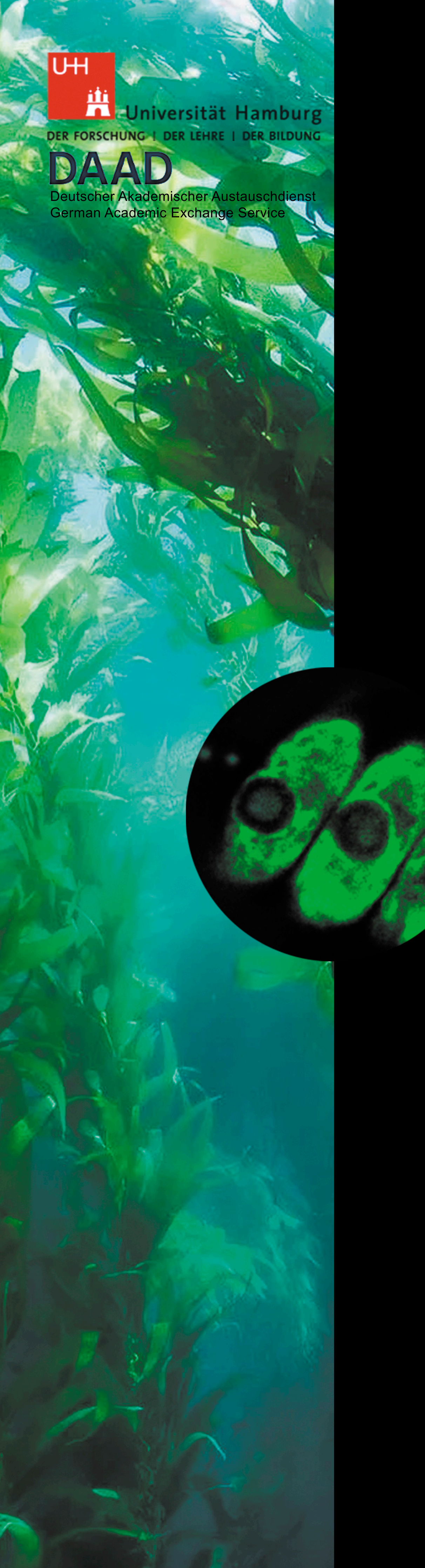

Microalgae and Bacteria Interaction

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Microalgae and Bacteria Interaction

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Eidesstattliche Versicherung - Declaration on oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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On oath, I declare that I have written the present dissertation on my own and have not used any other than the acknowledged sources and aids.

Hamburg, 13.07.2023

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Yekaterina Astafyeva

“Je n'ai fait celle-ci plus longue que parce que je n'ai pas eu le loisir de la faire plus courte.”
Les provinciales (1656), XVI de Blaise Pascal

“I have only made this letter longer because I have not had the time to make it shorter.”
The Provincial Letters (1657), Letter 16, Blaise Pascal

Contribution to the quoted articles

Astafyeva, Y., Alawi, M., Indenbirken, D., Danso, D., Grundhoff, A., Hanelt, D., Streit, W. R., and Krohn I. (2020). Draft genome sequence of the green alga *Scenedesmus acuminatus* SAG 38.81. Microbiology Resource Announcements, https://doi.org/10.1128/MRA.01278-19

— planning and performance of the microalga cultivation

— cultivation of the *Scenedesmus acuminatus* SAG 38.81 and extraction of the total genomic DNA

— reporting the draft whole-genome sequence of *Scenedesmus acuminatus* SAG 38.81

— contribution to the writing of the research article

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— planning, performance, and evaluation of the microalga-bacteria co-culturing

— investigation of the microbiota of *Scenedesmus quadricauda* MZCH 10104 and providing evidence of dominant species affiliated with the genera of *Variovorax*, *Porphyrobacter*, and *Dyadobacter*

— establishment of an artificial plant-bacteria system of the microalga *S. quadricauda* MZCH 10104 and the bacterial isolate *Dyadobacter* sp. HH091

— bacterial DNA and RNA extraction

— *Dyadobacter* sp. HH091 transformation with modified plasmid pBBR1MCS-5-eGFP

— physiological analyses, including microscopy investigations, pulse-amplitude-modulation (PAM) fluorometry, and fluorescence-activated cell sorting (FACS)

— contribution to bioinformatic evaluation and illustration of the transcriptome data

— contribution to experimental design and lab work of phylogenetic, metatranscriptomic, genomic, bioinformatic, and physiological analytical approaches

— contribution to the writing of the research article

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— planning, performance, and evaluation of experimental work

— examination of the bacterial colonisation and penetration into the cell wall of the algal host *M. radians* MZCH 672

— establishment of an artificial plant-bacteria system of the microalga *Micrasterias radians* MZCH 672 and the bacterial isolate *Dyadobacter* sp. HH091

— characterisation of the type IX secretion system (T9SS) mechanism implementing the attachment and invasion of microalga by the isolate HH091 and proposing the first model of the T9SS apparatus of *Dyadobacter* sp. HH091

— studying the isolate HH091 and its interaction with the microalga *M. radians* using transcriptome and extensive genome analyses

— prediction of polysaccharide utilising gene clusters of *Dyadobacter* sp. HH091 co-working with the T9SS conceivably involved in the algae-bacteria liaison

— contribution to bioinformatic evaluation and illustration of the transcriptome data

— contribution to experimental design and lab work of phylogenetic, metatranscriptome, genomic, bioinformatic, and physiological analytical approaches

— contribution to the writing of the research article

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— contribution to the evaluation and illustration of the bioinformatic data

— co-writing and proof-reading of the manuscript

Hamburg, 11.7.2023

Prof. Dr. Wolfgang R. Streit (Supervisor)

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ABSTRACT

Microalgae are among Earth's most prevalent life forms and are tightly associated with distinct and specialised microbiota. It is widely acknowledged that bacteria-algal interactions can enhance algal biomass production and enrich it with valuable compounds like lipids, carbohydrates, and pigments. However, a complete understanding of the complex interrelationships among various kingdoms and the distinct function of each organism remains elusive.

The following research topics were covered during this study:

(i) My study started with investigating the mutualistic collaboration of microalgae and bacteria, where my goal was to reveal the individual role of the members of the multispecies consortia. It was found that the algal microbiota, harbouring less than ten distinct microbial species, includes dominant species affiliated with *Variovorax*, *Porphyrobacter*, and *Dyadobacter* genera. The study provided strong evidence that *Dyadobacter* produces and releases polysaccharides degradation enzymes and leucine-rich repeat proteins; *Variovorax* supplies the consortium with auxins and vitamin B_{12}, while *Porphyrobacter* produces a broad spectrum of B vitamins. Studies have conclusively shown that microalgae and bacteria function synergistically, with bacteria employing quorum sensing and secretion system mechanisms for communication between themselves. The shared currency between partners appeared to be vitamins, microalgae growth-promoting substances, and dissolved carbon. Experimental and transcriptome-based evidence implied that *Dyadobacter* is a key to alga growth and fitness within this multispecies interaction and is highly adapted to the phycosphere. Through studying the synthetic plant-bacteria system, it was unveiled that the growth of the microalga *Scenedesmus quadricauda* was greatly stimulated by one of the bacterial isolates. This isolate was originally taken from the non-axenic algal culture of *S. quadricauda* and was identified as *Dyadobacter* sp. HH091. Further investigations were conducted using physiological methods after adding *Dyadobacter* sp. to axenic algal cultures. To perform the required investigations, I examined the samples using a microscope, measured the photosynthetic activity, and employed flow cytometry. The photosynthetic activity measurements by pulse-amplitude-modulation fluorometry demonstrated that the co-culturing of *S. quadricauda* with HH091 resulted in a 1.5-fold increase of the optimal quantum yield of photosystem II compared to control antibiotic-treated microalgae. Additional microscopic and fluorescence-activated cell sorting analyses verified that the number of viable and photosynthetic active cells in the presence of HH091 was much higher than in antibiotic-treated cultures.

(ii) Based on former observations, I established an artificial plant-bacteria system of the microalga *Micrasterias radians* MZCH 672 and the bacterial isolate *Dyadobacter* sp. HH091. I aimed to investigate microalgae cultivation and its symbiont utilising diverse physiological and omics-based techniques. The simultaneous identification and localisation of the isolate HH091 associated with the host microalga, either attached or intracellular, was performed using the combination of fluorescence labelling with confocal microscopy. The bacterial isolate has been found to colonise and penetrate the cell wall of *M. radians* MZCH 672. The isolate HH091, from the Bacteroidota phylum, significantly boosted microalgae growth in axenic algal cultures. For further advances, I studied the isolate and its interaction with the microalga *M. radians* using transcriptome and extensive genome analyses. Through the genome analysis of HH091, I identified gene clusters predicted to utilise polysaccharides and work alongside the type IX secretion system (T9SS) influencing investigated algae-bacteria liaison. These studies have gained a better understanding of how *Dyadobacter* sp. HH091 utilises the T9SS mechanism to attach to and invade microalgae. Omics analysis exposed T9SS genes: *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprF*, *sprT*, *porU* and *porV*. In addition, I identified genes that are crucial for gliding motility and protein secretion (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ*), despite not being conventionally categorised as T9SS components. Based on this extensive research, I proposed the first model for the T9SS apparatus in *Dyadobacter*.

(iii) To summarise, I provided a brief overview of microalgae microbiota and its use. I aimed to share the current knowledge of the health benefits of microalgae and the associated microbiota. In this chapter, I will discuss the current limitations hindering the full exploitation and development of technologies. To fully unlock the potential of microalgae and its microbiota, a more systematic approach can be created by addressing current limitations. While microalgae are well explored for the generation of biofuels, their potential as a source of antimicrobial and prebiotic substances has recently received increasing interest. Within this framework, microalgae may offer solutions to the societal challenge concerning the lack of antibiotics treating the growing level of antimicrobial-resistant bacteria and fungi in clinical settings. Furthermore, considering that most microalgae and associated microbiota remain unstudied, they may be a fascinating and rewarding source for novel, more sustainable antimicrobials, alternative molecules, and compounds. Finally, I contributed to describing the remaining issues and limitations and several promising research potentials to understand perspectives and future tasks for microalgae-bacteria research.

In summary, this data provided a valuable contribution to our understanding of how algae and bacteria interact. I thoroughly examined the mutually beneficial relationship between bacteria and algae, which also involves competition within the algal microbiome. This doctoral research used experimental and transcriptome-based evidence to examine how symbiotic bacteria interact with microalgae and enable their dominance.

ZUSAMMENFASSUNG

Mikroalgen gehören zu den am weitesten verbreiteten Lebensformen auf der Erde und sind eng mit unterschiedlichen und spezialisierten Mikrobiota verbunden. Es ist allgemein anerkannt, dass Wechselwirkungen zwischen Bakterien und Algen die Produktion von Algenbiomasse steigern und diese mit wertvollen Verbindungen wie Lipiden, Kohlenhydraten und Pigmenten anreichern können. Ein vollständiges Verständnis der komplexen Wechselbeziehungen zwischen verschiedenen Königreichen und der unterschiedlichen Funktion jedes einzelnen Organismus bleibt jedoch schwer zu erreichen.

Die folgenden Forschungsthemen wurden im Rahmen dieser Studie behandelt:

(i) Meine Studie begann mit der Untersuchung der gegenseitigen Zusammenarbeit von Mikroalgen und Bakterien, wobei mein Ziel darin bestand, die individuelle Rolle der Mitglieder der Multispezies-Konsortien aufzudecken. Es wurde festgestellt, dass die Algenmikrobiota, die weniger als zehn verschiedene Mikroorganismenarten beherbergt, dominante Arten umfasst, die mit den Gattungen *Variovorax*, *Porphyrobacter* und *Dyadobacter* verbunden sind. Die Studie lieferte starke Beweise dafür, dass *Dyadobacter* Enzyme zum Abbau von Polysacchariden und Leucin-reiche Wiederholungsproteine produziert und freisetzt; *Variovorax* versorgt das Konsortium mit Auxinen und Vitamin B_{12}, während *Porphyrobacter* ein breites Spektrum an B-Vitaminen produziert. Studien haben schlüssig gezeigt, dass Mikroalgen und Bakterien synergistisch funktionieren, wobei Bakterien Quorum-Sensing- und Sekretionssystemmechanismen für die Kommunikation untereinander nutzen. Die gemeinsame Währung zwischen den Partnern schien Vitamine, das Wachstum von Mikroalgen fördernde Substanzen und gelöster Kohlenstoff zu sein. Experimentelle und transkriptombasierte Daten deuten darauf hin, dass *Dyadobacter* ein Schlüssel zum Algenwachstum und zur Algenfitness innerhalb dieser Multispezies-Interaktion ist und sich in hohem Maße an die Phykosphäre angepasst hat. Durch die Untersuchung des synthetischen Pflanzen-Bakterien-Systems wurde festgestellt, dass das Wachstum der Mikroalge *Scenedesmus quadricauda* durch eines der Bakterienisolate stark stimuliert wurde. Dieses Isolat stammte ursprünglich aus der nicht-axenischen Algenkultur von *S. quadricauda* und wurde als *Dyadobacter* sp. HH091 identifiziert. Weitere Untersuchungen wurden mit physiologischen Methoden nach Zugabe von *Dyadobacter* sp. HH091 zu axenischen Algenkulturen durchgeführt. Um die erforderlichen Untersuchungen durchzuführen, habe ich die Proben mit einem Mikroskop untersucht, die photosynthetische Aktivität gemessen und Durchflusszytometrie eingesetzt. Die Messungen der photosynthetischen Aktivität

mittels Pulsamplitudenmodulations-Fluorometrie zeigten, dass die Co-Kultivierung von *S. quadricauda* mit HH091 zu einer 1,5-fachen Steigerung der optimalen Quantenausbeute von Photosystem II im Vergleich zu mit Antibiotika behandelten Kontroll-Mikroalgen führte. Zusätzliche mikroskopische und fluoreszenzaktivierte Zellsortierungsanalysen bestätigten, dass die Anzahl lebensfähiger und photosynthetisch aktiver Zellen in Gegenwart von HH091 viel höher war als in mit Antibiotika behandelten Kulturen.

