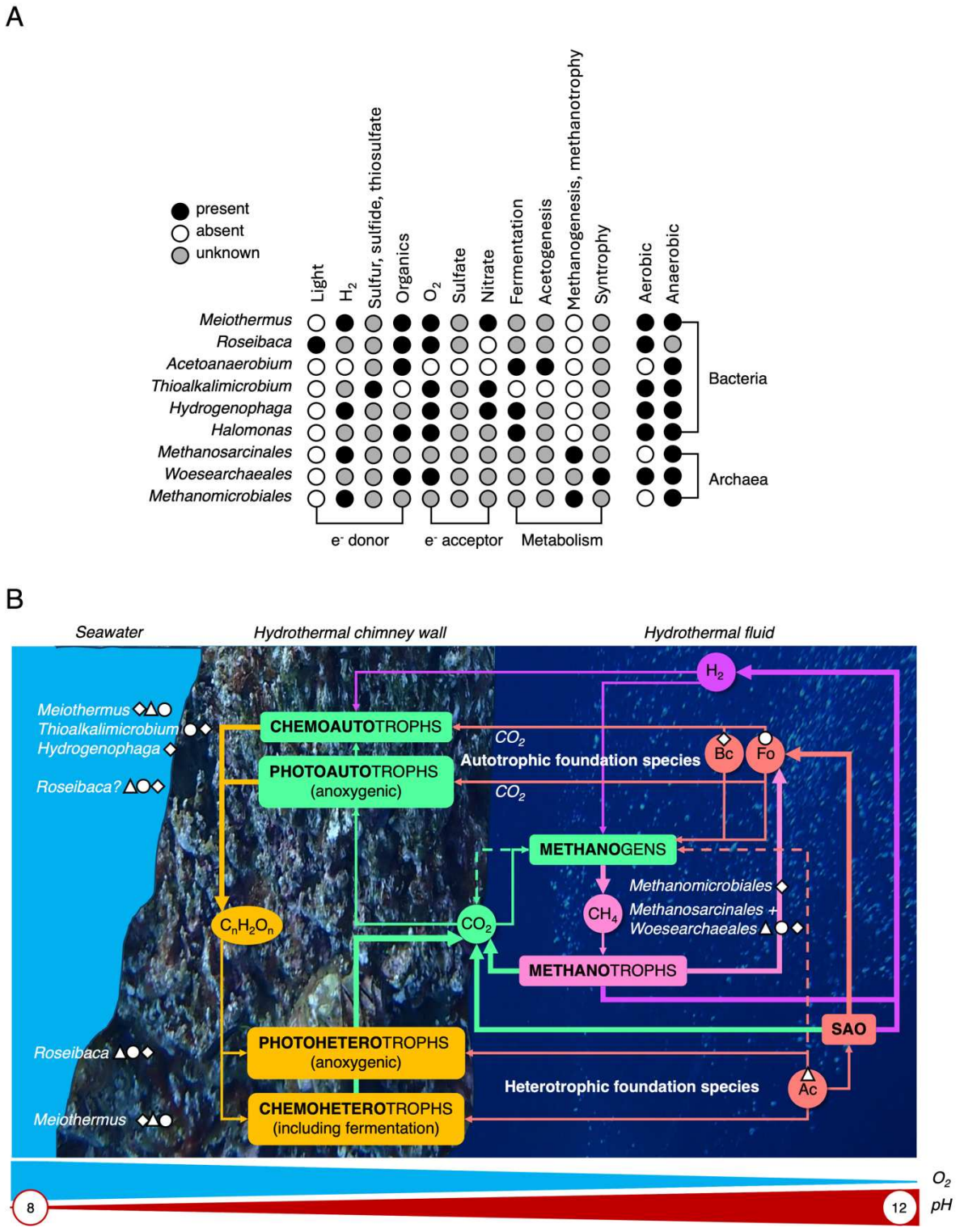
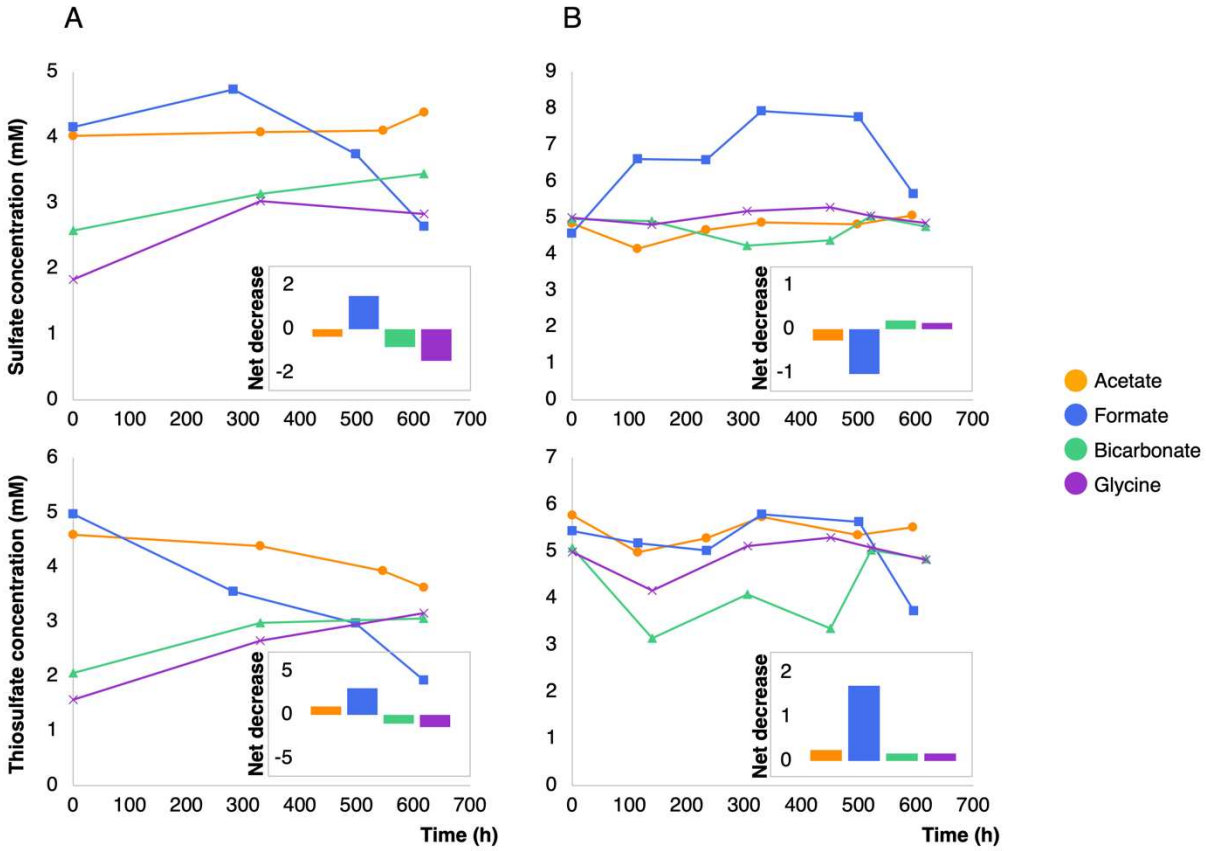
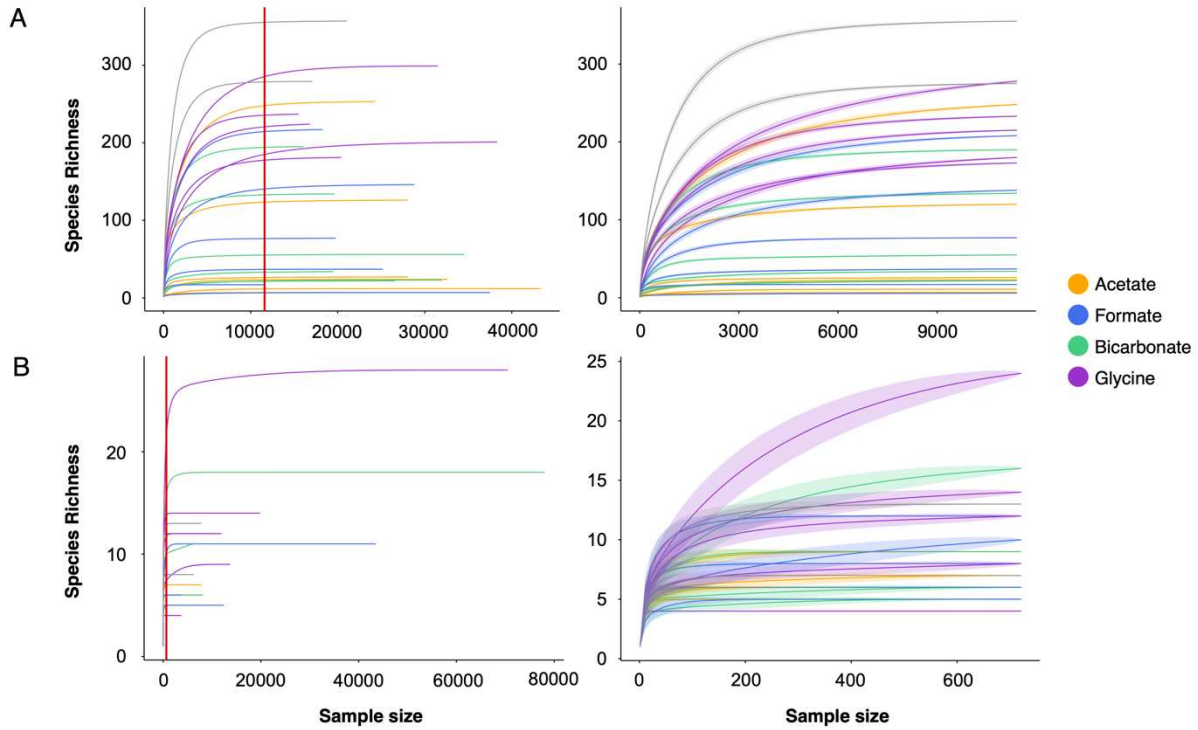


Figure 4-6. Proposed metabolic network of the cultivable Prony Bay hydrothermal community. A) Metabolic potential of enriched key taxa according to the references cited in the main text. B) Hypothesized trophic relationships. Putative foundation species are shown next to metabolic categories, with symbols indicating all culture conditions they were enriched in. Note the following abbreviations: SAO for syntrophic acetate oxidation, Bc for bicarbonate, Fo for formate, Ac for acetate

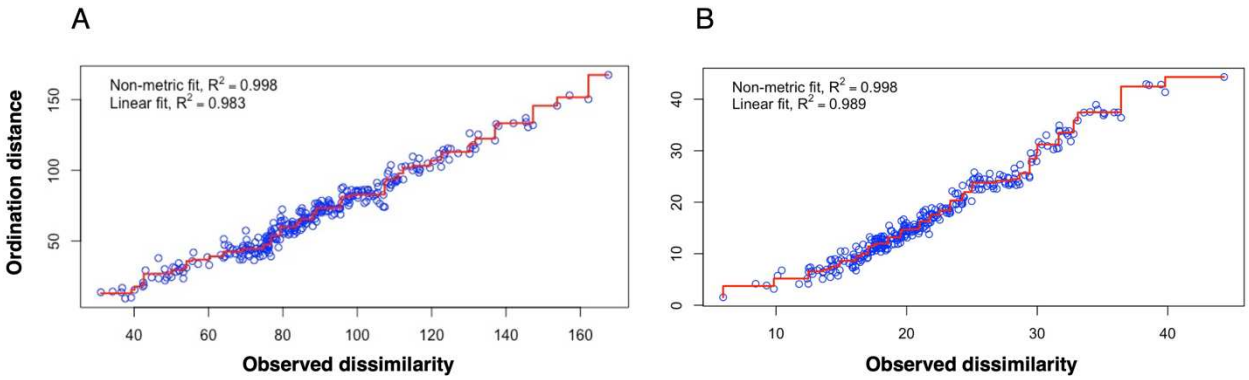
4.4 Supplementary material



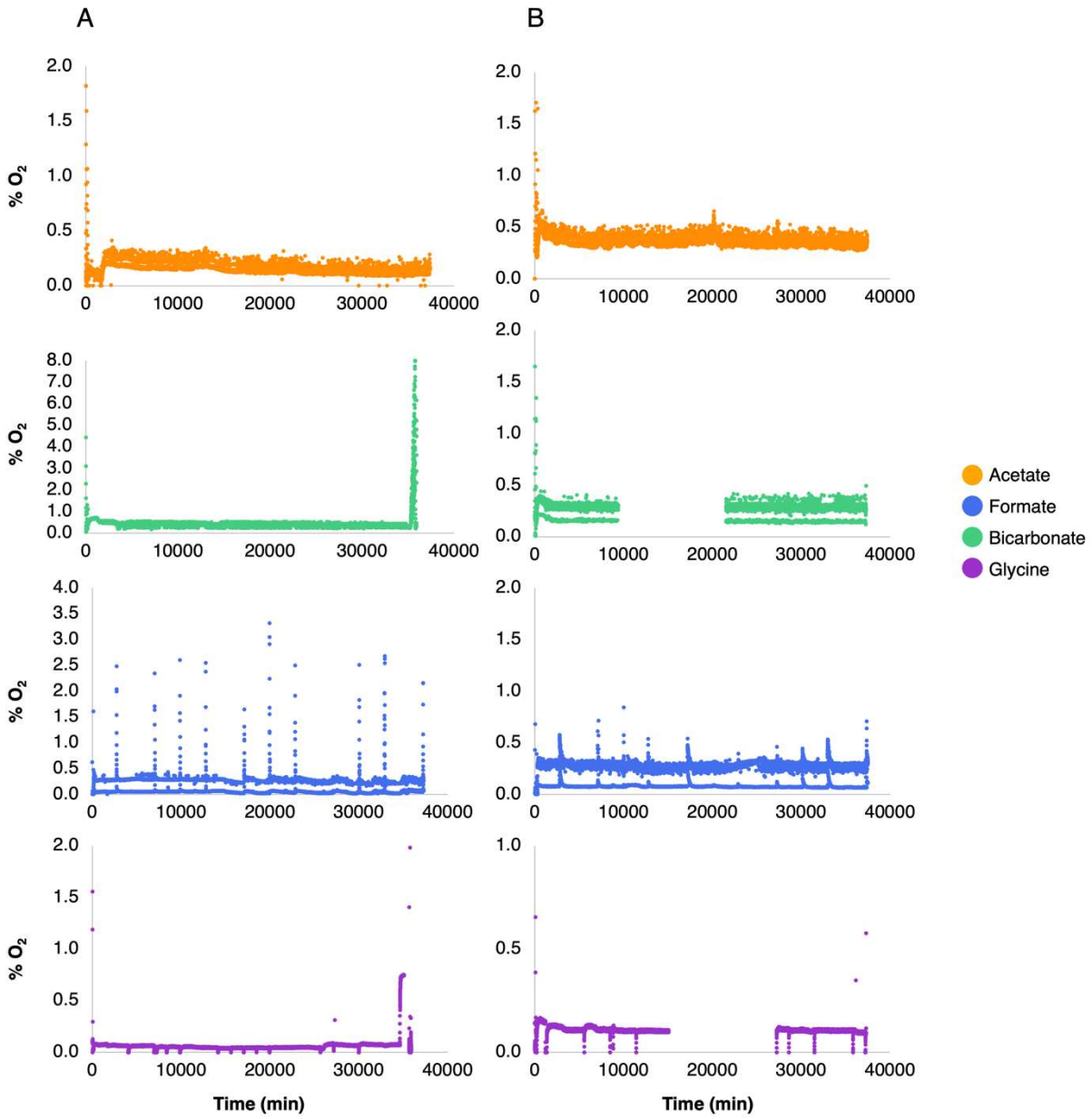
Supplementary Figure 4-1. Sulfate and thiosulfate consumption in the first (A) and second (B) experimental run in the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). The respective net decrease of sulfate or thiosulfate concentration, calculated as the difference between the first and last timepoint measured, is illustrated with barplots