(ii) Basierend auf früheren Beobachtungen habe ich ein künstliches Pflanzen-Bakterien-System aus der Mikroalge *Micrasterias radians* MZCH 672 und dem Bakterienisolat *Dyadobacter* sp. HH091 etabliert. Mein Ziel war es, die Mikroalgenkultivierung und ihre Symbionten mithilfe verschiedener physiologischer und Omics-basierter Techniken zu untersuchen. Die gleichzeitige Identifizierung und Lokalisierung des mit der Wirtsmikroalge assoziierten Isolats HH091, entweder angehängt oder intrazellulär, wurde mithilfe der Kombination von Fluoreszenzmarkierung und konfokaler Mikroskopie durchgeführt. Es wurde festgestellt, dass das Bakterienisolat die Zellwand von *M. radians* MZCH 672 besiedelt und durchdringt. Das Isolat HH091 aus dem Bacteroidota-Stamm steigerte das Mikroalgenwachstum in axenischen Algenkulturen erheblich. Für weitere Fortschritte habe ich das Isolat und seine Wechselwirkung mit der Mikroalge *M. radians* mithilfe von Transkriptom- und umfangreichen Genomanalysen untersucht. Durch die Genomanalyse von HH091 habe ich Gencluster identifiziert, von denen vorhergesagt wird, dass sie Polysaccharide nutzen und neben dem Typ-IX-Sekretionssystem (T9SS) zusammenarbeiten, um die untersuchte Algen-Bakterien-Verbindung zu beeinflussen. Diese Studien haben zu einem besseren Verständnis darüber geführt, wie *Dyadobacter* sp. HH091 den T9SS-Mechanismus nutzt, um sich an Mikroalgen anzuheften und in diese einzudringen. Durch die Omics-Analyse wurden T9SS-Gene freigelegt: *gldK, gldL, gldM, gldN, sprA, sprE, sprF, sprT, porU* und *porV*. Darüber hinaus habe ich Gene identifiziert, die für die Gleitmotilität und Proteinsekretion entscheidend sind (*gldA, gldB, gldD, gldF, gldG, gldH, gldI, gldJ*), obwohl sie herkömmlicherweise nicht als T9SS-Komponenten kategorisiert werden. Basierend auf dieser umfangreichen Forschung habe ich das erste Modell für den T9SS-Apparat in Dyadobacter vorgeschlagen.

(iii) Zusammenfassend habe ich einen kurzen Überblick über die Mikroalgen-Mikrobiota und ihre Verwendung gegeben. Mein Ziel war es, das aktuelle Wissen über die gesundheitlichen Vorteile von Mikroalgen und der damit verbundenen Mikrobiota zu

teilen. In diesem Kapitel werde ich die aktuellen Einschränkungen diskutieren, die die vollständige Nutzung und Entwicklung von Technologien behindern. Um das Potenzial von Mikroalgen und ihrer Mikrobiota voll auszuschöpfen, kann ein systematischerer Ansatz geschaffen werden, indem aktuelle Einschränkungen angegangen werden. Während Mikroalgen für die Herstellung von Biokraftstoffen gut erforscht sind, stößt ihr Potenzial als Quelle antimikrobieller und präbiotischer Substanzen in letzter Zeit zunehmend auf Interesse. In diesem Rahmen können Mikroalgen Lösungen für die gesellschaftliche Herausforderung bieten, die sich aus dem Mangel an Antibiotika zur Behandlung der wachsenden Zahl antimikrobiell resistenter Bakterien und Pilze im klinischen Umfeld ergibt. Wenn man außerdem bedenkt, dass die meisten Mikroalgen und die damit verbundene Mikrobiota noch unerforscht sind, könnten sie eine faszinierende und lohnende Quelle für neuartige, nachhaltigere antimikrobielle Mittel, alternative Moleküle und Verbindungen sein. Abschließend habe ich dazu beigetragen, die verbleibenden Probleme und Einschränkungen sowie mehrere vielversprechende Forschungspotenziale zu beschreiben, um Perspektiven und zukünftige Aufgaben für die Mikroalgen-Bakterien-Forschung zu verstehen.

Zusammenfassend stellten diese Daten einen wertvollen Beitrag zu unserem Verständnis der Interaktion von Algen und Bakterien dar. Ich habe die für beide Seiten vorteilhafte Beziehung zwischen Bakterien und Algen eingehend untersucht, was auch die Konkurrenz innerhalb des Algenmikrobioms mit sich bringt. Diese Doktorarbeit nutzte experimentelle und transkriptombasierte Erkenntnisse, um zu untersuchen, wie symbiotische Bakterien mit Mikroalgen interagieren und deren Dominanz ermöglichen.

1 INTRODUCTION

1.1 Microalgae-bacteria interactions: multifaceted synergistic relations

Close associations of microalgae and bacteria have resulted in a complex network of these cross-kingdom interactions and a potential specialisation of various organisms. Phylogenetically microalgae belong to a highly heterogeneous group of eukaryotic and prokaryotic microorganisms that are unicellular photosynthetic organisms growing in marine and aquatic environments (1, 2). Microalgal cells are surrounded by a phycosphere, a region rich in organic material released by the algae creating the critical interface in which algae and other organisms tightly interact (3). Thus, algae and bacteria synergistically affect each other's physiology and metabolism, impacting the ecosystems and representing all modes of interactions between various organisms, ranging from mutualism to parasitism (4, 5).

Orchestrated nutrient exchange, mutual support of growth factors, and quorum sensing mediation define a broad spectrum of associations at highly complex assemblages of unicellular microalgae and associated bacteria (3). The positive effects of algae-bacteria interaction on algal growth and flocculation processes have changed the scenario of considering bacteria as mere contamination of algae cultures (6). Several studies have verified that the microbiome of microalgae consists mainly of species of the phyla Bacteroidota, Flavobacteria, α-Proteobacteriota, β-Proteobacteriota, and λ-Proteobacteriota (2, 7). Many non-culturable organisms can also be detected, but the overall abundance of microorganisms is often limited to approximately 30 species (2, 8). It is still unclear whether all these bacteria are essential for the algal health and survival of the community. During the screening, it was found that there are some invasive or pathogenic species with limited potential, but they seem to coexist harmoniously within the community. This can be explained by a selection pressure favouring the microalgae-bacteria consortia (9, 10).

However, for applications in aquaculture, the potential of the interactions between microalgae and microorganisms may improve algal biomass production and enrich the biomass with compounds of biotechnological interest, such as lipids, carbohydrates, and pigments. Consequently, the growth and photosynthetic activity of bacteria profit from the interaction with microalgae, especially by adhesion, clumping factor, motility, chemotaxis, secretion systems, quorum sensing, and quorum quenching systems and synthesis of growth promoters (5, 11–13). Intra- and interspecies communication among microbes occurs via a complex set of signal molecules secreted during beneficial and harmful interactions that coordinate and control the behaviour of microorganisms in mixed communities. Other microbial community members will recognise the production and release of signal molecules, initiate the communication causing up- or down-regulation of gene expression, and alter the activity and physiology of the recipient (8, 14, 15). Bacterial communication regulates virulence-associated factors, propagation, population density,

change in metabolic rate, and various bacterial functions, including biofilm formation, motility, adhesion, and coordination between microbial communities (16).

1.2 Mutualism and commensalism as a primary survival concept in phycosphere

One of the goals of this study was a detailed insight into the mutualistic collaboration of microalgae and bacteria, including the involvement of competitive interplay between bacteria. Since there is currently limited knowledge about the individual members of the multispecies consortia, I set out to determine the role of one of the bacterial isolates from the non-axenic algal culture, *Dyadobacter* sp. HH091, in the interplay with the other dominant bacterial species and the alga. To achieve this goal, I made the previously isolated bacterium genetically accessible and investigated its effects using physiological and omics-based techniques. After conducting this study, I investigated the interplay between the isolated symbiont *Dyadobacter* sp. HH091 and the microalgae *Micrasterias radians*.

It is widely recognised that the interaction between algae and bacteria is highly beneficial, as they work together to influence ecosystems and exhibit a variety of modes of collaboration (4). The positive effect of bacteria on algal growth in biotechnology has changed the central concept of mere contamination of algal cultures, considering bacteria as an essential driver in this interaction (11, 17). Strong associations between microalgae and bacteria have evolved a complex network of these cross-kingdom interactions and narrow specialisation of different organisms (2, 3, 18, 19).

The potential of the microalgae-bacteria liaison is determined by its applicability in aquaculture, which aims to improve algal biomass production and enrich this biomass with compounds of biotechnological interest, such as lipids, carbohydrates, and pigments. In addition, bacteria may alter the algal microenvironment in ways that stimulate algal functions. The general bacterial attributes that may profit the interaction with microalgae and which might affect their growth and photosynthetic activity include adhesion, clumping factor, motility, chemotaxis, different secretion systems, quorum sensing and quorum quenching systems, and synthesis of growth promoters (11, 13, 19, 20).

Previous research on microalgae- and photobioreactors biofilm-associated bacteria indicated that most observed microorganisms were affiliated with α-Proteobacteriota, β-Proteobacteriota, and Bacteroidota (2, 7, 8, 21). Further investigations have characterised the biotic interaction of microalgae and bacteria using metagenomic, transcriptomic, and proteomic approaches. In this study, the microbiomes of microalgae have been sequenced, and various bacterial strains affiliated with the algae have been isolated to answer if the associated microbiota is specific for the microalgae and which role individual bacterial taxa play (18). It was observed that effector

molecules known from plant-microbe interactions as inducers for innate immunity are already relevant at this evolutionary early plant-microbiome level. Essential genes involved in plant-microbe interactions were mainly affiliated with different mechanisms, including vitamin biosynthesis, transport and secretion systems, signal transduction, carbohydrate, and lipid modification. The metatranscriptome analysis indicated that the transcriptionally most active bacteria, concerning crucial genes commonly involved in plant-microbe interactions, in the microbiome of the *Chlorella* (Trebouxiophyceae), *Scenedesmus* (Chlorophyceae) and *Micrasterias* (Zygnematophyceae) belong to the phylum of the α-Proteobacteriota and Bacteroidota (18).
The crucial vital features of overall plant-bacteria interaction covered different mechanisms with the involvement of transport and secretion systems (e.g., T6SS, T9SS), quorum quenching proteins, leucine-rich repeat proteins (LRR) and enzymes related to bacterial reactive oxygen species (ROS) tolerance, and the biosynthesis of vitamins (B_1, B_2, B_5, B_6, B_7, B_9 and B_{12}). Furthermore, the metatranscriptome analysis demonstrated that within the microbiota of *S. quadricauda,* the dominant species were affiliated with the *Variovorax*, *Porphyrobacter*, and *Dyadobacter* genera. Furthermore, experimental and transcriptome-based evidence implied that *Dyadobacter* was a key to alga growth and fitness within this multispecies interaction and is highly adapted to the phycosphere (19).

1.3 Exploring the potential of microalgae and the associated microbiota

Since microalgae are active photosynthetic organisms, which can be grown under various conditions, they are highly attractive for the biotechnological production of different chemical compounds. They are mainly well known for their use in producing advanced biofuels (e.g., drop-in biofuels and fourth-generation biofuels) and, to some extent, for producing bioplastics (22–28). Recently, it has become clear that algae and their microbiota harbour a large and diverse set of genes to synthesise molecules that suppress bacterial pathogens (2, 18). For instance, sterols with anti-inflammatory capacity, like diacylglycerols, triacylglycerols, and phytosterols (29–31). In this respect, they have great potential to significantly contribute to critical societal needs linked to treating infections due to human, animal, and plant pathogenic microorganisms. Furthermore, the appearance of untreatable antibiotic-resistant microorganisms in clinical settings is a significant concern to human health (32, 33). Thus, there is a need to develop novel antimicrobials distinct in their mode of action from those already known and on the market.
Quorum sensing and quorum quenching play an essential role in the expression of virulence (34, 35). Quorum quenching and various quorum sensing interference mechanisms have been

outlined and discussed as possible strategies to prevent and treat microbial biofilm formation (34–37).

Microalgae and their associated bacterial microbiota may be valuable tools to verify this concept further. Microalgae microbiomes offer both quorum quenching enzymes and a wide variety of quorum sensing molecules that have been shown to interfere with pathogens (38, 39). In this framework, the screening of 19 strains of microalgae reported that one microbial community of *Chlorella saccharophila* and one of *Chlorella vulgaris* both degraded N-acyl homoserine lactones (AHLs), resulting in the inhibition of violacein production in the reporter strain (40). When using *E. coli* (JB523)-strain sensitive to N-(3-oxohexanoyl)-L-homoserine lactone, the microbiome associated with *C. saccharophila* was found to significantly suppress bacterial quorum sensing and further inhibit AHL-regulated bioluminescence in the pathogen *Vibrio harveyi* (40). AHL degradation can occur indirectly, such as in cultures of *Tetraselmis suecica* and *Chaetoceros muelleri, associated* with AHL-degrading bacteria belonging to the genera *Bacillus* and *Pseudomonas* (41). These bacterial isolates were found to degrade AHL molecules, and *Bacillus* sp. was reported to suppress the quorum sensing system of *Vibrio campbelli* and thus protected the larvae of the giant river prawn (*Macrobrachium rosenbergii*) from infection, improving survival from 42 to 67% during an infection challenge.

Using the analysis of algae and microalga microbiomes available at IMG/MER https://img.jgi.doe.gov, I identified numerous quorum quenching genes. The study of metagenomes of *Scenedesmus quadricauda*, *Chlorella saccharophila*, *Chlorella sorokiniana* and *Micrasterias crux-melitensis* revealed dienelactone hydrolases, imidazolonepropionases, 6-phosphogluconolactonases and metal-dependent hydrolases, associated with quorum quenching, known as potential candidates for overexpression experiments and biotechnological studies.

A further interesting fact is that many microalgae communities can produce polysaccharides, which are well known to exert a broad spectrum of biological activities, especially antiviral properties (42). In the framework of this research, these polysaccharides were found among numerous algal microbiomes, including *S. quadricauda* (IMG ID 3300005759), *C. saccharophila* (IMG ID 3300008885), *C. sorokiniana* (IMG ID 3300042370) and *M. crux-melitensis* (IMG ID 3300008886) (43).

It is widely known that microalgae contain several compounds with antioxidant properties. Based on that, I analysed data sets from IMG/MER and discovered genes that code for antioxidative superoxide dismutase, catalases, and rhodanese-related sulfurtransferases. Furthermore, studying algal genomes (*C. variabilis* NC64A and *C. subellipsoidea* C-169) demonstrated the presence of genes coding for the biosynthesis of known antioxidants, such as chlorophyll, carotenoid, lutein and astaxanthin. These genomes are available under the accession number IMG ID 2507525016.

The awareness in the scientific community about the largely unexplored potential of microalgae has led to increased interest during the last decade, as evidenced by an exponential increase in the number of publications and patents about microalgae and health. However, compared to many microalgal species, our knowledge remains sparse. Therefore, further research requires a focused and more systematic approach to explore this promising resource better, emphasising human, animal, and plant health and well-being.

1.4 Aim of this study

Within this framework, I addressed the following questions in the current study. First, which role do secretion systems play in these remarkable interactions? Is direct cell-to-cell contact between the interaction partners required, and what influence does bacterial quorum sensing have? To answer these questions, I used fluorescence labelling of bacteria and 4′-6-diamidino-2-phenylindole (DAPI) staining with confocal microscopy to determine the physical association of microalga cells with the *Dyadobacter* isolate HH091.

Further, to get a deeper insight into this synthetic bacteria-microalgae model system, I characterised the interactions of the isolate *Dyadobacter* sp. HH091 with the microalga *M. radians* MZCH 672 using transcriptome and genome analyses (44). These data expand our understanding of species-species interactions and identify several genes involved in the molecular basis of bacteria-alga interactions that can serve as an established synthetic plant-bacteria system (19). Therefore, the genome and metabolic potential of the bacterium *Dyadobacter* sp. HH091 is of particular interest in understanding bacteria-algae interactions.

To summarise, I provided a comprehensive review of the current information regarding the health advantages of microalgae and their microbiota. My goal was to provide an understanding of the current knowledge on this topic. I also discussed the challenges that hinder the complete utilisation and advancement of related technologies (43).