Supplementary Figure 4-2. Rarefaction curves for bacterial (A) and archaeal (B) sequences from the microbial consortia grown on acetate (yellow), formate (blue), bicarbonate (green) or glycine (purple). Bacterial sequences were normalized to 11420 reads, which corresponds to the minimal sample size, archaeal sequences to 721 reads. The rarefaction depth is indicated with a red vertical line



Supplementary Figure 4-3. Stressplots of the non-metric multidimensional scaling matrices calculated for bacterial (A) and archaeal (B) sequences.



Supplementary Figure 4-4. Percentage of oxygen in the bioreactor gas phase during (A) the first and (B) the second experimental run. In the second replicate of the culture grown on bicarbonate and glycine, data acquisition was temporally interrupted due to a technical problem. In the cultures grown on formate, the oxygen peaks correspond to sampling timepoints. Initial and final oxygen peaks in all cultures correspond to the startup and shutdown of the bioreactors and do not influence the samples discussed in the article



CHAPTER 5
Discussion and perspectives

5.1 Preface

In this chapter, we join the results from the metagenomic and the cultivation approach and examine the emerging picture of Prony Bay's surface and shallow subsurface foundation consortia. Furthermore, we discuss how deep subsurface communities could be targeted by reducing methodological bias from both approaches, expanding the scope of assessed microbial consortia and considering other carbon sources than the ones tested here. The thesis is concluded by re-establishing the link between our research and the origin of life, placing our findings in a broader context.

5.2 Foundation species from the surface and shallow subsurface

The metagenomic and the cultivation approach complemented each other not only from a methodological point of view, but also in terms of results: While the metagenomic analysis provided insight into the base of the trophic network in Prony Bay's surface environments, the cultivation experiments gave access to the anaerobic shallow subsurface communities. Combining the two approaches, we are able to propose a draft of the near-surface serpentinite-hosted foundation community along a steep gradient of O_2 concentrations and pH values from the ambient environment to the hydrothermal fluid (Figure 5-1). Together, the identified autotrophic, heterotrophic and mixotrophic taxa might explain the development of a near-surface ecosystem in the DIC-depleted environment of Prony Bay.

Both approaches suggest that Prony Bay's ecosystem does not rely on glycine as a primary carbon source. Neither surface nor shallow subsurface communities featured the genetic make-up or the capability to growth on this compound. While there might be methodological biases in this respect (as discussed in chapter three and four), this can be considered a relatively clear result for the time being. The surface communities were able to metabolize bicarbonate and acetate, and cultivated shallow subsurface communities were able to grow on formate in addition to these two compounds. This is consistent with results from other serpentinite-hosted environments and especially Lost City, where subsurface communities are expected to be largely dependent on formate fixation (Lang et al., 2018; McGonigle et al., 2020; Brazelton et al., 2022).

The identification of bicarbonate, formate and acetate as putative carbon sources supports the prevalence of both autotrophic and heterotrophic foundation species (Figure 5-1). Overall, we identified a greater diversity of autotrophs but given the significant growth on acetate it is likely that at least in the shallow subsurface, heterotrophs play an equally important role. Autotrophic surface foundation species include the gammaproteobacterial bicarbonate-fixing *Xanthomonadales* and UBA7966. Autotrophic shallow subsurface foundation species include the gammaproteobacterial *Hydrogenophaga* (bicarbonate-fixing) and *Thiolkalimicrobium* (formate-fixing), as well as a recently proposed *Meiothermus* genotype (predominantly bicarbonate-fixing) and a putative photautotrophic genotype of *Roseibaca* (predominantly formate-fixing). In addition, a bicarbonate-fixing methanogenic subsurface foundation species associated with the *Methanomicrobiales* was identified.

Heterotrophic, acetate-assimilating foundation species include a *Cyanobacteriia* taxon in surface environments and the standard *Meiothermus* as well as *Roseibaca* genotypes in the shallow subsurface. Furthermore, a potential symbiosis between acetate-oxidizing *Woesearchaeales* strain and a *Methanosarcinales* member closely related to the Lost City phylotype was observed in anaerobic cultivation experiments.

In the surface environment, we additionally identified two mixotrophic foundation species which featured the genetic make-up to both fix bicarbonate and assimilate acetate: The *Rhodobacterales* (which include the *Roseibaca* genus) and the *Burkholderiales*. While two distinct genotypes of were described for *Meiothermus*, it is possible that the *Roseibaca* taxon we cultivated is actually mixotrophic instead of having diverged into separate genotypes as proposed in chapter four.

A surprising number of putative autotrophic and even mixotrophic (see the discussion on the *Rhodobacteraceae* MAG in chapter three) foundation species relied on the oxidation of reduced sulfur species despite the environmental abundance of H_2. This energy source was most likely abundant in hydrothermal vent settings on Early Earth and might testify to the deep-branching nature of the serpentinite-hosted community. In addition, phototrophs relying on light as an energy source contribute to our hypothesized foundation consortia. While this is not surprising in surface and shallow subsurface environments, a potential significance for deep subsurface communities is discussed in section 5.3.2.

Regarding electron acceptors, we mostly identified putative foundation species reducing O_2 and oxidized nitrogen species. These compounds are classical electron acceptors of surface and shallow subsurface communities, and it is likely that they are replaced by oxidized sulfur species and organic compounds with increasing depth. As the bioreactors were exclusively alimented with sulfate and thiosulfate, it remains unclear which electron acceptors were used by the cultivated microbial consortia.

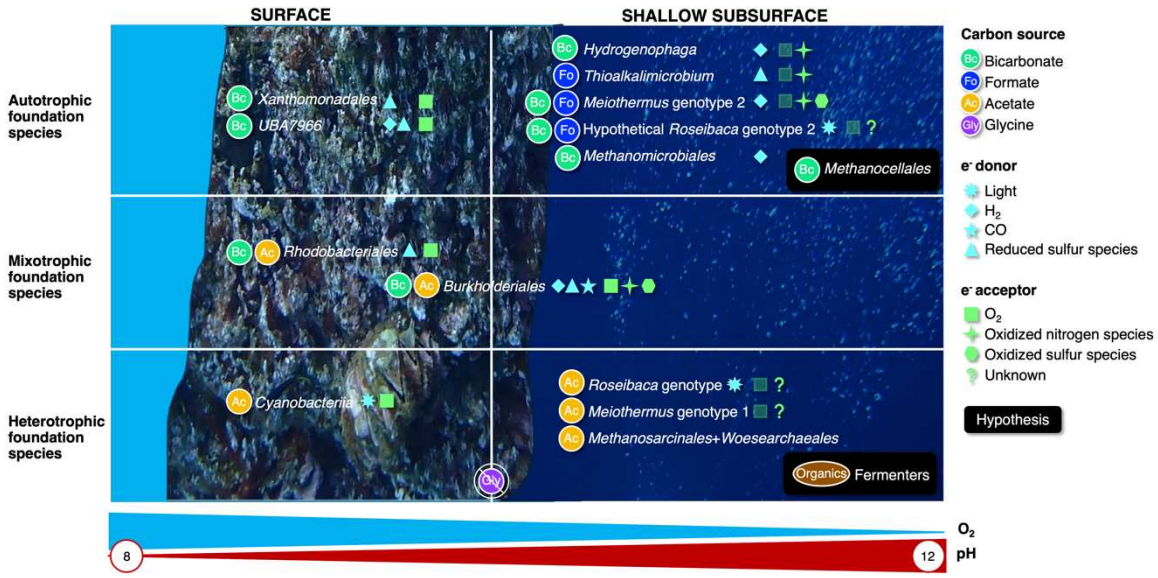


Figure 5-1. Hypothesized foundation species consortia in Prony Bay's surface (metagenomic approach) and subsurface (cultivation approach) environment. Carbon source, electron donors and electron acceptors used by each putative foundation taxon are depicted with color-coded symbols. Hypotheses on deep-surface foundation species are shown in black boxes.

5.3 Tracking down foundation species from the deep subsurface

While the surface and shallow subsurface communities are an integral part of Prony Bay's ecosystem, they are not fully representative of the deep-branching serpentinite-hosted community that is a potential Early Earth analog. To further pursue research questions related to the origin of life, it is necessary to assess the subsurface community on a larger scale than we have achieved here. This includes a revision of the techniques used in this thesis, ideally in an attempt to target a larger proportion of the natural trophic network, as well as a potential expansion of the pool of potential primary carbon sources that community members are tested for.

5.3.1 Minimizing methodological bias

Accessing the deep subsurface communities might be possible by adjusting both the metagenomic and the cultivation approach to minimize methodological bias that might have obscured respective taxonomic groups. The optimization of sample collection and environmental DNA extraction protocols, as well as the development of experimental techniques might help in achieving this goal.

5.3.1.1 Metagenomics: Optimization of sample collection and DNA extraction

The metagenomic approach proved to be an effective method for determining the community's genetic potential. In order to access the deep subsurface, however, it would not only be necessary to target a greater diversity of bacterial taxa, but also to recover more archaeal sequences which are typically associated with deep serpentinite-hosted environments. The average protein oxidation state and the taxonomic composition of ST0711 confirms that the submarine ST07 site is a good candidate to study the subsurface community. However, the comparison with ST0714, which was collected at the same site three years later and mostly represents the ambient marine community, illustrates the difficulty of obtaining a good-quality sample of the anaerobic part of the community. Sample collection is a challenging process that requires experience and great care to avoid contamination with seawater. Divers need to identify an active chimney emitting a strong flow of hydrothermal fluid, which needs to replace the seawater completely before the waterproof bag is closed around the hydrothermal chimney. An alternative possibility is the recovery of pure fluid samples by inserting a tube into the hydrothermal chimney and pumping the hydrothermal fluid into a sterile bottle. The collected fluid can then be filtered through a Sterivex unit, and DNA extractions directly conducted on the filter. This technique has been tested by our team during the latest field campaign in 2023 and preliminary 16S rRNA data confirms the recovery of taxonomic groups typically associated with the serpentinite-hosted deep subsurface. Notably, this included a significant proportion of

Meiothermus (unpublished data). Metagenomic analyses of these samples are planned to be conducted in the future.

Obtaining sufficient amounts of good-quality DNA for WGS sequencing from pure fluid samples is likely challenging due to the overall low biomass in the subsurface and the nature of the sample, which does not allow local concentration of cells by accumulation in biofilms or on solid particles. The initial storage of samples for DNA extraction has been proven to be crucial step that strongly influences the quality of isolated genomic material. The samples assessed in chapter three remained in storage for several years, which likely had a negative impact. Conservation in liquid preservatives such as RNAlater during transport to the laboratory and rapid sample processing on arrival, if possible, might thus lead to improved results. In addition, the development of advanced DNA extraction protocols may aid the recovery of sufficient material. While a great number of commercial kits is available for samples from various environments (of which we tested several in the frame of this thesis), a curated method specifically adapted to the system of origin might facilitate the extraction of extremophile DNA. A critical step is the lysis of the cell, which can be challenging especially when targeting archaea as their cell wall is usually more rigid than in mesophile organisms. The implication of physical, enzymatic and chemical lysing techniques such as freeze-thawing, bead beating, proteinase K treatment, and treatments with sodium dodecyl sulfate or concentrated sodium hydroxide at 98 °C has been proven successful on samples from deep subseafloor environments (Natarajan et al., 2016).

5.3.1.2 Cultivation: Illuminating the bioreactor "black box"

The cultivation experiments provided first insight into the subsurface microbial community. Despite the enrichment of taxa such as the autotrophic *Meiothermus* genotype and the *Methanosarcinales*, however, we cannot fully extrapolate our results on Prony Bay's deep subsurface. This would require the use of light-proof bioreactors, which might be achieved by wrapping the double-jacket in opaque material such as aluminum foil.

The cultivation technique used here presents a solid proof of concept but needs adjustments: Cultivated consortia came with some surprises (notably the enrichment of normally aerobic taxa in anaerobic conditions) and left some questions open. This can be attributed to the fact that each bioreactor constitutes a "black box", where we know the initial microbial community and the enriched microbial consortium at different points in time but can only hypothesize how the community interacted and evolved over multiple generations of a metabolically diverse trophic network.