In conclusion, the results of this research will serve as an efficient tool in further investigations of symbiotic microalgae-bacteria interactions. While microalgae are well explored for the generation of biofuels, their potential as a source of antimicrobial and prebiotic substances has also received increasing interest. In this context, microalgae may offer solutions to the societal challenge of the lack of antibiotics for treating bacteria and fungi resistant to antimicrobial drugs in clinical settings. The remarkable benefit of co-cultivation of microalgae and bacteria will have commercial and environmental positive impacts on microalgal cultivation.

2 MICROALGAE AND BACTERIA INTERACTION – EVIDENCE FOR THE DIVISION OF DILIGENCE IN THE ALGA MICROBIOTA

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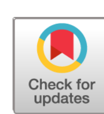

Microalgae and Bacteria Interaction—Evidence for Division of Diligence in the Alga Microbiota

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ABSTRACT Microalgae are one of the most dominant forms of life on earth that is tightly associated with a distinct and specialized microbiota. We have previously shown that the microbiota of *Scenedesmus quadricauda* harbors less than 10 distinct microbial species. Here, we provide evidence that dominant species are affiliated with the genera of *Variovorax*, *Porphyrobacter*, and *Dyadobacter*. Experimental and transcriptome-based evidence implies that within this multispecies interaction, *Dyadobacter* is a key to alga growth and fitness and is highly adapted to live in the phycosphere. While presumably under light conditions the alga provides the energy source to the bacteria, *Dyadobacter* produces and releases mainly a large variety of polysaccharides modifying enzymes. This is coherent with high-level expression of the T9SS in alga cocultures. The transcriptome data further imply that quorum-quenching proteins (QQ) and biosynthesis of vitamins B_1, B_2, B_5, B_6, and B_9 are expressed by *Dyadobacter* at high levels in comparison to *Variovorax* and *Porphyrobacter*. Notably, *Dyadobacter* produces a significant number of leucine-rich repeat (LRR) proteins and enzymes involved in bacterial reactive oxygen species (ROS) tolerance. Complementary to this, *Variovorax* expresses the genes of the biosynthesis of vitamins B_2, B_5, B_6, B_7, B_9, and B_{12}, and *Porphyrobacter* is specialized in the production of vitamins B_2 and B_6. Thus, the shared currency between partners are vitamins, microalgae growth-promoting substances, and dissolved carbon. This work significantly enlarges our knowledge on alga-bacteria interaction and demonstrates physiological investigations of microalgae and associated bacteria, using microscopy observations, photosynthetic activity measurements, and flow cytometry.

IMPORTANCE The current study gives a detailed insight into mutualistic collaboration of microalgae and bacteria, including the involvement of competitive interplay between bacteria. We provide experimental evidence that Gram-negative bacteria belonging to the *Dyadobacter*, *Porphyrobacter*, and *Variovorax* are the key players in a *Scenedesmus quadricauda* alga-bacteria interaction. We impart strong evidence that *Dyadobacter* produces and releases polysaccharides degradation enzymes and leucine-rich repeat proteins; *Variovorax* supplies the consortium with auxins and vitamin B_{12}, while *Porphyrobacter* produces a broad spectrum of B vitamins. We show not only that the microalgae collaborate with the bacteria and vice versa but also that the bacteria interact with each other via quorum-sensing and secretion system mechanisms. The shared currency between partners appears to be vitamins, microalgae growth-promoting substances, and dissolved carbon.

KEYWORDS microalgae and microbiota interaction, synthetic plant-bacteria system, phycosphere biofilm, *Scenedesmus quadricauda*, *Dyadobacter* sp.

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The term microalgae is a collective phylogenetical expression for a highly heterogeneous group of eukaryotic and prokaryotic microorganisms that are unicellular photosynthetic organisms growing in marine and aquatic environments, respectively (1, 2). In general, microalgal cells are surrounded by a phycosphere, which is a region rich in organic material released by the algae creating the key interface in which algae and other organisms tightly interact (3). Thus, algae and bacteria synergistically affecting each other's physiology and metabolism together have an impact on the ecosystems and represent all modes of interactions between various organisms, ranging from mutualism to parasitism (4, 5).

Tight associations of microalgae and bacteria have resulted in the evolution of a complex network of these cross-kingdom interactions and a potential specialization of various organisms. Orchestrated nutrient exchange, mutual support of growth factors, and quorum-sensing mediation define a wide spectrum of associations at highly complex assemblages of unicellular microalgae and associated bacteria (3). The positive effects of algae-bacteria interaction on algal growth and flocculation processes has changed the scenario of considering bacteria as mere contamination of algae cultures (6). Several studies have verified that the microbiome of microalgae consists mainly of species of the phyla Bacteroidota, Flavobacteria, Alphaproteobacteriota, Betaproteobacteriota, and Gammaproteobacteriota (2, 7). Many noncultivatable organisms can be detected as well, but the overall abundance of microorganisms is often limited to approximately 30 species (2, 8). It is still unclear whether all of these bacteria are important for algal health and survival of the community. Interestingly, limited potential invasive or pathogenic species have been also detected during the screening but somehow appear to live in a balanced manner in the community, which can be explained by a selection pressure that favors the microalgae-bacteria consortia (9–12).

However, for applications in aquaculture, the potential of the interactions between microalgae and microorganisms may improve algal biomass production and may enrich the biomass with compounds of biotechnological interest such as lipids, carbohydrates, and pigments. Consequently, growth and photosynthetic activity of bacteria profit from the interaction with microalgae, especially by adhesion, clumping factor, motility, chemotaxis, secretion systems, quorum-sensing (QS), and quorum-quenching (QQ) systems and synthesis of growth promoters (5, 13–15). Intra- and interspecies communication among microbes occurs via a complex set of signal molecules secreted during both beneficial and harmful interactions that coordinate and control the behavior of microorganisms in mixed communities. Production and release of signal molecules will be recognized by other members of the microbial community, will initiate the communication causing up- or downregulation of gene expression, and will alter the activity and physiology of the recipient (8, 16, 17). Bacterial communication provides the regulation of virulence-associated factors, propagation, population density, and change in metabolic rate, various bacterial functions, including biofilm formation, motility, adhesion, and coordination between microbial communities (18).

The goal of the study was detailed insight into mutualistic collaboration of microalgae and bacteria, including the involvement of competitive interplay between bacteria. Since not much is known about the individual role of the individual members of the multispecies consortia, we set out to determine the role of one of the bacterial isolates from nonaxenic algal culture, *Dyadobacter* sp. HH091, in the interplay with the other dominant bacterial species and the alga. For this purpose, we isolated the bacterium from the microbiome, made it genetically accessible, and studied its impact using physiological and omics-based methods.

RESULTS

Previous studies have shown that microalgae obtained from strain collections are associated with various bacterial communities with a rather low diversity (2, 8). In this research, we concentrated on the microalga *Scenedesmus quadricauda* and its bacterial community. In this community, we observed bacteria affiliated with the genera *Dyadobacter* (phylum

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Bacteroidota) and *Variovorax* and *Porphyrobacter* (phylum Proteobacteriota) as the dominant species (2, 9, 19). To gain insight into the physiological role of the microbiota, we set out to isolate individual strains and used them in cocultivation studies. Using classical enrichment strategies, we were able to cultivate a single *Dyadobacter* affiliated with a *S. quadricauda* laboratory nonaxenic culture on tryptone yeast extract salts (TYES) medium as described in the Materials and Methods section. The isolation of *Porphyrobacter* and *Variovorax* from the same *S. quadricauda* laboratory nonaxenic culture was unsuccessful.

The obtained *Dyadobacter* isolate was designated *Dyadobacter* sp. HH091 (from here on called "HH091"). Its phylogenetic affiliation with the genus *Dyadobacter* (phylum Bacteriodota) was initially verified by using 16S rRNA gene amplification and DNA sequencing. Following this, the organism's chromosomal DNA was extracted and sent out to establish its genome sequence. The HH091 genome draft consisted of 30 contigs and has a size of 7,837,776 bp with a G+C content of 43.87% (Table S1; Fig. S1). Annotation identified 6,628 genes, 6,565 of which are protein coding (Table S1). The HH091 genome was deposited at Integrated Microbial Genomes & Microbiomes (IMG/M) (https://img.jgi.doe.gov) under accession IMG ID 2842103827.

Dyadobacter is well adapted for life in multispecies communities and in the vicinity of microalga, which is confirmed by the metatranscriptomic activity of genes affiliated with competitive and plant-bacteria interaction. Its genome codes for several fascinating genetic features, such as multiple QS and QQ loci, which play important roles in cell signaling, and surviving in competitive conditions. Notably, we observed 23 possible QQ genes and 41 *luxR* solos. Further, it codes for complete secretion systems of type 4 (T4SS), 5 (T5SS), 6 (T6SS), and 9 (T9SS). In addition, a remarkable wealth of glycosyl hydrolases (GHs) was observed. A total of 69 GHs, 47 polysaccharide lyases (PLs) and 50 carbohydrate esterases (CEs) were predicted. Table 1 and Tables S1 to S5 give an overview of the genomic features of HH091.

Dynamics of the bacterial colonization of the microalgae studied by confocal microscopy. Based on the above-made observations, we were interested to analyze the effects of HH091 on the microalga in cocultures (Fig. 1). We were able to electroporate and stably maintain the plasmid pBBR1MCS-5-eGFP in HH091. This plasmid carries an enhanced green fluorescent protein (eGFP) under the control of the lac promoter and allowed the detection of HH091 on the surface and inside the algal cells using confocal laser scanning microscope (CLSM). In our experiments, we could not observe any growth inhibition of *Dyadobacter* sp. HH091 (wild type [WT]) and the plasmid-expressing bacteria. Additional high-resolution CLSM images of *S. quadricauda* incubated with HH091 implied that the bacterium was often tightly associated with the algal cells (Fig. 1). Fig. 1C presents the middle layer of a Z-Steck image of *S. quadricauda* with HH091, in which the bacteria is found inside the algal cells. We examined cocultures of HH091 grown together with *S. quadricauda* and compared its photosynthetic activity and relative fitness with the antibiotic-treated algal control cultures over a time period of 13 days (Fig. 2A). To identify the difference in the growth of algal cultures (with and without HH091), we used the optical density measurement (Fig. 2A). In these tests, the first hints of visible difference were observed after 2 to 3 days. The photosynthetic activity measurements by pulse-amplitude-modulation (PAM) fluorometry demonstrated that the coculturing of *S. quadricauda* with HH091 resulted in 1.5-fold increase of the optimal quantum yield of Photosystem II (PS II) in comparison to control antibiotic-treated microalgae (Fig. 2A). Additional microscopic and fluorescence-activated cell sorting (FACS) analyses verified that the number of viable and photosynthetic active cells in the presence of HH091 was much higher in comparison to antibiotic-treated cultures (Fig. 2B and C). In these tests, the algae culture (with and without HH091) were analyzed for 13 days after inoculation and by monitoring the chlorophyll autofluorescence of the algal population or the low-level autofluorescence of the bacteria in the FL2 channel (Fig. 2B). In a FSC X FL2 density blot diagram, three specific populations were detectable. These populations could be assigned as follows: population I, bacteria; population II, dead and lysed algal cells; and; population III, healthy microalgae (Fig. 2B; Fig. S2). In the beginning of the experiment, the content of living algal

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Table 1 Possible interaction pathways of *Dyadobacter* sp. HH091 genome[a]

Selected key features	*Dyadobacter* sp. HH091
Transporter, efflux pumps, and secretion systems	
Transport proteins and efflux pumps	138
MFS, ABC, and biopolymer transporters	189
T4SS	3
T5SS	9
T6SS	9
T9SS	41
Sec-independent protein secretion pathway	2
Sec-SRP	5
Signal transduction and regulation mechanisms	
Response regulators (NarL/FixJ, LytR, OmpR)	116
ECF sigma factors	54
Polysaccharides degradation	
Auxiliary activities	16
Carbohydrate esterases	50
Glycoside hydrolases	69
Glycosyl transferases	69
Polysaccharide lyases	47
Carbohydrate-binding modules	21
Peptidases	139
Competitive interactions	
Potential antibiotic substances	16
Endonucleases and exonucleases	60
Permeases	157
Proteases	69
Heme synthesis	9
Quorum quenching	23
Bacteria-plant interaction pathways	
Vitamins biosynthesis	224
Invasion-associated proteins	2
LRR proteins	2
ROS tolerance	13

[a]The table shows the key features of possible competitive and plant-bacteria interaction pathways of the *Dyadobacter* sp. HH091 genome, using Integrated Microbial Genomes (IMG) function search. The data are shown as the total number of hits. Major facilitator superfamily (MFS), ATP-binding cassette (ABC)-transporters, type 4 secretion system (T4SS), type 5 secretion system (T5SS), type 6 secretion system (T6SS), type 9 secretion system (T9SS), secretion (Sec), secretion-signal recognition particle (Sec-SRP), extracytoplasmic function (ECF), leucine-rich repeat (LRR), reactive oxygen species (ROS).

cells in an antibiotic-treated culture was 59.4% (±0.5), whereas 5.56% (±0.6) of the population included bacteria with 13.2% (±0.8) of lysed algal cells. In the end of the experiment, we identified 70.8% (±1.6) of healthy microalgae, 18.8% (±0.5) of lysed algal cells, and 3.49% (±0.5) of bacteria in a coculture with *Dyadobacter*. In general, we

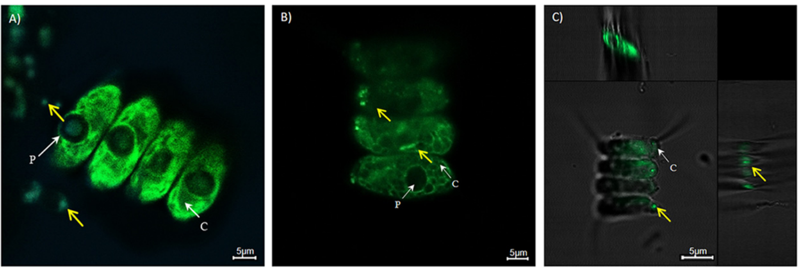

FIG 1 Confocal microscope images of the strain *Dyadobacter* sp. HH091 expressing enhanced green fluorescent protein (eGFP) (yellow arrows) in coculture with *S. quadricauda* MZCH 10104. A confocal laser scanning microscope (CLSM) Axio Observer.Z1/7 LSM 800 (Carl Zeiss Microscopy GmbH, Jena, Germany) with ZEN software (version 2.3; Carl Zeiss Microscopy GmbH) was used. (A) Three-day culture. (B) seven-day culture; (C) seven-day culture, Z-Steck image. An autofluorescence quenching kit was used to lower the autofluorescence of chlorophyll of the microalga. c = chloroplast; p = pyrenoid. Bar = 5 μm.