To confirm putative foundation species, more information on their individual metabolic activity is needed. The identification of foundation species from the cultivation experiments is based on their enrichment on the different carbon sources. While the community structure at different timepoints suggests that these taxa were indeed primary producers of the cultivated microbial consortia, we cannot fully exclude the possibility that they were enriched on the metabolic products of other organisms obscured by the bioreactor "black box". This is particularly complicated when assessing putative foundation species grown on formate and acetate: Both of these compounds can be produced biologically through acetogenesis and fermentation, and respective taxa such as *Acetoanaerobium* were present in the respective consortia, albeit in moderate abundances (Figure 4-5). To confirm the uptake of abiotic formate

and acetate by putative foundation species, it would be necessary to track their way through the trophic network. In theory, this is possible by performing DNA stable isotope probing (SIP). In DNA-SIP, substrates of interest are labelled with the heavier isotope of carbon, oxygen, nitrogen or another element of interest, which allows to follow their consumption in a cultivated microbial consortium. The community DNA is extracted and subsequently divided into two fractions via ultracentrifugation; the heavier fraction containing the labelled DNA will sink to the bottom of the centrifugation tube and can be recovered for amplicon or WGS sequencing (Neufeld et al., 2007). It was planned to conduct DNA-SIP in the frame of this thesis: In the second experimental run, we used ^{13}C labelled carbon substrates (sodium acetate-2-^{13}C, sodium formate-^{13}C, glycine-1-^{13}C, sodium bicarbonate-^{13}C) instead of the regular compounds. On a side note, this might explain that overall growth rates were decreased by a factor of ten in the second experimental run relative to the first one, as biological ^{13}C uptake is usually less favorable than the uptake of ^{12}C (Abraham et al., 1998). Unfortunately, we were unable to recover sufficient amounts of DNA to proceed with this analysis. As the unlabeled DNA fraction is omitted, DNA-SIP generally requires more starting material than regular genomic analyses. Future attempts might extend the incubation period to obtain more biomass (as discussed in chapter four, this might also help to re-evaluate glycine uptake); the growth curves of our consortia did not reach a plateau and acetate as well as formate were not fully consumed after three weeks, hence it might be possible to increase the cultivated cellular density.

Alternatively, the metabolic activity of hypothesized foundation species might be evaluated in axenic culture. The isolation of single serpentinite-hosted organisms has been proven challenging, most likely due to unknown symbiotic relationships which constitute an important survival strategy in oligotrophic environmental conditions. Nevertheless, our team has successfully isolated several strains from Prony Bay over the last decade and it might be possible to target at least some of the proposed foundation species in the future (Ben Aissa et al., 2014, 2015; Mei et al., 2014, 2016; Bes et al., 2015; Postec et al., 2021).

Furthermore, the consortia's metatranscriptomes could be analyzed. While this does not allow differentiation between the uptake of abiotic or biological formate and acetate, it constitutes a possibility to verify that our putative foundation species actively perform the hypothesized metabolic pathways and consume the respective carbon source. This would be especially interesting in the case of *Roseibaca*, which were strongly enriched on formate but are classified as photoheterotrophs. During the second experimental run, we collected provisional samples for RNA extraction at the end of each experiment. These samples were collected and preprocessed on ice before immediately being stored at -80 °C. As it already proved challenging to collect enough material to perform the targeted gene approach discussed in this thesis, it is unlikely that these samples contain enough material for metatranscriptomic analysis. In the frame of the PhD project, we attempted to extract RNA from culture samples which are not presented here but based on the same Prony Bay sites as the ones discussed in this thesis. The cultures samples featured relatively high cellular densities, but RNA yields were so low that WGS sequencing was only successful for a handful of samples. It might however be worth to attempt RNA extractions and WGS sequencing using protocols adapted to low-quality and low-quantity samples (Wu et al., 2011; Schuierer et al., 2017). In case this fails, it might be possible to reverse-transcribe isolated RNA into complementary DNA (cDNA) using random primers that can bind anywhere on the template. Subsequently, the full-length cDNA

can be amplified to increase its quantity (Stewart, 2013). Alternatively, a gene capture approach might be used to target transcripts of the genes discussed in chapter three. Our team is collaborating with the CaptGEN platform (Clermont-Ferrand, France) that is specialized on the design of hybridization probes. These short, synthetic sequences specifically bind to the region of interest but can be challenging to design for divergent enzyme families that are characterized by a high diversity of sequences (Gasc et al., 2016); an example for this the carbonic anhydrase which catalyzes the reversible conversion of bicarbonate to CO_2 (Kanehisa et al., 2016) .

Another bias that needs to be discussed in view of future experiments is the fact that the inoculum is derived from crushed hydrothermal chimney fragments and thus contains bicarbonate. In the experiments presented here, this bias is compensated by the fact that the consortia grown on bicarbonate featured lower growth rates that the consortia grown on formate and acetate: It is thus unlikely that bicarbonate from the inoculum was the (main) driver of growth in these culture conditions. In the opposite case, however, this issue would be very problematic. Future experiments should thus be inoculated with samples that are free from carbonaceous material. To achieve this, the hydrothermal chimney samples may be washed in buffer solution to detach the cells from solid particles. For example, a combination of three different resuspension buffers proved successful on cave rock samples (Schultz et al., 2023). This method might have the simultaneous advantage of removing auto-fluorescent particles from microscopy samples (which can complicate cell counting) and potential inhibitors of DNA and RNA extraction. However, it needs to carefully be tested to recover not only free-living cells but also cells from biofilms. Alternatively, cultures might be directly established from pure hydrothermal fluid samples. This approach has been pursued by our team with the samples from the latest field campaign to Prony Bay in 2023 and preliminary results are promising, especially concerning deep subsurface taxa (unpublished data).

5.3.1.3 Extrapolating results to the ecosystem scale

The conclusions drawn in this thesis are based on genomic regions that are registered in protein databases, as well as on cultivable microbial consortia. Both approaches are thus limited to a small excerpt of the natural community that is most likely not representative of the ecosystem as a whole. Especially regarding the deep subsurface, this poses issues as little is known on the dimension and constitution of such environments in general. In the future, larger-scale approaches might allow to draw a more accurate picture of the natural trophic network.

In metagenomics, such analyses would need to target the functional dark matter, i.e. the protein coding regions that have not yet been annotated, to expand the scope of assessed metabolic functions. The rapid development of computational resources and bioinformatic techniques might help to achieve this goal in the future. One possibility is the graphic clustering of unknown protein coding sequences into novel protein families by calculating sequence similarities. The prediction of protein tertiary structures can then aid the functional prediction of these protein families, and taxonomic annotation can be performed based on the data available for the clustered sequences. A recent study was able to double the amount of protein families obtained from a dataset with this approach (Pavlopoulos et al., 2023). The reconstruction of novel gene families would be especially interesting in the case of RuBisCo, which was missing from all putative bicarbonate-fixing foundation species presented in chapter three that otherwise featured an almost complete CBB cycle. A larger pool of metabolic

functions derived from a sample might aid the reconstruction of non-canonical metabolic pathways and facilitate the modelling of trophic networks on the ecosystem level. This could be achieved by the implementation of artificial intelligence techniques such as machine learning, which is a powerful tool to predict the complex interactions between biological, chemical, geological and physical factors in an ecosystem (Strannegard et al., 2022).

The best cultivation approach to assess global metabolic activity might be the conduction of microcosm experiments that preserve the natural environment of sampled organisms. As developed under 5.3.1.2, the implementation of isotopically labelled substate can provide insight into metabolic activity and interaction in a microbial consortium. During the field campaign to Prony Bay in 2022, we incubated microcosms containing chimney samples, hydrothermal fluid and ^{13}C-labelled bicarbonate, formate, acetate and glycine under a N_2 and H_2 rich atmosphere in hermetically sealed serum bottles. The isotopic composition of the headspace was analyzed via gas chromatography after several weeks to determine the isotopic composition of CH_4 and CO_2. Unfortunately, the concentration of these gases remained below the detection limit. As discussed in chapter three and four, similar experiments were conducted on communities from others serpentinite-hosted environments with mixed results (Crespo-Medina et al., 2014; Morrill et al., 2014; Kohl et al., 2016). Future studies might attempt the installation of *in situ* microcosms to further approach the natural environment, even though this might be challenging when targeting the deep subsurface. Overall, interdisciplinary studies coupling the analysis of the geological, chemical and biological environment are needed to assess the ecosystem scale.

5.3.2 Looking into alternative carbon sources and metabolic types

Besides the optimization of bioinformatic and experimental protocols, the identification of deep subsurface foundation species might require looking into alternative carbon sources. Experiments conducted in the context of origin of life research have revealed a relatively broad range of organic compounds that can be produced abiotically in a laboratory setting. These include the Miller-Urey experiment, Strecker synthesis and Butlerow's formose reaction mentioned in the introduction of this thesis, which yielded various amino acids and simple sugars (Strecker, 1850; Butlerow, 1861; Miller and Urey, 1959; Müller et al., 2022). While it is not proven that these reactions can occur in a natural setting, they could hypothetically aliment fermentative foundation species in the deep subsurface. Fermentation is defined as an anaerobic catabolic metabolism where organic compounds simultaneously act as both electron donors and electron acceptors. This usually does not yield as much metabolic energy as the use of classic electron acceptors with higher redox potentials. Nevertheless, fermentation is a successful survival strategy in anaerobic environments where electron acceptors are scarce, and a vast diversity of fermentative reactions and organisms has been described (Hackmann, 2024). Electron acceptor availability in serpentinite-hosted environments largely depends on supply from seawater, which diminishes with increasing depth. Consequently, fermenting taxa such as *Clostridiales, Paceibacteria* and *Bipolaricaulia* have been shown to be abundant in Prony Bay (Postec et al., 2015; Frouin et al., 2018). Most cases of sugar and amino acid fermentation produce CO_2 as a by-product and would thus be candidates for foundation pathways. Examples

include alcohol, mixed acid, butanediol, glutamate, lysine, ornithine, tryptophan, methionine, histidine fermentation and the Stickland reaction where pairs of amino acids are being consumed. It might be worth to measure the isotopic signature of fermentation precursors in serpentinite-hosted environments to determine their origin and assess the presence of fermentative pathways. This would also make sense in the light of the carbon sources discussed in this thesis, as acetate is one of the most common products of fermentation (Hackmann, 2024). Assessing and quantifying the occurrence of potential fermentative foundation species might thus also shed light on whether the identified acetate-assimilating (and formate-fixing) taxa are actual foundation species or rather part of the first trophic level.

Next to *Gammaproteobacteria* (the most common bacterial class is serpentinite-hosted environments), the prevalence of phototrophs in our hypothesized foundation consortia is remarkable. This metabolic type has not been considered much when assessing serpentinite hosted environments, but our results suggest that it plays an important role not only in surface but also in shallow subsurface environments. With increasing depth, chlorophyll pigments are replaced by bacteriochlorophyll which can absorb near-infrared wavelengths (Hanada, 2016). It has been suggested that infrared utilizing phototrophs colonize the deep subsurface of hydrothermal vents by using thermal radiation emitted by the hot fluids (Díaz et al., 2013). This is supported by the fact that a green sulfur bacterium was isolated from a deep-sea volcanic hydrothermal vent in 2005 (Beatty et al., 2005). In addition to fermentative organisms, it might thus be interesting to examine a potential occurrence of phototrophs in Prony Bay's deep subsurface.

5.4 Links to the origin of life

The identification of both heterotrophic and autotrophic foundation species in a system that is potentially analogous to Early Earth evokes the debate on a heterotrophic versus autotrophic origin of life. The heterotrophic theory implies that organic compounds came before primordial metabolism, either from comets or from abiotic chemistry. The "primitive soup" theory has dominated the research field for a long time and is still one of the leading concepts today. However, it has several weaknesses, most importantly the fact that it does not align with the chemistry of modern cells and that it is difficult to reconcile with thermodynamic laws (Schönheit et al., 2016; Duval et al., 2021). This is why many modern approaches are currently centered around an autotrophic origin of life. The general idea is that minerals containing transition metals (notably greigite, mackinawite, fougerite and pyrite) catalyzed self-sustaining autotrophic carbon fixation reactions at the emergence of life, harvesting energy from a continuous electrochemical disequilibrium just like modern life does. All of these minerals are found in serpentinite-hosted environments and play a role in Russel's alkaline hydrothermal vent theory (Russell et al., 2013; Russell and Ponce, 2020). Primordial metalloenzymes could also explain the origin of early heterotrophic pathways. In the frame of autotrophic theories of the origin of life, it has for example been suggested that the first heterotroph evolved in environments with lower H_2 partial pressures by fermenting amino acids, bases and ribose derived from autotrophs. Notably, a RuBisCo variant is present in deep-branching fermenters and might constitute a bridge between early autotrophs and heterotrophs (Schönheit et al., 2016).

Both carbon and carbohydrate metabolism involve pathways that are potentially ancient and include reactions that can be catalyzed without enzymes. Autotrophic carbon metabolism relies on the carbon fixation pathways discussed in chapter three. While the CBB cycle is believed to have evolved early, it probably only emerged after the great oxygenation event and is thus not relevant for the origin of life (Ward and Shih, 2019). On the contrary, the Wood-Ljungdahl pathway and rTCA cycle have been proposed to constitute a direct link between prebiotic chemistry and metabolism. The Wood-Ljungdahl pathway is employed by deep-branching acetogens and methanogens and yields electrons from H_2, rendering it the ideal candidate for an autotrophic origin of life in a hydrothermal setting. It also includes a large proportion of metalloenzymes which could be derived from non-enzymatic mineral catalysts (although this has not been demonstrated for all pathway reactions) (Muchowska et al., 2020). Likewise, metal-based cofactors play a central role in the rTCA cycle. It is assumed that a simplified version of the rTCA pathway might have arisen at the origin of life, and also the prebiotic carbon fixation pathway proposed by Wächtershäuser (mentioned in the introduction of this thesis) concerns an iron-sulfur based variation of the rTCA cycle (Wächtershäuser, 1992; Muchowska et al., 2020). Both the Wood-Ljungdahl pathway and the rTCA cycle were absent from the MAGs reconstructed in chapter three. As the metagenomic results were limited to surface foundation taxa, however, this is not surprising. The improvement of sampling, DNA

extraction and bioinformatic techniques as discussed under 5.3 might allow a reassessment of the occurrence of these pathways in anaerobic deep subsurface communities.

Heterotrophic carbohydrate metabolism includes glycolysis, the breakdown of glucose into pyruvate to generate ATP, the pentose phosphate pathway, and gluconeogenesis, the synthesis of glucose from non-carbohydrate precursors. While only one non-enzymatic reaction has been demonstrated for gluconeogenesis so far, the enzymes involved in this pathway are conserved across deep-branching organism and certain anionic groups of its intermediates might point to a prebiotic origin. Concerning glycolysis and the pentose phosphate pathway, it has been shown that many of their reactions can be catalyzed by iron (Muchowska et al., 2020). While we did not specifically assess gluconeogenesis, glycolysis and the pentose phosphate pathway in this thesis, they are fundamental pillars of heterotrophy and likely operate in the putative heterotrophic foundation species we identified.