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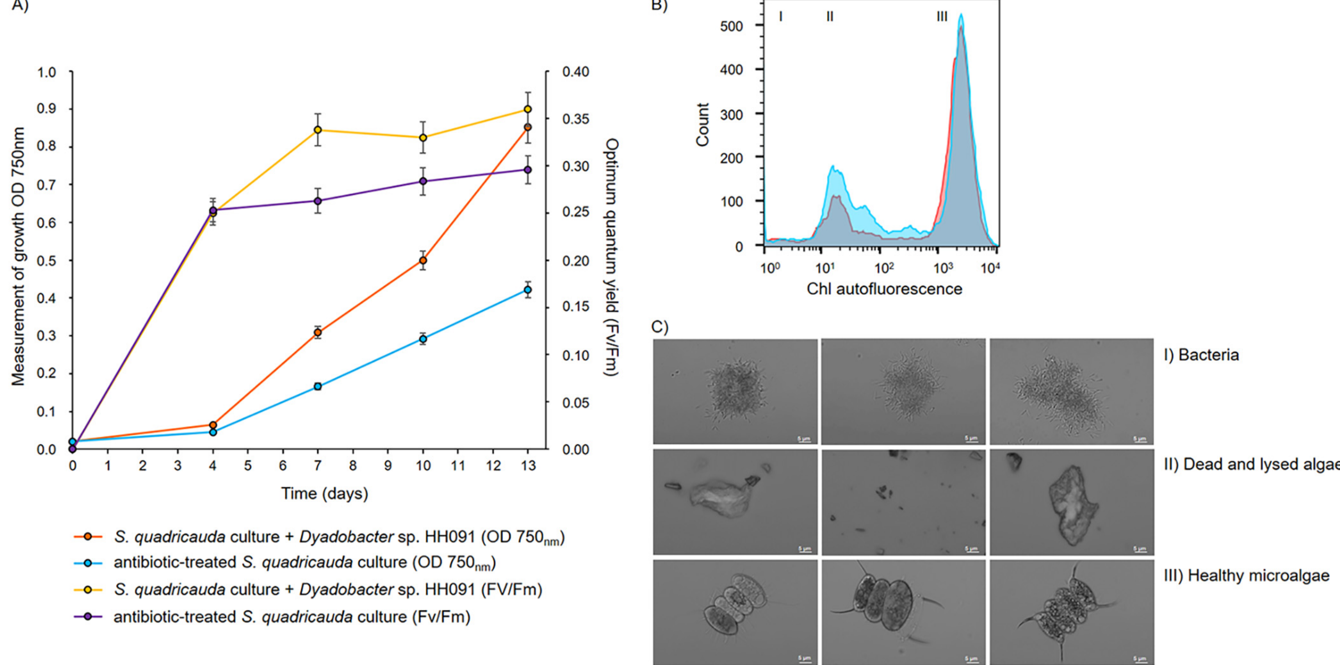

FIG 2 (A, B) Photosynthetic activity and growth measurement (A) and fluorescence-activated cell sorting (FACS) analyses (B) of *S. quadricauda* MZCH 10104 in coculture with the strain *Dyadobacter* sp. HH091. (A) Increased photosynthetic activity and growth rate (optical density at 750 nm [OD 750 nm]) can be observed in the coculture with HH091. (B) Comparison of populations, based on chlorophyll intensity. Orange shading indicates a stable distribution of bacteria (population I), dead and lysed algae (population II), and healthy microalgae (population III) in the coculture at the end of the experiment (13 days). Blue shading shows how the proportion of dead and lysed algae is increased in the culture without HH091. The raw data are available in Fig. S2. (C) Images of cell sorting acquired with CLSM Axio Observer.Z1/7 LSM 800 (Carl Zeiss Microscopy GmbH, Jena, Germany) and ZEN software (version 2.3; Carl Zeiss Microscopy GmbH). Bar = 5 μm. Pulse-amplitude-modulation (PAM) fluorometry and FACS analyses demonstrated the improved fitness of *S. quadricauda* cocultured with HH091.

count approximately 10 to 15 bacterial cells for 1 microalgal cell. Comparison of populations, based on chlorophyll intensity, indicates a stable distribution of bacteria (population I), dead and lysed algae (population II), and healthy microalgae (population III) in the coculture at the end of the experiment (13 days), whereas in the culture without HH091, the proportion of dead and lysed algae is increased (3.24% of bacteria [population I], 27.4% of lysed algal cells [population II], and 57.7% of algae cells [population III]) (Fig. 2B; Fig. S2).

Transcriptome sequencing (RNA-Seq) global analysis of multispecies bacterial consortia and microalga transcriptomes. We hypothesize that more than one compound is essential for a healthy algae growth, which is not known yet. To further analyze the role of HH091 in this specific cross-kingdom interaction, we set out to analyze the transcriptomes of this bacterium in the background of the native multispecies microbiota. We chose to use the original *S. quadricauda* lab culture, from which HH091 was isolated, as this would most likely resemble the native situation in a multispecies community. Thus, we analyzed the multispecies transcriptome of the microbiota at exponential and stationary growth phases of *S. quadricauda* cultures (Table S6; Fig. 3). Sequences obtained for this study were submitted to the European Nucleotide Archive (ENA; PRJEB23338).

In total, we obtained 42 million (mio) reads of bacteria data after trimming. The data are the results of three replicates with each replicate producing between 19 and 25 mio reads. The trimmed reads were assembled into contigs for the exponential and stationary phase experiments (Table S6). In total, the RNA-Seq data covered a significant portion of overall bacterial genomes and the affiliated pathways.

Fig. 3 summarizes the transcriptomics for the exponential (A) and the stationary (B) growth phases. In all treatments, the largest fraction of transcribed genes had no function assigned (20 to 25%). In the bacteria, the second largest fraction was associated

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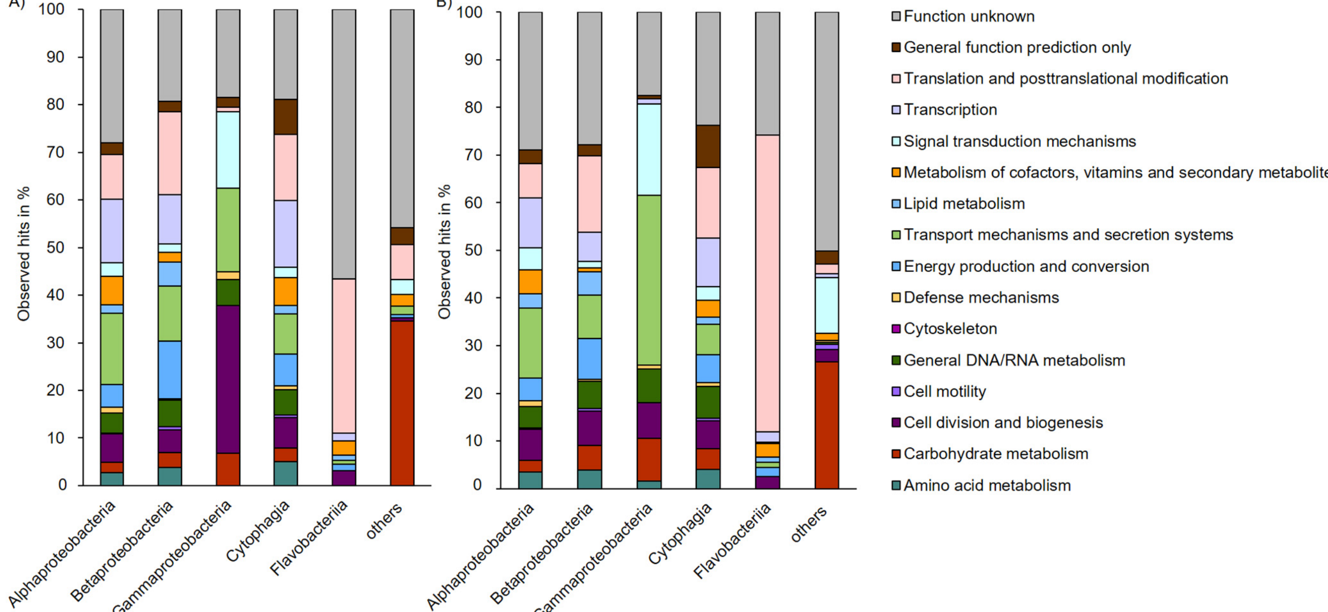

FIG 3 Expressed genes of bacteria in coculture with the microalga *S. quadricauda* during the exponential (A) and stationary (B) growth phases. The generated sequence data (approximal 42 mio reads) were mapped to the available genomes and metagenomes (8). The most dominant species of the microbiome are affiliated with the phyla of the Proteobacteriota (*Variovorax* and *Porphyrobacter*) and Bacteroidota (*Dyadobacter*).

with transport mechanisms and secretion systems during the exponential phase (15% Alphaproteobacteriota, 11.5% Betaproteobacteriota, 17.5% Gammaproteobacteriota, 8.5% Cytophagia, 0.73% Flavobacteriia, and 1.64% others). Further, a large fraction was connected to translation and posttranslational modification (9.5% Alphaproteobacteriota, 17% Betaproteobacteriota, 1% Gammaproteobacteriota, 14% Cytophagia, 32.5% Flavobacteriia, and 7.5% others). Notably, the bacteria showed relatively high levels of transcription of genes linked to the biosynthesis of cofactors, vitamins, and secondary metabolites (6% Alphaproteobacteriota, 2% Betaproteobacteriota, 6% Cytophagia, 3% Flavobacteriia, and 2.5% others).

The transcriptionally most active bacteria were *Dyadobacter* (3 mio reads mapped), *Porphyrobacter* (2.4 mio reads mapped), and *Variovorax* (1 mio reads mapped) at the exponential growth phase. This was similar at the stationary growth phase with *Dyadobacter* (6.9 mio reads), *Porphyrobacter* (5.5 mio reads), and *Variovorax* (1.3 mio reads). The data sets were normalized for the further analyses as outlined in the Materials and Methods section.

RNA-Seq identifies highest bacterial transcribed genes related to carbohydrate degradation, competitive, and plant-bacteria interaction. In the following, we highlight some of the most relevant findings of the whole data sets. Notably, we included those genes that had more than 500 counts/gene. The detailed analyses of the most strongly expressed genes are summarized in Fig. 4. The most strongly expressed genes affiliated with *Dyadobacter* were related to carbohydrate degradation, competitive, and plant-bacteria interaction. Genes, most strongly expressed in *Porphyrobacter* and *Variovorax*, included competitive interaction mechanisms, vitamins biosynthesis, secretion systems, and fatty acids biosynthesis (Fig. 4).

Nevertheless, the transcriptome data set hinted toward a metabolic symbiosis between microalgae/bacteria and bacteria/bacteria. Fig. 4 reflects the expression of genes affiliated with transporter and secretion systems, signal transduction and regulation mechanisms, polysaccharide degradation, competitive interactions, and interaction pathways of the microbiome of *S. quadricauda* during the exponential (A) and the stationary (B) growth phases.

Correlation between transcriptome and genome analyses exposes multifaceted synergistic cooperation. To examine the distribution of specific protein families across the transcriptionally most active bacterial genomes, we performed a protein

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FIG 4 Heat map reflecting the relative expression of genes affiliated with overall plant-bacteria interaction pathways of the bacterial metagenome of *S. quadricauda* during the exponential (A) and stationary (B) growth phases. Red shading indicates a high expression level, and white indicates a low expression level. The numbers indicate the total amount of hits during RNA-Seq. Genes have more than 500 counts/gene.

family comparison across the microbiome of *S. quadricauda* MZCH 10104 (Table S7). The protein families were filtered in affiliation with overall plant-bacteria interaction pathways.

Crucial key features of overall plant-bacteria interaction pathways highlighted in metatranscriptomic analysis were mapped to the reference genomes of *Porphyrobacter* sp. AAP82 (IMG 2551306481), *Variovorax paradoxus* S110 (IMG 644736413), and *Dyadobacter* sp. HH091 (IMG 222279) (Fig. 5). For *Porphyrobacter* and *Variovorax*, we used publicly available reference genome IMG 2551306481 (GenBank accession number ANFX00000000) and IMG 644736413 (GenBank accession numbers NC012791 and NC012792), respectively, and mapped the transcriptome data on these genomes. These two genomes were chosen as reference genomes because they have high-quality annotations and are originally isolated from aquatic/plant habitats (20, 21). The transcriptome data obtained for *Dyadobacter* were mapped onto the genome of HH091 (IMG 222279). The circular mapping was generated using the Circular Genome Viewer tool within PATRIC, the Pathosystems Resource Integration Center (www.patricbrc.org). Moving inward, the subsequent two rings show coding DNA sequences (CDSs) in forward (brown) and reverse (green) strands. Blue and yellow plots indicate GC content and a GC skew [(GC)/(G+C)]. Key features of possible competitive and plant-bacteria interaction pathways are marked with the following colors: purple for transporter, efflux pumps, and secretion systems; orange for signal transduction and regulation mechanisms; turquoise for polysaccharide degradation; red for competitive interactions; and blue for bacteria-plant interaction pathways (Fig. 5).

B vitamins are key drivers in bacteria-alga and bacteria-bacteria interactions. Thiamin biosynthesis genes were mainly expressed in *Dyadobacter* involving the whole cluster of required biosynthesis genes. *Dyadobacter* and *Porphyrobacter* are most likely auxotrophic for B_{12} biosynthesis (2). Biosynthesis of cobalamin requires approximately 30 enzymatic steps for its complete *de novo* construction. Among two existing distinct biosynthetic pathways, which are termed the aerobic and anaerobic routes (22), *Variovorax* codes in its genome for the anaerobic pathway of the vitamin B_{12} biosynthesis. Thus, it is likely that vitamin B_{12} was provided by *Variovorax* and not by any of the two other bacteria, as they lack these genes. A riboflavin (B_2) biosynthesis cluster is

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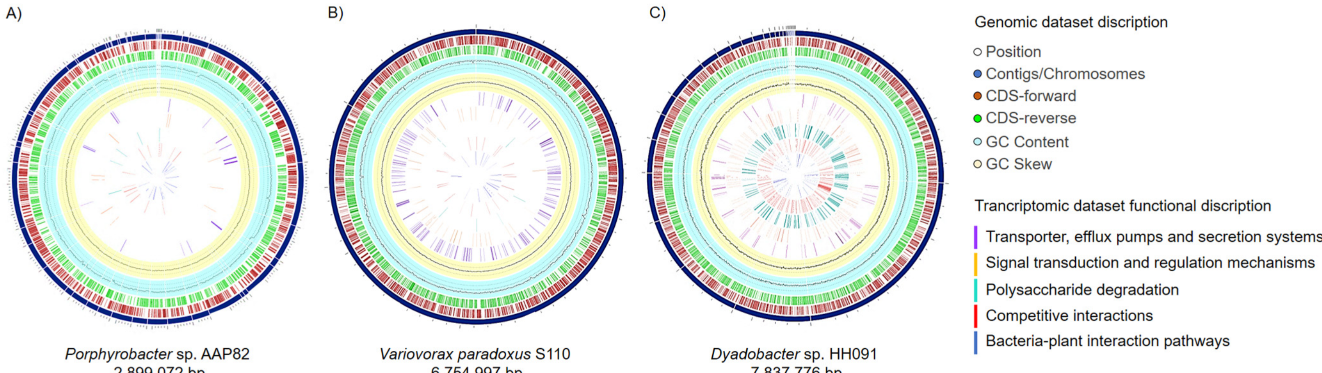

FIG 5 Metatranscriptome mapping to the reference genomes of *Porphyrobacter* sp. AAP82 (IMG 2551306481) (A), *Variovorax paradoxus* S110 (IMG 644736413) (B), and *Dyadobacter* sp. HH091 (IMG 222279) (C). Moving inward, the subsequent two rings show coding DNA sequences (CDSs) in forward (brown) and reverse (green) strands. Blue and yellow plots indicate GC content and a GC skew ([GC]/[G+C]]. Key features of possible competitive and plant-bacteria interaction pathways are marked with the following colors: purple, transporter, efflux pumps and secretion systems; orange, signal transduction and regulation mechanisms; green, polysaccharide degradation; red, competitive interactions; blue, bacteria-plant interaction pathways.

presented in all three bacteria, while biotin (B_7) pathway can be referred just to *Variovorax* (Fig. 4; Table S7).