Metalloenzymes are not only important in carbon and carbohydrate metabolism, but also in lithotrophic energy metabolism and fermentation. The most prominent examples are NiFe and FeFe hydrogenases, whose functions include the uptake and fermentative production of H_2. While it is still under debate whether the early atmosphere was reducing (H_2-rich) or neutral (Cleaves et al., 2008), H_2 metabolism is generally assumed to be an early feature of bioenergetics (Vignais and Billoud, 2007). Several putative surface foundation species identified through the metagenomic approach featured NiFe hydrogenases, and we enriched some taxa know to be hydrogenotrophic in the anaerobic cultivation experiments, indicating that the surface and shallow subsurface communities are not entirely irrelevant with regard to the origin of life (Figure 5-1). When assessing deep subsurface foundation species, it may be interesting to re-evaluate the formate dehydrogenase, which shares structural and functional similarities with FeFe hydrogenases and is part of the Wood-Ljungdahl pathway (Morra, 2022). In addition, it might be valuable to target fermenters with FeFe hydrogenases.

A special case regarding bioenergetics at the emergence of life is flavin-based electron bifurcation. This mechanism couples thermodynamically favorable reactions with unfavorable ones and is employed by certain enzymes to maximize energetic efficiency. The active flavin center of such enzymes can accept electrons from a single electron donor and simultaneously transfer them to a high-potential and a low-potential electron acceptor. The free energy from the exergonic redox reaction is thereby used to catalyze the endergonic one. Enzymes performing flavin-based electron bifurcation include hydrogenases, the nitrogenase complex and enzymes involved in fermentation, thus both autotrophic and heterotrophic processes (Buckel and Thauer, 2018). Even though the corresponding enzyme has not yet been described, it is believed that the transition metals tungsten and molybdenum can perform electron bifurcating reactions. Molybdenum is released during serpentinization and might have catalyzed prebiotic processes in interaction with fougerite, before forming early enzymes with simple organic pterin ligands. The latter exhibit high structural similarity with flavin, which might thus be a particularly ancient organic molecule (Baymann et al., 2018). While none of the putative foundation species identified in chapter three feature a bifurcating hydrogenase (which is not surprising as they are mostly aerobic), several other high-abundance MAGs did (Figure 3-5); it might be interesting to further look into their metabolism.

As Schönheit (2016) put it, the autotrophic origin of life offers “a simple, stable, and ‘down to Earth’ starting point for the further evolution of microbial physiology”. Nevertheless, the

results presented in this thesis suggest that not only autotrophs, but also heterotrophs play a fundamental role in the trophic network of a system that can be interpreted as an Early Earth analog. In this case, delving deeper into the subject could be taken quite literally: Prony Bay's deep subsurface might provide clearer answers.

5.5 References

Abraham, W.-R., Hesse, C., and Pelz, O. (1998). Ratios of Carbon Isotopes in Microbial Lipids as an Indicator of Substrate Usage. *Appl. Environ. Microbiol.* 64, 4202–4209. doi: 10.1128/AEM.64.11.4202-4209.1998

Baymann, F., Schoepp-Cothenet, B., Duval, S., Guiral, M., Brugna, M., Baffert, C., et al. (2018). On the Natural History of Flavin-Based Electron Bifurcation. *Front. Microbiol.* 9, 1357. doi: 10.3389/fmicb.2018.01357

Beatty, J. T., Overmann, J., Lince, M. T., Manske, A. K., Lang, A. S., Blankenship, R. E., et al. (2005). An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. *Proc. Natl. Acad. Sci.* 102, 9306–9310. doi: 10.1073/pnas.0503674102

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2014). Vallitalea pronyensis sp. nov., isolated from a marine alkaline hydrothermal chimney. *Int. J. Syst. Evol. Microbiol.* 64, 1160–1165. doi: 10.1099/ijs.0.055756-0

Ben Aissa, F., Postec, A., Erauso, G., Payri, C., Pelletier, B., Hamdi, M., et al. (2015). Characterization of Alkaliphilus hydrothermalis sp. nov., a novel alkaliphilic anaerobic bacterium, isolated from a carbonaceous chimney of the Prony hydrothermal field, New Caledonia. *Extremophiles* 19, 183–188. doi: 10.1007/s00792-014-0697-y

Bes, M., Merrouch, M., Joseph, M., Quéméneur, M., Payri, C., Pelletier, B., et al. (2015). Acetoanaerobium pronyense sp. nov., an anaerobic alkaliphilic bacterium isolated from a carbonate chimney of the Prony Hydrothermal Field (New Caledonia). *Int. J. Syst. Evol. Microbiol.* 65, 2574–2580. doi: 10.1099/ijs.0.000307

Brazelton, W. J., McGonigle, J. M., Motamedi, S., Pendleton, H. L., Twing, K. I., Miller, B. C., et al. (2022). Metabolic Strategies Shared by Basement Residents of the Lost City Hydrothermal Field. *Appl. Environ. Microbiol.* 88, e00929-22. doi: 10.1128/aem.00929-22

Buckel, W., and Thauer, R. K. (2018). Flavin-Based Electron Bifurcation, Ferredoxin, Flavodoxin, and Anaerobic Respiration With Protons (Ech) or NAD+ (Rnf) as Electron Acceptors: A Historical Review. *Front. Microbiol.* 9, 401. doi: 10.3389/fmicb.2018.00401

Butlerow, A. M. (1861). Bildung einer zuckerartigen Substanz durch Synthese. *Justus Liebigs Ann. Chem.* 120, 295–298.

Cleaves, H. J., Chalmers, J. H., Lazcano, A., Miller, S. L., and Bada, J. L. (2008). A reassessment of prebiotic organic synthesis in neutral planetary atmospheres. *Orig. Life Evol. Biosphere J. Int. Soc. Study Orig. Life* 38, 105–115. doi: 10.1007/s11084-007-9120-3

Crespo-Medina, M., Twing, K. I., Kubo, M. D. Y., Hoehler, T. M., Cardace, D., McCollom, T., et al. (2014). Insights into environmental controls on microbial communities in a continental serpentinite aquifer using a microcosm-based approach. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00604

Díaz, N., Cardenas, R., Martín, O., and Leiva Mora, M. (2013). The potential for photosynthesis in hydrothermal vents: A new avenue for life in the Universe? *Astrophys. Space Sci.* 346. doi: 10.1007/s10509-013-1460-z

Duval, S., Zuchan, K., Baymann, F., Schoepp-Cothenet, B., Branscomb, E., Russell, M. J., et al. (2021). "5 MINERALS AND THE EMERGENCE OF LIFE," in *Metals, Microbes, and Minerals - The Biogeochemical Side of Life*, eds. P. Kroneck and M. Sosa Torres (De Gruyter), 135–158. doi: 10.1515/9783110589771-011

Frouin, E., Bes, M., Ollivier, B., Quéméneur, M., Postec, A., Debroas, D., et al. (2018). Diversity of Rare and Abundant Prokaryotic Phylotypes in the Prony Hydrothermal Field and Comparison with Other Serpentinite-Hosted Ecosystems. *Front. Microbiol.* 9, 102. doi: 10.3389/fmicb.2018.00102

Gasc, C., Peyretaillade, E., and Peyret, P. (2016). Sequence capture by hybridization to explore modern and ancient genomic diversity in model and nonmodel organisms. *Nucleic Acids Res.* 44, 4504–4518. doi: 10.1093/nar/gkw309

Hackmann, T. J. (2024). The vast landscape of carbohydrate fermentation in prokaryotes. *FEMS Microbiol. Rev.* 48, fuae016. doi: 10.1093/femsre/fuae016

Hanada, S. (2016). Anoxygenic Photosynthesis —A Photochemical Reaction That Does Not Contribute to Oxygen Reproduction—. *Microbes Environ.* 31, 1–3. doi: 10.1264/jsme2.ME3101rh