Cell-cell communication as a key component of microbe-alga interaction. In the *Dyadobacter* genome the *darA* and *darB* genes, coding for the autoinducer synthase, were highly transcribed during exponential and stationary growth phases (6,376 and 12,094 hits). These genes are involved in the dialkylresorcinol synthesis gene cluster of *Dyadobacter*. Dialkylresorcinols are known as novel and widespread bacterial signaling molecules (23). Further analysis of the cell-cell communication as a key component of microbe-alga interaction identified two autoinducer synthase genes (i.e., *luxI* homologues) coding for *N*-acyl-L-homoserine lactone synthases in *Variovorax*.

Additional searches for QQ genes identified 19 genes that were mostly transcribed at the stationary growth phase. A potential dienelactone hydrolase from *Dyadobacter* revealed 1,110 counts and a *Porphyrobacter* homologue had 1,212 counts of mRNA reads.

Overall, among other components of cell signaling, we identified four *luxR* solo coding genes being highly expressed in *Dyadobacter* and having 4,429 counts. Similarly, in *Variovorax*, three *luxR* genes were found with 2,761 mRNA read counts during the stationary growth phase. A similar pattern was observed in the exponentially growing cultures (Fig. 4; Table S7).

Secretion systems are most relevant for competitive interaction. The analysis revealed secretion systems, which are known to be beneficial for bacterial surface colonization and for the algal-bacterial interactions (24, 25). T4SS, T6SS, and T9SS were expressed by the different bacteria. A more detailed inspection of the genomes revealed that *Dyadobacter* coded for T5SS, T6SS, and T9SS, whereas the genomes of *Porphyrobacter* and *Variovorax* coded for T2SS, T4SS, T5SS, and T6SS. Genes affiliated with T1SS and T3SS were not found during this analysis.

The T9SS was strongly expressed by *Dyadobacter*, with 12,967 hits in the exponential and 58,578 hits in the stationary growth phases. Other components of transport and secretion systems were upregulated during the stationary growth phase, often with gene hits that were twice as high. In our data set, *Variovorax* was the only bacterium that expressed the T2SS. While counting 1,843 hits during the exponential growth phase, this number gets doubled during the stationary phase. Gene counts for the T4SS found in *Porphyrobacter* during the exponential growth phase increased by a factor of 7 in the stationary phase.

***Dyadobacter* expresses many polysaccharide-modifying enzymes.** The Bacteroidetes phylum is known for its ability to degrade a wide range of polysaccharides, a trait that has enabled its dominance in many diverse environments, including plants and algal polysaccharides (26). As a member of this phylum, HH091, codes for a wealth of

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polysaccharide-modifying enzymes, many of which were highly expressed (Table 1; Fig. 4). α-L-Fucosidases of GH29 and GH95 families were observed in the metatranscriptome. Highly transcribed genes also included a β-fructosidase, which belongs to the GH32 family. Additionally, genes predicted to be involved in chitin, xylan, xyloglucan, and galactoglucomannan utilization were highly expressed. Since HH091 was the most active organism to encode polysaccharide utilization loci (PULs), it revealed a remarkably high-level transcription of these genes. PULs of HH091 were expressed with 253,760 RNA-Seq reads at exponential and 486,271 at the stationary growth phase (Fig. 4).

Mutualism and commensalism as the main survival concepts in phycosphere. Fig. 4 reflects the expression of genes affiliated with competitive interaction of the microbiota. Genes that are responsible for competitive interaction pathways included potential antibiotic substance biosynthesis pathways, nucleases, permeases, proteases, and genes involved in heme biosynthesis. In addition, 128,759 hits were related to genes linked to reactive oxygen species (ROS) tolerance in the HH091 data set. During stationary growth phase, 89,165 hits versus 39,594 hits of exponential growth phase were observed (Fig. 4). The occurrence and expression of ROS tolerance genes in bacteria are essential for resistance to oxidative stress caused by the host (27).

The expression of invasion-associated proteins during the exponential growth phase is mostly negligible but gains importance during the stationary growth phase, as observed for *Variovorax* and *Dyadobacter* (Fig. 4). Further, we observed a relatively high number of hits for leucine-rich repeat (LRR) and invasion-associated proteins belonging to *Dyadobacter* and *Variovorax*. It is known that bacteria use LRR and invasion proteins as signaling and detecting components for the establishment of the interaction with the plant innate immune system (28).

Phytohormones biosynthesis systems essential for plant-bacteria liaison. Phytohormones play a vital role in plant growth and development as a regulator of numerous biological processes that can positively influence the growth and development of microalgae (8). To determine the involvement of the microalgae-growth-promoting substances, we have analyzed the enzymatic systems responsible for phytohormones biosynthesis. While *Dyadobacter* and *Porphyrobacter* appear to lack any genes affiliated with plant hormone production, the *Variovorax* genome codes for two enzymatic systems, nitrilase and nitrile hydratase/amidase, that are predicted to convert indole-3-acetonitrile to the plant hormone indole-3-acetic acid. *Variovorax* lacks tryptophan 2-monooxygenase, although it can produce indole-3-acetic acid using indole-3-acetonitrile as the precursor. In the transcriptome data, a total of 9,915 counts was linked to this pathway, implying that auxins are potentially produced and released. These data imply that *Variovorax* produces auxin-like molecules, possibly stimulating the growth of the microalga. Taken together, these data imply that each of the three bacteria transcribes a unique set of genes that are of relevance for synthesis of common goods and growth and survival of the whole community.

DISCUSSION

A comprehensive understanding of the composition of the microbial community, as well as competitive interaction, is required to create scientific and theoretical fundamentals of interaction mechanisms between microalgae and other microorganisms, including the development of effective processes for simultaneous algal cultivation with enhancing the efficiency of microalgae biomass growth and associated valuable compounds production.

Specialized distribution of assignments at algae-bacterial phycosphere. The evaluation of the top 250 gene hits resulted in the identification of genes related to the general metabolic activities, carbohydrate degradation, biofilm formation, transport mechanisms, and secretion systems, which support the distribution of assignments at the microbial consortium. Former reports on the relationships within the microalga-bacteria consortia have also provided a blueprint for the construction of mutually beneficial synthetic ecosystems, in which the general metabolic activities played a significant role (29–31).

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Many pathogenic bacteria are known to use secretion systems for the facilitation of their proliferation and survival inside eukaryotic hosts, typically by the secretion of protein effectors or protein-DNA complexes (32). The presence of T6SS suggests the distribution of tasks among members of studied consortia, providing the fitness and colonization advantages, which are not restricted to virulence. T9SS machinery components, established for *Dyadobacter* within the Bacteroidota phylum, represents the assembly of the gliding motility apparatus and possible external release of proteins with various functions, including cell surface exposition, attachment, and other virulence factors (33, 34). The widespread occurrence of gliding motility genes was previously revealed among the members of the same phylum, the gliding bacterium *Flavobacterium johnsoniae*, and the nonmotile oral pathogen *Porphyromonas gingivalis*. *F. johnsoniae* uses T9SS as the gliding motility apparatus and for the secretion of a chitinase that is required for chitin digestion, and the *P. gingivalis* secretes through this system gingipain protease virulence factors. The same route likely secretes other polysaccharide-digesting enzymes produced by other members of the phylum. The mechanisms underlying the processes of gliding motility and cell surface machinery to utilize polysaccharides remain unclear (35, 36). Nevertheless, it is known that T9SS machinery secretes most of CAZymes, such as GHs, PLs, CEs, and accessory proteins (37, 38).

Complex relationships through competition and synergy in algae-bacterial phycosphere biofilms. Studying the interactions between members of alga-bacterial phycosphere, we investigated the hypothesis that dominant bacterial members possess the role of superior competitors. Members of the microbiome of *S. quadricauda* participate in the consortium niche in a competitive way that is reflected in a heat map with the correspondence to genes, affiliated with potential antibiotic substances, endonucleases and exonucleases, permeases, proteases, heme synthesis, and QQ (Fig. 4), which are known as important factors required for biofilm formation, virulence, and competition (39–42). The analysis of the microbiome of *S. Quadricauda* revealed different proteins supposed to be beneficial during competition for space and nutrients on surfaces in biofilms. Bacterial dominance can be attributed to the ability of these organisms to rapidly form microcolonies and their ability to produce extracellular antibacterial compounds (43). The *S. quadricauda* microbiome is composed of single-species populations or mixed populations with various levels of interaction, depending on the exponential or stationary growth phase. *Porphyrobacter*, *Dyadobacter*, and *Variovorax* were found to be the dominant producers of numerous antibacterial proteins, which can possibly eliminate other microorganisms or exhibit strong inhibitory activity against them.

The signaling molecules related to QS and QQ activity were affiliated with the Alphaproteobacteriota and Bacteroidota. Among metatranscriptome data sets proteins predicted as QQ included dienelactone hydrolase, imidazolonepropionase, 6-phosphogluconolactonase, gluconolactonase, oxidoreductases, and metal-dependent hydrolases of the β-lactamase superfamily, related to QQ activity. Highly transcribed genes were observed, mostly at the stationary growth phase, that fulfill the competitive needs of bacteria to comprise one of the dominant heterotrophic bacterial groups in aquaculture, which are represented in Fig. 4. Dienelactone hydrolase, known as a QQ enzyme that degrades or modifies *N-Acyl* homoserine lactones (AHLs) (44, 45), was established for *Dyadobacter* and *Porphyrobacter*. Gluconolactonases, reported as quorum-quenching enzymes (46), were mapped to *Dyadobacter*. Another class of enzyme, oxidoreductase, established to catalyze the oxidation or reduction of acyl side chain (33, 34), originated in *Variovorax* and *Dyadobacter*. The analysis also revealed several phenotypes beneficial for bacterial surface colonization, including motility, exopolysaccharide production, biofilm formation, and toxin production (Fig. 4), which are often regulated by QS (8, 16, 17).

Simultaneously, several members of the *S. quadricauda* microbiome appeared to be the main suppliers of vitamins to microalga. Genes involved in thiamin, cobalamin, biotin, and riboflavin synthesis were established for Alphaproteobacteriota and Cytophagaceae, which confirms the strong evidence that alga-associated bacteria are responsible for the supply of the essential vitamins to alga (19). Thus, our study shows that this interaction involves the strong collaboration between members of the alga-bacterial phycosphere

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with the support of nutritive components and the synergetic exchange of biosynthetic compounds.

A significant number of genes of high importance for root colonization, biofilm formation, invasion (47, 48), virulence, and pathogenicity were identified (49–51). Fig. 4 describes genes affiliated with overall plant-bacteria interaction pathways, including ROS tolerance, LRR proteins, and invasion-associated proteins. Numerous genes responsible for the ROS tolerance were highly transcribed at the stationary phase, which explains the necessity of bacteria to protect itself from massive amounts of reactive oxygen species released by microalgae, which was previously suggested to expose them to pathogens (28). It is supposed that dominating microorganisms can use LRR and invasion proteins as a signaling and detecting components for the establishment of the interaction with the possible innate immune system of the *S. quadricauda*.

In summary, the current study gives a detailed insight into mutualistic collaboration of microalgae and bacteria, including the involvement of competitive interplay between bacteria. Future work will now have to unravel the signaling between the bacteria and eukaryotes, as well as the detailed nutrient exchange and mutual support in different aspects of cross-kingdom synergistic network.

MATERIALS AND METHODS

Microorganisms used in this study and cultivation media. *S. quadricauda* MZCH 10104 was obtained from the Microalgae and Zygnematophyceae Collection Hamburg (MZCH) and cultivated in BG11 medium at 20 ± 1°C and 100 ± 10 μmol photons m^{-2} s^{-1} with a 14-h light/10-h dark schedule (19, 52, 53). To maintain the axenity of the algal culture, *S. quadricauda* was treated with the antibiotic cocktail: penicillin G, streptomycin sulfate, and gentamicin sulfate (100, 25, and 25 mg/L, respectively) (54–56).

The media for cultivation of individual bacterial isolates derived from the microalgae-associated community were prepared as follows. R2A medium and TYES were prepared as described previously (57, 58), and M9, TSB, and NB media were prepared according to the method of Sambrook and Russell (59). To stimulate microbial growth, the media were in part supplemented with algal culture extracts during the exponential and stationary growth phase of the microalgae ranging from 5 to 50% (vol/vol). The inoculated plates were incubated for 5 to 7 days at 22°C under aerobic and anaerobic conditions.

Dyadobacter sp. HH091 was isolated during this work from a laboratory culture of *S. quadricauda* MZCH 10104. The isolate was routinely grown in 5 mL of tryptone yeast extract salts (TYES) broth at 22°C for 3 to 4 days at 200 rpm (60).

Coculturing procedure and conditions. *S. quadricauda* MZCH 10104 and *Dyadobacter* sp. HH091 were cocultured in BG11 medium at 20 ± 1°C and 100 ± 10 μmol photons m^{-2} s^{-1} with a 14-h light/10-h dark schedule over a time period of 13 days. Therefore, 1 mL of *S. quadricauda* was treated with an antibiotic cocktail of $penicillin_{100}$, $streptomycin_{25}$, and $gentamycin_{25}$ in 50 mL of BG11 medium to remove all bacteria. The antibiotic treatment was performed for 1 day. Afterwards, the microalga was centrifuged (5,000 rpm for 10 min) and washed two times with 1 mL BG11 and finally resuspended in 50 mL of BG11 medium, where it was grown for 13 days. At the start of the experiment, each flask contained 50 mL of BG11, *S. quadricauda* (optical density at 750 nm [OD_{750nm}] = 0.03), and *Dyadobacter* (OD_{600nm} = 0.05).

Bacterial RNA extraction and sequencing. The hot phenol method with minor modifications was used to extract the total RNA (19, 61). RNA quality was checked using a 2100 Bioanalyzer with the RNA 6000 Nano kit (Agilent Technologies). The RNA integrity number (RIN) for all samples was ≥7. Equal amounts of the remaining transcripts and kit components were used for cDNA library construction. Libraries suitable for sequencing were prepared from 400 and 275 ng of total RNA with oligo(dT) capture beads for poly(A) mRNA enrichment using the TruSeq stranded mRNA library preparation kit (Illumina) according to the manufacturer's instructions. After 14 cycles of PCR amplification, the size distribution of the barcoded DNA libraries was estimated ~300 bp by electrophoresis on Agilent DNA HS bioanalyzer microfluidic chips.

Sequencing of pooled libraries spiked with 5% PhiX control library was performed at 8 million reads/sample in paired-end mode with 150-nucleotide (nt) read length on the NextSeq 500 platform (Illumina) using a High Output 400M sequencing kit. Demultiplexed FASTQ files were generated with bcl2fastq2 v2.20.0.422 (Illumina).

Processing and analysis of RNA-Seq reads. Fastp (v0.21.0) was used to remove artificial and low-quality (Phred quality score below 15) sequences from the 3′-end of sequence reads (62). Putative base calling errors located in regions were two reads of a read pair overlap were corrected (option: –correction). Kraken2 (v2.1.2) was used in combination with Bracken (v2.6.2) to assess the taxonomic composition (63, 64). Additionally, reads were assembled with Trinity (v2.13.2) (65). Assembly statistics were assessed with Quast (v5.0.2) (66). The resulting contigs were aligned to sequences present in the UniProtKB/Swiss-Prot database (release 2021_04) and taxonomically annotated accordingly (67). Both were achieved with Mmseqs2 (version ad5837b3444728411e6c90f8c6ba9370f665c443) in "easy taxonomy" mode (–lca-mode 4) (68).

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Total bacterial DNA extraction. Genomic DNA of pure cultures of the strain *Dyadobacter* sp. HH091 was extracted using the peqGOLD bacterial DNA kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions.

***Dyadobacter* sp. HH091 transformation.** The strain HH091 was transformed with modified plasmid pBBR1MCS-5-eGFP by electroporation according to standard methods, which resulted in bright green fluorescent colonies as observed by fluorescence microscopy (59). The plasmid contains the broad-host-range vector pBBR1MCS-5, providing a gentamicin resistance and the expression of GFP. Gentamycin was provided at 100 μg/mL, and the bacteria were grown for 3 to 4 days at 22°C in liquid medium or on a plate.

Bacterial genome sequencing, *de novo* assembly, and binning. Total genomic DNA of *Dyadobacter* sp. HH091 was extracted for a genomic analysis using the NucleoBond high-molecular-weight genomic DNA kit for microorganisms (Macherey-Nagel, Germany) following the manufacturer's instructions and a previously published enzymatic cell lysis protocol with some modifications, including freezing in liquid nitrogen, bead beating, and an additional lysis pretreatment with proteinase K and lysozyme for 24 h at 55°C (2). The extracted DNA was sequenced on an Illumina NextSeq 500 platform using rapid sequencing by synthesis (SBS) chemistry v2 (Illumina, San Diego). For this, the DNA library was constructed applying the NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs) according to the manufacturer's protocol. Initial fragmentation of DNA was performed on the Bioruptor NGS (Diagenode) with 30 s on/30 s off for 16 cycles. Sequencing of the metagenomic DNA library was performed on the NextSeq 500 platform (Illumina) as paired-end run (2 $\times$ 150 cycles) with $\sim$60 mio reads. Fastp (v0.21.0) was used to remove artificial and low-quality (Phred quality score below 15) sequences from the 3′-end of sequence reads. Putative base calling errors located in regions were two reads of a read pair overlap were corrected (Fastp option: –correction). Reads shorter than 40 bp were discarded. Sequence reads were than assembled using SPAdes (v3.15.3) (69). The final draft assembly consists of 7,862,706 bp with a GC content of 43.81%. The final genome assembly resulted in 2,018 contigs (N50, 607,803 bp; L50, 10), with a largest contig size of 1,195,963 bp.

Physiological analyses. (i) Microscopy investigations. *Dyadobacter* sp. HH091 expressing eGFP was cocultured with *S. quadricauda* MZCH 10104 and studied using a confocal laser scanning microscope (CLSM) Axio Observer.Z1/7 LSM 800 (Carl Zeiss Microscopy GmbH, Jena, Germany), including Z-Stack microscope techniques. The analysis of the CLSM images were done with ZEN software (version 2.3; Carl Zeiss Microscopy GmbH). An improved 4′-6-diamidino-2-phenylindole (DAPI) staining procedure with some modifications was used in microscopy investigations (70). Modifications included the treatment with the TrueVIEW autofluorescence quenching kit (Vector Labs, SP-8400), which was employed to enhance staining and to lower the autofluorescence of chlorophyll of the microalga known to be troublesome. Background autofluorescence occurring in the 600- to 700-nm range makes it impossible to detect the bacteria transformed with plasmids expressing fluorescent proteins. The TrueVIEW Quencher is an aqueous solution of a hydrophilic molecule, which binds to chlorophyll electrostatically and lowers the fluorescence (71).

(ii) Pulse-amplitude-modulation (PAM) fluorometry. The photosynthetic activity of microalgae was measured by pulse-amplitude-modulation (PAM) fluorometry. The measured parameters represent the optimal quantum yield of PS II photochemistry (F_v/F_m), with the fluorescence (F_0) measured during the illumination of a pre-dark-adapted sample with open reaction centers and under saturating light with closed reaction centers is the maximum fluorescence (F_m). The difference $F_m - F_0$, is called variable fluorescence (F_v), representing the amount of light energy that can be used by PS II (72).

(iii) Fluorescence-activated cell sorting (FACS). Flow cytometry was applied to analyze the chlorophyll content of *S. quadricauda* cocultivated with *Dyadobacter* sp. HH091. An antibiotic-treated algae culture without *Dyadobacter* sp. served as a control. Growth of *S. quadricauda* was monitored over 13 days after inoculation (start OD_{750nm} = 0.090) with and without *Dyadobacter* sp. (OD_{600nm} = 0.05). Culture samples with volumes of 1 mL (with and without *Dyadobacter*) were withdrawn in triplicate for the experiments every 3 days. For every measurement, we used 0.5 mL of algal culture diluted in 0.5 mL of BG11 medium and filtered through a 35-μm Strainer cap. The samples were subjected to flow cytometry using the S3e cell sorter (Bio-Rad, Hercules, CA) equipped with a 488-nm excitation laser and detectors for side and forward scatter (i.e., SSC and FSC area), FL1 (525/30-nm band pass filter), and FL2 (560-nm long-pass filter). Data analysis was carried out with the FlowJo software package (v10.6.1; BD Life Science, Ashlan, OR). The FL2 detector allowed detection of the chlorophyll autofluorescence of *S. quadricauda*, and the culture samples were analyzed in a two-dimensional density plot of FL2 area versus FSC area to identify algal cells with elevated and reduced chlorophyll levels, and bacteria to determine the relative ratio of each population. The identity (algae or bacteria) and physiological state (live or dead algae) of the populations, detected in the FL2-FCS area plot, was confirmed by microscopy after sorting the respective subpopulations with the cell sorting function (purity mode) of the cell sorter instrument. For each sample the measurement was normalized until 15,000 events.

Data availability. Sequences obtained for this study were submitted to the European Nucleotide Archive (ENA). They are publicly available under accession number PRJEB23338. Assembly of the *Dyadobacter* sp. HH091 genome is available via IMG/M (https://img.jgi.doe.gov) using IMG ID 2842103827.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.7 MB.

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We declare no conflict of interest.

Y.A. and I.K. contributed to experimental design; lab work of phylogenetic, metatranscriptomic, genomic, bioinformatic, and physiological analytical approaches; and writing of the research article. D.I. and L.B. contributed to lab work of the genomic approaches. M.G. and E.K. contributed to lab work of metatranscriptomic approaches. M.A. and M.Q. contributed to assembly of metatranscriptomic data sets and bioinformatics approaches of the genomic data set. M.G., S.R., and I.d.G. contributed to lab work of physiological analytical approaches. D.H., W.R.S., and I.K. contributed to general experimental design and writing of the research article. All authors contributed to manuscript revision, and all read and approved the submitted version.

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3 INTERPLAY BETWEEN THE MICROALGA *MICRASTERIAS RADIANS* AND ITS SYMBIONT *DYADOBACTER* SP. HH091

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Interplay between the microalgae *Micrasterias radians* and its symbiont *Dyadobacter* sp. HH091

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Based on previous research, related to detailed insight into mutualistic collaboration of microalga and its microbiome, we established an artificial plant-bacteria system of the microalga *Micrasterias radians* MZCH 672 and the bacterial isolate *Dyadobacter* sp. HH091. The bacteria, affiliated with the phylum Bacteroidota, strongly stimulated growth of the microalga when it was added to axenic algal cultures. For further advances, we studied the isolate HH091 and its interaction with the microalga *M. radians* using transcriptome and extensive genome analyses. The genome of HH091 contains predicted polysaccharide utilizing gene clusters co-working with the type IX secretion system (T9SS) and conceivably involved in the algae-bacteria liaison. Here, we focus on characterizing the mechanism of T9SS, implementing the attachment and invasion of microalga by *Dyadobacter* sp. HH091. Omics analysis exposed T9SS genes: *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprF*, *sprT*, *porU* and *porV*. Besides, *gld* genes not considered as the T9SS components but required for gliding motility and protein secretion (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ)*, were also identified at this analysis. A first model of T9SS apparatus of *Dyadobacter* was proposed in a course of this research. Using the combination of fluorescence labeling of *Dyadobacter* sp. HH091, we examined the bacterial colonisation and penetration into the cell wall of the algal host *M. radians* MZCH 672.

KEYWORDS

***Dyadobacter* sp. HH091, *Micrasterias radians*, microalgaebacteria interaction, synthetic early plant-bacteria system, symbiotic relations**

Introduction

Algae and bacteria synergistically collaborate with each other, influence ecosystems, and represent various modes of interactions between organisms (Ramanan et al., 2016). The positive effect of bacteria on algal growth in the field of biotechnology, has changed the main concept of a mere contamination of algal cultures, considering bacteria as an important driver in this interaction (Lee et al., 2015; Shen and Benner, 2018). Strong

associations between microalgae and bacteria have resulted in the evolution of a complex network of these cross-kingdom interactions and narrow specialization of different organisms (Krohn et al., 2013; Krohn-Molt et al., 2017; Cirri and Pohnert, 2019; Astafyeva et al., 2022).

Nowadays, it is recognized that the potential of the interactions between microalgae and microorganisms, determined by special applicability in aquaculture, aims to improve algal biomass production and to enrich this biomass with compounds of biotechnological interest such as lipids, carbohydrates, and pigments. The algal microenvironment may be altered by bacteria in ways that stimulate algal functions. The general bacterial attributes that may profit the interaction with microalgae, and which might affect their growth and photosynthetic activity, include adhesion, clumping factor, motility, chemotaxis, different secretion systems, quorum sensing and quenching systems, and synthesis of growth promoters (Luo and Moran, 2014; Brameyer et al., 2015; Shen and Benner, 2018; Astafyeva et al., 2022).

Previous research of microalgae-and photobioreactors-associated biofilm bacteria, identified that the majority of the observed microorganisms were affiliated with α-Proteobacteriota, β-Proteobacteriota, and Bacteroidota (Mouget et al., 1995; Davies et al., 1998; Krohn et al., 2013; Whitman et al., 2018). Further investigations have characterized the biotic interaction of microalgae and bacteria using metagenomic, transcriptomic, and proteomic approaches. In this research the microbiomes of microalga have been sequenced, and various bacterial strains affiliated with the algae have been isolated to answer, if the associated microbiota is specific for the microalgae and which role individual bacterial taxa play (Krohn-Molt et al., 2017). Thereby it was observed that effector molecules known from plant–microbe interactions as inducers for the innate immunity are already of relevance at this evolutionary early plant-microbiome level. Key genes involved in plant–microbe interactions were mostly affiliated with different mechanisms, including vitamin biosynthesis, transport and secretion systems, signal transduction, carbohydrate and lipid modification. The metatranscriptome analysis indicated that the transcriptionally most active bacteria, with respect to key genes commonly involved in plant–microbe interactions, in the microbiome of the *Chlorella* (Trebouxiophyceae), *Scenedesmus* (Chlorophyceae) and *Micrasterias* (Zygnematophyceae) belong to the phylum of the α-Proteobacteriota and Bacteroidota (Krohn-Molt et al., 2017).

Recent studies unveiled tight associations of microalga *Scenedesmus quadricauda* and bacteria using metatranscriptomic analysis, including physiological investigations, microscopy observations, photosynthetic activity measurements and flow cytometry. The crucial key features of overall plant-bacteria interaction covered different mechanisms with the involvement of transport and secretion systems (e.g., T6SS, T9SS), quorum quenching proteins (QQ), leucine-rich repeat proteins and enzymes (LRR) related to bacterial reactive oxygen species (ROS) tolerance, as well as the biosynthesis of vitamins (B_1, B_2, B_5, B_6 B_7, B_9 and B_{12}). The metatranscriptome analysis demonstrated that within the microbiota of *S. quadricauda* the dominant species were affiliated with the genera of *Variovorax*, *Porphyrobacter* and *Dyadobacter*. Experimental and transcriptome-based evidences implied that within this multispecies interaction *Dyadobacter* was a key to alga growth and fitness, and is highly adopted to live in the phycosphere (Astafyeva et al., 2022).

Within this framework, we addressed the following questions in the current study. Which role do secretion systems play in these remarkable interactions? Is a direct cell-to-cell contact between the interaction partners required and what influence does bacterial QS have? To answer these questions, we used fluorescence labeling of bacteria and 4′-6-diamidino-2-phenylindole (DAPI) staining with confocal microscopy to determine the physical association of microalga cells with the *Dyadobacter* isolate HH091. Further, to get a deeper insight in this fascinating synthetic bacteria-microalgae model system, we have characterized the interactions of the isolate *Dyadobacter* sp. HH091 (Astafyeva et al., 2022), with the microalga *M. radians* MZCH 672 using transcriptome and genome analyses. These data expand our understanding of species-species interactions and identify several genes involved in the molecular basis of bacteria-alga interactions that can serve as an established synthetic plant-bacteria system. Therefore, the genome and metabolic potential of the bacterium *Dyadobacter* sp. HH091 is of particular interest in understanding bacteria-algae interactions.

Materials and methods

Microorganisms used in this study and cultivation media

Micrasterias radians MZCH 672 was obtained from the Microalgae and Zygnematophyceae Collection Hamburg (*MZCH*) and cultivated in WHM medium (Stein, 1973) at 20 ± 1°C and 100 ± 10 µmol photons m^{-2} s^{-1} with a 14/10-h light/dark period. To maintain the axenity of the algal culture, *M. radians* was treated with the antibiotic cocktail: penicillin G, streptomycin sulfate and gentamycin sulfate (100/25/25 mg/l) (Droop, 1967; Andersen, 2005; Lee et al., 2015; Astafyeva et al., 2022).

Dyadobacter sp. HH091 was isolated previously from a laboratory culture of *S. quadricauda* MZCH 10104 (Krohn-Molt et al., 2017; Astafyeva et al. 2022). The isolate was routinely grown in 5 ml of tryptone yeast extract salts (TYES) broth (Reasoner and Geldreich, 1985; Holt, 1993), at 22°C for 3–4 days at 200 rpm.