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 44, D457–D462. doi: 10.1093/nar/gkv1070

Kohl, L., Cumming, E., Cox, A., Rietze, A., Morrissey, L., Lang, S. Q., et al. (2016). Exploring the metabolic potential of microbial communities in ultra-basic, reducing springs at The Cedars, CA, USA: Experimental evidence of microbial methanogenesis and heterotrophic acetogenesis. *J. Geophys. Res. Biogeosciences* 121, 1203–1220. doi: 10.1002/2015JG003233

Lang, S. Q., Früh-Green, G. L., Bernasconi, S. M., Brazelton, W. J., Schrenk, M. O., and McGonigle, J. M. (2018). Deeply-sourced formate fuels sulfate reducers but not methanogens at Lost City hydrothermal field. *Sci. Rep.* 8, 755. doi: 10.1038/s41598-017-19002-5

McGonigle, J. M., Lang, S. Q., and Brazelton, W. J. (2020). Genomic Evidence for Formate Metabolism by *Chloroflexi* as the Key to Unlocking Deep Carbon in Lost City Microbial Ecosystems. *Appl. Environ. Microbiol.* 86, e02583-19. doi: 10.1128/AEM.02583-19

Mei, N., Postec, A., Erauso, G., Joseph, M., Pelletier, B., Payri, C., et al. (2016). Serpentinicella alkaliphila gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from

the serpentinite-hosted Prony hydrothermal field, New Caledonia. *Int. J. Syst. Evol. Microbiol.* 66, 4464–4470. doi: 10.1099/ijsem.0.001375

Mei, N., Zergane, N., Postec, A., Erauso, G., Ollier, A., Payri, C., et al. (2014). Fermentative hydrogen production by a new alkaliphilic Clostridium sp. (strain PROH2) isolated from a shallow submarine hydrothermal chimney in Prony Bay, New Caledonia. *Int. J. Hydrog. Energy* 39, 19465–19473. doi: 10.1016/j.ijhydene.2014.09.111

Miller, S. L., and Urey, H. C. (1959). Organic Compound Synthesis on the Primitive Earth. *Science* 130, 245–251. doi: 10.1126/science.130.3370.245

Morra, S. (2022). Fantastic [FeFe]-Hydrogenases and Where to Find Them. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.853626

Morrill, P. L., Brazelton, W. J., Kohl, L., Rietze, A., Miles, S. M., Kavanagh, H., et al. (2014). Investigations of potential microbial methanogenic and carbon monoxide utilization pathways in ultra-basic reducing springs associated with present-day continental serpentinization: the Tablelands, NL, CAN. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00613

Muchowska, K. B., Varma, S. J., and Moran, J. (2020). Nonenzymatic Metabolic Reactions and Life's Origins. *Chem. Rev.* 120, 7708–7744. doi: 10.1021/acs.chemrev.0c00191

Müller, F., Escobar, L., Xu, F., Węgrzyn, E., Nainytė, M., Amatov, T., et al. (2022). A prebiotically plausible scenario of an RNA–peptide world. *Nature* 605, 279–284. doi: 10.1038/s41586-022-04676-3

Natarajan, V. P., Zhang, X., Morono, Y., Inagaki, F., and Wang, F. (2016). A Modified SDS-Based DNA Extraction Method for High Quality Environmental DNA from Seafloor Environments. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00986

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W., et al. (2007). DNA stable-isotope probing. *Nat. Protoc.* 2, 860–866. doi: 10.1038/nprot.2007.109

Pavlopoulos, G. A., Baltoumas, F. A., Liu, S., Selvitopi, O., Camargo, A. P., Nayfach, S., et al. (2023). Unraveling the functional dark matter through global metagenomics. *Nature* 622, 594–602. doi: 10.1038/s41586-023-06583-7

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00857

Postec, A., Quéméneur, M., Lecoeuvre, A., Chabert, N., Joseph, M., and Erauso, G. (2021). Alkaliphilus serpentinus sp. nov. and Alkaliphilus pronyensis sp. nov., two novel anaerobic alkaliphilic species isolated from the serpentinite-hosted Prony Bay Hydrothermal Field (New Caledonia). *Syst. Appl. Microbiol.* 44, 126175. doi: 10.1016/j.syapm.2020.126175

Russell, M. J., Nitschke, W., and Branscomb, E. (2013). The inevitable journey to being. *Philos. Trans. R. Soc. B Biol. Sci.* 368, 20120254. doi: 10.1098/rstb.2012.0254

Russell, M. J., and Ponce, A. (2020). Six “Must-Have” Minerals for Life’s Emergence: Olivine, Pyrrhotite, Bridgmanite, Serpentine, Fougerite and Mackinawite. *Life Basel Switz.* 10, 291. doi: 10.3390/life10110291

Schönheit, P., Buckel, W., and Martin, W. F. (2016). On the Origin of Heterotrophy. *Trends Microbiol.* 24, 12–25. doi: 10.1016/j.tim.2015.10.003

Schuierer, S., Carbone, W., Knehr, J., Petitjean, V., Fernandez, A., Sultan, M., et al. (2017). A comprehensive assessment of RNA-seq protocols for degraded and low-quantity samples. *BMC Genomics* 18, 442. doi: 10.1186/s12864-017-3827-y

Schultz, J., Modolon, F., Peixoto, R. S., and Rosado, A. S. (2023). Shedding light on the composition of extreme microbial dark matter: alternative approaches for culturing extremophiles. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1167718

Stewart, F. J. (2013). “Chapter Ten - Preparation of Microbial Community cDNA for Metatranscriptomic Analysis in Marine Plankton,” in *Methods in Enzymology*, ed. E. F. DeLong (Academic Press), 187–218. doi: 10.1016/B978-0-12-407863-5.00010-1

Strannegard, C., Engsner, N., Eisfeldt, J., Endler, J., Hansson, A., Lindgren, R., et al. (2022). Ecosystem Models Based on Artificial Intelligence., in *2022 Swedish Artificial Intelligence Society Workshop (SAIS)*, (Stockholm, Sweden: IEEE), 1–9. doi: 10.1109/SAIS55783.2022.9833026

Strecker, A. (1850). Ueber die künstliche Bildung der Milchsäure und einen neuen, dem Glycocoll homologen Körper. *Justus Liebigs Ann. Chem.* 75, 27–45. doi: 10.1002/jlac.18500750103

Vignais, P. M., and Billoud, B. (2007). Occurrence, Classification, and Biological Function of Hydrogenases: An Overview. *Chem. Rev.* 107, 4206–4272. doi: 10.1021/cr050196r

Wächtershäuser, G. (1992). Groundworks for an evolutionary biochemistry: The iron-sulphur world. *Prog. Biophys. Mol. Biol.* 58, 85–201. doi: 10.1016/0079-6107(92)90022-X

Ward, L. M., and Shih, P. M. (2019). The evolution and productivity of carbon fixation pathways in response to changes in oxygen concentration over geological time. *Free Radic. Biol. Med.* 140, 188–199. doi: 10.1016/j.freeradbiomed.2019.01.049

Wu, J., Gao, W., Zhang, W., and Meldrum, D. R. (2011). Optimization of whole-transcriptome amplification from low cell density deep-sea microbial samples for metatranscriptomic analysis. *J. Microbiol. Methods* 84, 88–93. doi: 10.1016/j.mimet.2010.10.018