Analysis of the flexirubin pigments in *Dyadobacter* sp. HH091

We experimentally validate the production of flexirubin by *Dyadobacter* sp. HH091 by exposing them to 50 µl 10 M KOH, which resulted in a change from yellow to orange/red

if flexirubin pigments were present, followed by a neutralization step with 42 µl 12 M HCl, which resulted in a return to yellow pigmentation.

Co-culturing procedure and conditions

Micrasterias radians MZCH 672 and *Dyadobacter* sp. HH091 were co-cultured in WHM medium at 20 ± 1°C and 100 ± 10 µmol photons m^{-2} s^{-1} with a 14/10 h light/dark period over a time period of 12 days. Therefore, 1 ml of *M. radians* was treated with an antibiotic cocktail of penicillin G, streptomycin sulfate and gentamycin sulfate in 50 ml of WHM medium to remove all bacteria. The antibiotic treatment was performed for 1 day. Afterwards, the microalga was centrifuged (5,000 rpm, 10 min) and washed two times with 1 ml WHM medium and finally resuspended in 50 ml of medium, where it was grown for 20 days. At the start of the experiment, each flask contained 50 ml of WHM, *M. radians* (OD_{750nm} = 0.007) and *Dyadobacter* sp. (OD_{600nm} = 0.05).

Dyadobacter sp. HH091 transformation

The strain HH091 was transformed with modified plasmid pBBR1MCS-5-eGFP by electroporation according to standard methods, which resulted in bright green fluorescent colonies as observed by fluorescence microscopy (Sambrook and Russell, 2001). The plasmid contains the broad-host-range vector pBBR1MCS-5, providing a gentamycin resistance and the expression of GFP. Gentamycin was applied at 100 µg/ml, and the bacteria were grown as described previously (Droop, 1967; Andersen, 2005; Lee et al., 2015; Astafyeva et al., 2022).

Confocal laser scanning microscopy

Dyadobacter sp. HH091 expressing eGFP was co-cultured with *M. radians* MZCH 672 and studied using a confocal laser scanning microscope (CLSM) Axio Observer.Z1/7 LSM 800 (Carl Zeiss Microscopy GmbH, Jena, Germany), which also included Z-Stack microscope techniques. The analysis of the CLSM images were done with ZEN software (version 2.3; Carl Zeiss Microscopy GmbH). DAPI staining procedure was used in microscopy investigations as described previously (Astafyeva et al., 2022). Modifications included the treatment with TrueVIEW Autofluorescence Quenching Kit (Vector Labs, SP-8400), which was employed to enhance staining and to lower the autofluorescence of chlorophyll of the microalga. Background autofluorescence occurring in the 600–700 nm range, makes it impossible to detect the bacteria transformed with plasmids expressing fluorescent proteins. The TrueVIEW Quencher is an aqueous solution of a hydrophilic molecule, which binds to chlorophyll electrostatically and lowers the fluorescence (Karpishin, 2018).

Bacterial RNA isolation and sequencing

Dyadobacter sp. HH091 cells, separated by dialysing bags (Roth, Germany), were co-cultured with microalga for 1 week. Then bacterial cells were subsequently harvested, treated with RNAlater (Sigma, Germany) and frozen at −80°C. The samples were processed by Eurofins (Constance, Germany), where the RNA was isolated and assessed for QC. The RNA Integrity Number (RIN) for all samples was ≥8. Strand-specific cDNA library preparation from polyA enriched RNA (150 bp mean read length) and RNA sequencing was performed using the genome sequencer Illumina HiSeq technology in NovaSeq 6000 S4 PE150 XP sequencing mode. For further analysis fastq-files were provided.

Bacterial RNA data analysis

RNA-seq analysis was performed using PATRIC, the Pathosystems Resource Integration Center.[1] Trim Galore 0.6.5dev was used to remove adapters (Phred quality score below 20) (Krueger, 2012). RNA-Seq data was processed by the tuxedo strategy (Trapnell et al., 2012). All genes were selected with|log2 (fold change)| ≥ 1,5. The differentially expressed genes (DEGs) dataset was collected and used for further analysis. The volcano plot of the distribution of all DEGs was generated using A Shiny app ggVolcanoR (Mullan et al., 2021).

Carbohydrate-active enzymes were screened through local Blastp search in the database of carbohydrate-active enzymes (CAZymes).[2] The database compiles categories of enzymes that act on carbohydrates, e.g., glycoside hydro-lases (GHs), polysaccharide lyases (PLs), glycosyltransferases (GTs) (Levasseur et al., 2013). Domain guided annotation based on conserved domains in *Dyadobacter* sp. HH091 was performed within the STRING database (Szklarczyk et al., 2021).

Sequences obtained and GenBank submissions

RNA sequences obtained for this study were submitted to the European Nucleotide Archive (ENA). They are publicly available under accession PRJEB54772. Assembly of the *Dyadobacter* sp. HH091 genome is available *via* IMG/MER[3] using the IMG ID 2842103827.

1 www.patricbrc.org
2 www.cazy.org
3 https://img.jgi.doe.gov

Results

Symbiont *Dyadobacter* sp. HH091 attached to the surface of *Micrasterias radians* MZCH 672

Based on our previous research, we were intrigued to examine the bacterial colonisation of the microalga *M. radians* MZCH 672. CLSM was used to observe the interaction process between *Dyadobacter* sp. HH091 and *M. radians*. The co-culture of *M. radians* with *Dyadobacter* sp. expressing eGFP are shown in Figure 1. In addition, Z-Stack microscopy was employed to generate a more detailed and higher resolution image of the microalgal contact site with its symbiont. Our results showed, that symbiotic bacterial cells were found in close proximity of the alga after 1 day of incubation (Figure 1A). More nearby contacts were identified *via* CLSM between the host microalga and its symbiont on the third day of incubation (Figure 1B). At Figure 1A bacterial cells are found close to algal cells, while Figure 1B demonstrates the penetration of the symbiont into its host's cell wall. These experiments revealed the presence of direct contacts between *M. radians* and symbiotic *Dyadobacter* sp. HH091 cells through their surrounding and tight interaction, promising the mutual exchange of various substances between the two partners.

We examined co-cultures of HH091 grown together with *M. radians* and compared its relative growth performance with the antibiotic-treated algal control cultures over a time period of 20 days (Supplementary Figure S1). To identify the difference in the growth of algal cultures (with and without HH091) we used the optical density measurement (Supplementary Figure S1). In these tests first hints of visible difference were observed after 3–4 days.

RNA seq identifies active genes for host-symbiont interaction pathways

Transcriptome analysis was applied to indicate highly active genes involved into bacteria-algal interaction. In total, we obtained 43 million (mio) reads of bacteria data after trimming. The data are the result of three replicates with each replicate producing between 4 and 8 mio reads (Supplementary Table S1). The RNAseq data covered a significant portion of the bacterial genome and the affiliated pathways. During data preprocessing low quality transcripts were filtered, resulting in 1,530 genes to be studied (Supplementary Table S2). RNA-Seq analysis was performed using the Tuxedo strategy, the heatmap (Figure 2) was generated using the Expression Import Service of the Pathosystems Resource Integration Center, PATRIC, the absolute value of $\log_2$ Ratio > 1.5 (Kim et al., 2013, 2015; McClure et al., 2013).

The expression levels of the DEGs response of *Dyadobacter* sp. HH091 in co-culture with *M. radians* are depicted in the heatmap (Figure 2). The heatmap reflects the expression of genes affiliated with overall mechanisms described in categories. The highest number of transcripts belongs to carbohydrate transport and metabolism, inorganic ion transport and metabolism, signal

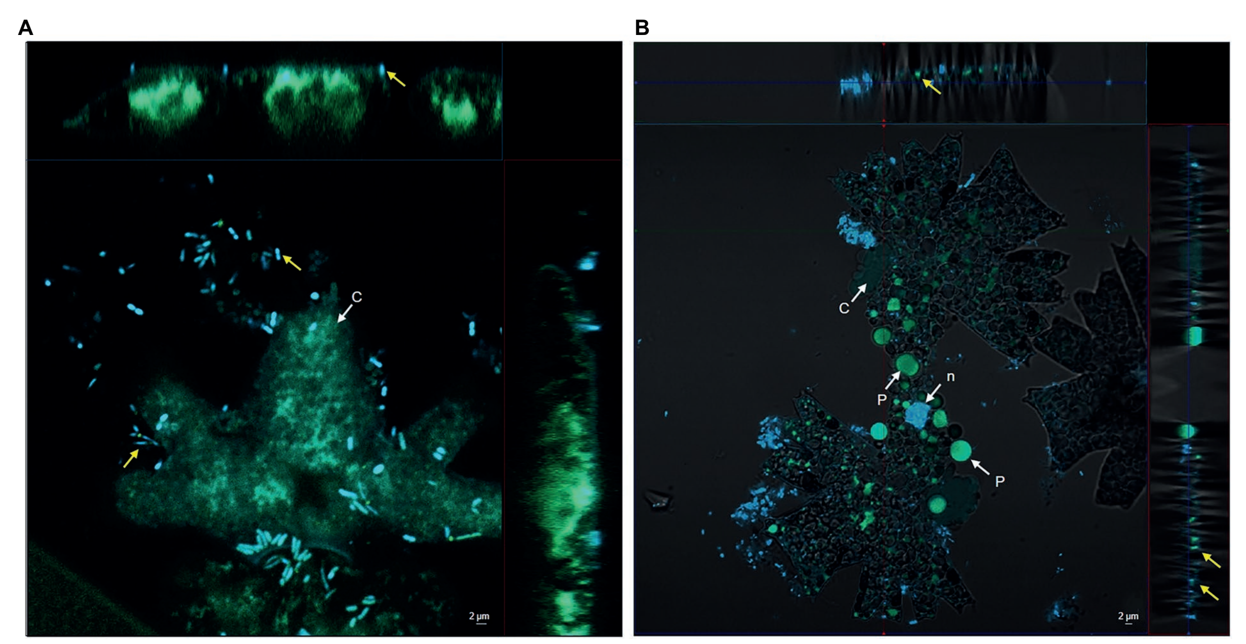

FIGURE 1
Confocal microscope including Z-Stack images of *Dyadobacter* sp. HH091 expressing eGFP (yellow arrows) found in a close proximity to *Micrasterias radians* MZCH 672. Autofluorescence Quenching Kit was used to lower the autofluorescence of chlorophyll of the microalga. Structures: c chloroplast, n nuclear region, p pyrenoid. Scale bar=2μm in each micrograph. **(A)** First day of incubation. **(B)** Third day of incubation.

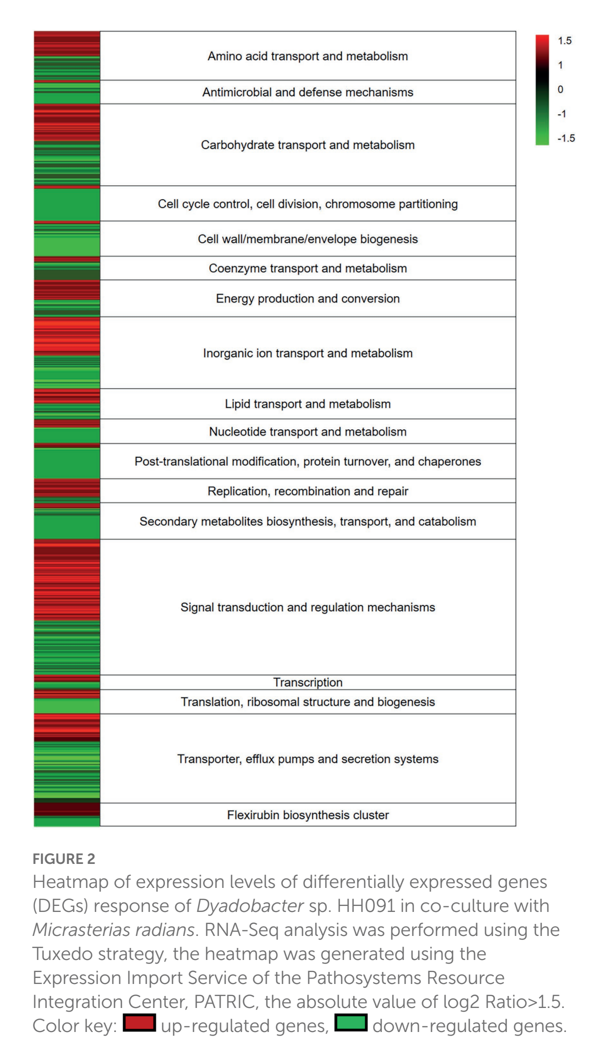

FIGURE 2
Heatmap of expression levels of differentially expressed genes (DEGs) response of *Dyadobacter* sp. HH091 in co-culture with *Micrasterias radians*. RNA-Seq analysis was performed using the Tuxedo strategy, the heatmap was generated using the Expression Import Service of the Pathosystems Resource Integration Center, PATRIC, the absolute value of log2 Ratio>1.5. Color key: up-regulated genes, down-regulated genes.

transduction and regulation mechanisms, and transporter, efflux pumps and secretion systems.

The distribution of gene expression between *Dyadobacter* sp. HH091 co-cultured with *M. radians* and control samples is represented by the volcano plot (Figure 3A). The volcano plot was constructed to compare the two groups using ggVolcanoR. A total of 1,530 differentially expressed genes (DEGs) were identified from the dataset (Figure 3A). Among them, 612 and 918 genes were up-regulated and downregulated, respectively, between two groups according to their $\log_2$FC and *p*-values. Function profile of the DEGs in *Dyadobacter* sp. HH091 is shown in Figure 3B. The studying of the transcriptome of the strain HH091 co-cultured with its microalgal host unveiled the multifaceted combination of mechanisms required for and/or affiliated with T9SS, as well as T9SS cargo proteins, Sus proteins (SusC and SusD), TonB-dependent receptors, cAMP-binding proteins, oxidoreductases, aminotransferases, cytochrome c, numerous transcriptional regulators, including LuxR solos, and flexirubin biosynthesis. The highest number of up-regulated genes belongs to T9SS cargo proteins (42), transcriptional regulators (56), Sus proteins (SusC (10) and SusD (6)), permeases (14), and oxidoreductases (13). Most down-regulated genes are related to oxidoreductases (11), T9SS cargo proteins (24), SusC (10) and SusD proteins (14), T9SS components and Gld proteins (35), permeases (16), and transcriptional regulators (20). Intriguingly, flexirubin biosynthesis mechanism involved 13 up-regulated and 4 down-regulated genes.

Transcriptome analysis indicated highly active genes of T9SS mechanism and flexirubin biosynthesis cluster

By a combination of comparative genome and transcriptome analyses we identified a cluster of genes presumably involved in flexirubin biosynthesis, which was performed using the STRING database (Szklarczyk et al., 2021). This cluster includes two genes, *darA* and *darB*, with likely roles in flexirubin synthesis, and other genes that could be involved in localization of flexirubin pigments (Supplementary Table S3). The flexirubin biosynthesis cluster of *Dyadobacter* sp. HH091 consists of the *dar* operon and a neighboring gene encoding LuxR solo (NarL/FixJ). NarL/FixJ shares 46% identity and 47% similarity with the LuxR solo PluR of *Photorhabdus luminescens* (Brameyer et al., 2015). In *P. luminescens* PluR performs as a LuxR-type receptor serving for QS. Based on these observations we proposed the model of flexirubin/dialkylresorcinol (DAR) biosynthesis in HH091, which consists of QS circuit genes possibly up-regulating several mechanisms like T9SS, gliding motility and protein secretion (Figure 4). These QS circuit genes are found to be adjacent to T9SS genes, genes affiliated with gliding motility and protein secretion (genes coding for gliding motility-associated-like proteins, T9SS type A sorting domain-containing proteins, chitin binding proteins, peptidoglycan-associated proteins, and PorT family protein).

Additional studying of homologs showed the presence of these genes in the representative genomes of the phylum Bacteriodota *Flavobacterium johnsoniae*, *Flavobacterium psychrophilum* (McBride et al., 2009) and *Chitinophaga pinensis* (Schöner et al., 2014), and among the members of the phylum Proteobacteriota *Photorhabdus asymbiotica* (Brameyer et al., 2015) and *Pseudomonas aurantiaca* (Nowak-Thompson et al., 2003). Responsible for flexirubin biosynthesis, genes *darA* and *darB* are similar to *F. johnsoniae*, which were previously identified to be engaged in biosynthesis of 2-hexyl-5-propyl-alkylresorcinol (McBride et al., 2009). In addition to *darA* and *darB*, other genes in this cluster are predicted to encode enzymes involved in lipid synthesis and some of these enzymes likely have roles in flexirubin synthesis (Supplementary Table S3). This cluster includes numerous genes, such as acyl carrier protein, (3-oxoacyl)-acyl carrier protein synthase, acyl-CoA thioester hydrolase, histidine ammonia-lyase, 1-acyl-sn-glycerol-3-phosphate acyltransferase, beta-ketoacyl synthases, and beta-hydroxyacyl-(acyl carrier protein) dehydratase, including several

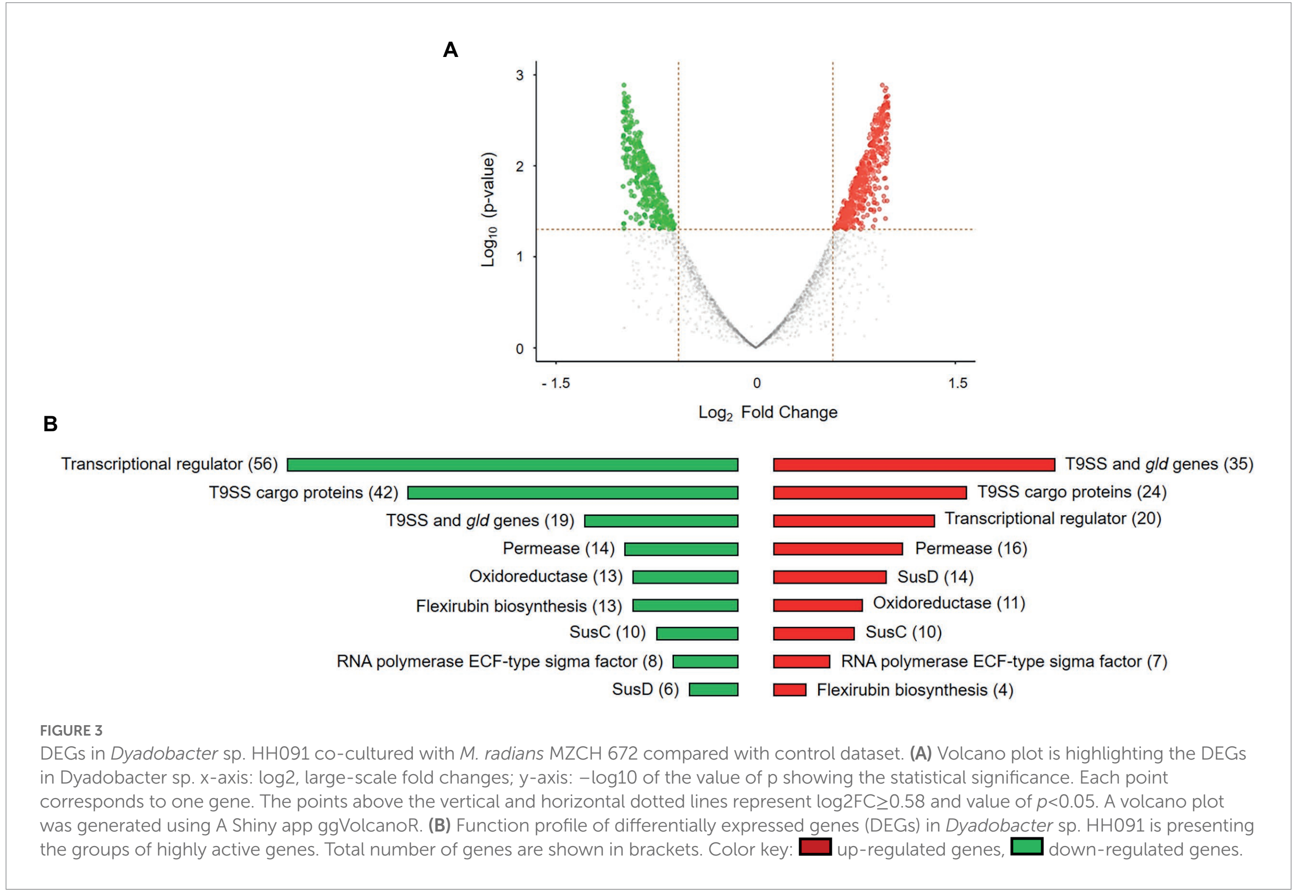

FIGURE 3
DEGs in *Dyadobacter* sp. HH091 co-cultured with *M. radians* MZCH 672 compared with control dataset. **(A)** Volcano plot is highlighting the DEGs in Dyadobacter sp. x-axis: log2, large-scale fold changes; y-axis: −log10 of the value of p showing the statistical significance. Each point corresponds to one gene. The points above the vertical and horizontal dotted lines represent log2FC≥0.58 and value of $p<0.05$. A volcano plot was generated using A Shiny app ggVolcanoR. **(B)** Function profile of differentially expressed genes (DEGs) in *Dyadobacter* sp. HH091 is presenting the groups of highly active genes. Total number of genes are shown in brackets. Color key: up-regulated genes, down-regulated genes.

ABC-2-type transporters known to be entangled in the localization of flexirubin (McBride et al., 2009).

Experimental identification and validation of flexirubin confirmed its production by *Dyadobacter* sp. HH091 (Supplementary Figure S2). Cells were photographed before treatment (I), after exposure to 50 µl of 10 M KOH (II), and after exposure to KOH followed by exposure to 42 µl 12 M HCl (III). Flexirubin-positive cells were yellow at neutral pH (I and III) and orange/red under alkaline conditions (II).

Proposed model of T9SS in *Dyadobacter* sp.

Highly active genes within this transcriptome belong to T9SS mechanism and gliding motility (Supplementary Table S4). Overall, 18 genes (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprF*, *sprT*, *porU* and *porV*), required for gliding motility and protein secretion, and/or involved in T9SS (Hunnicutt and Mcbride, 2000; McBride and Braun, 2004; Braun et al., 2005; Lauber et al., 2018; McBride, 2019; Hennell James et al., 2021; Trivedi et al. 2022; Veith et al., 2022), were identified among DEGs (Supplementary Table S2).

Besides that, a high number of transcripts was observed among genes responsible for polysaccharides utilization. That can also elucidate the up-regulation of genes coding for T9SS, while in commensal and environmental bacteroidotal species the T9SS is characteristically used to secrete enzymes that enable the organisms to utilize complex polysaccharides as a carbon source (Veith et al., 2013; Hennell et al., 2021).

Among up-regulated genes we identified different GHs and cell surface glycan-binding lipoproteins, known to be involved into plant and algal cell wall degradation mechanisms (Giovannoni et al., 2020). That included cellulose-degrading endoglucanases, hemicellulose-degrading xylosidases, pectin degradation proteins, starch-degrading enzymes, β-glucuronyl hydrolases, SusC and SusD family cell surface glycan-binding lipoproteins (Supplementary Table S2).

Being concentrated on the components of T9SS, we identified highly active genes by transcriptome analysis of the strain HH091 co-cultured with its microalgal host. Domain guided annotation is based on conserved domains detected by STRING analysis of *Dyadobacter* sp. HH091 primary sequences against the genome of *Flavobacterium* spp. (Supplementary Table S4). Based on this analysis and previous researches (McBride and Zhu, 2013; Veith et al., 2013; Astafyeva et al., 2022), we proposed a model of T9SS including gliding motility proteins in *Dyadobacter* sp. HH091 (Figure 5). Intriguingly, genes, transcribing for the Gld motor proteins, were mostly down-regulated (*gldKLMN*), while genes coding for gliding motility-associated ABC transporter ATP-binding proteins were up-regulated. The transcriptome analysis suggests an explanation for this finding, because the symbiont possibly uses the T9SS not only for gliding motility, but

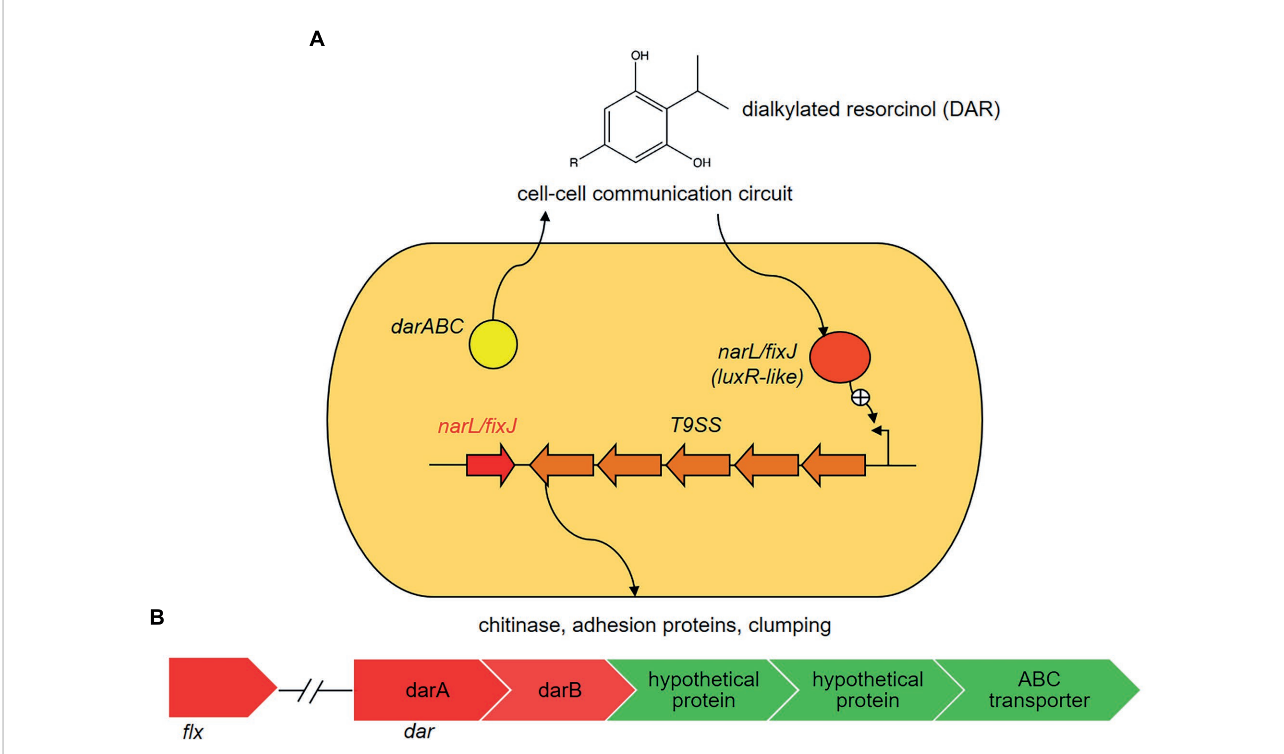

FIGURE 4
Proposed model of flexirubin or dialkylresorcinol (DARs) biosynthesis in *Dyadobacter* sp. HH091. In the proposed model, *Dyadobacter* sp. HH091 communicates *via* DARs and represents a novel quorum sensing (QS) circuit (Brameyer et al., 2015). It consists of the *dar* operon and a neighboring gene encoding a *luxR* solo (*narL/fixJ*). NarL/FixJ shares 46% identity and 47% similarity with the LuxR solo PluR of *P. luminescens* (IMG 2597490348), LuxR-type receptor serving for QS. The proposed QS circuit genes, adjacent to T9SS genes, genes affiliated with gliding motility and protein secretion, possibly upregulates several mechanisms, including T9SS, gliding motility and protein secretion. **(A)** Expression levels of DEGs involved into flexirubin biosynthesis: *dar* and *flx* clusters, and transport systems (ATP binding cassettes (ABC) and hypothetical proteins). Color key: up-regulated genes, down-regulated genes. **(B)** Expression levels of differentially expressed genes (DEGs) involved into flexirubin biosynthesis: *dar* and *flx* clusters, and transport systems (ATP binding cassettes (ABC)-transporters and hypothetical proteins).

also for the secretion of other proteins. Recent results by McBride and Saiki showed that nonmotile bacteroidotal members, such as *P. gingivalis*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *P. distasonis*, and *Salinibacter ruber*, have homologs of genes, that have functions essential for protein secretion, but not for motility (Saiki and Konishi, 2007; McBride et al., 2009). Figure 5 represents a model of the T9SS including proteins required for gliding motility and/or protein secretion of *Dyadobacter* sp. HH091. This model includes the T9SS category (GldK, GldL, GldM, GldN, SprA, SprE, SprF, SprT, PorU, PorV), multiple PorXY-SigP signalling system components, and further Gld proteins (GldA, GldB, GldD, GldF, GldG, GldH, GldI, GldJ).

Most of the *gld* and T9SS genes are found to be adjacent to genes coding for proteins, involved into biosynthesis of glycosyltransferases, cell surface proteins, lipoprotein export proteins, as well as antibacterials, adhesion factors, microcolonization development, and EPS production. Interestingly, the up-regulated adjacent genes are also affiliated with cargo proteins of the T9SS. T9SS cargoes possess a conserved C-terminal domain (CTD) and an N-terminal signal peptide, and carry a CTD as a secretion signal, which is cleaved and replaced with anionic lipopolysaccharide by transpeptidation for extracellular anchorage to the outer membrane (OM) (Kulkarni et al., 2017; Mizgalska et al., 2021, 22; Gorasia et al., 2022). In this research, DEGs covered 42 up-regulated and 24 down-regulated genes affiliated with T9SS cargo proteins (Supplementary Table S5).

Along this detailed dataset investigation, the high activity of genes related to secretion systems and other entangled mechanisms underline the ability of *Dyadobacter* to perform the interaction with microalga and enable its dominance in many diverse environments.

Discussion

The most comprehensive and fundamental understanding of microbial metabolic pathways in a multispecies system, as well as symbiotic and competitive interactions, is required to provide scientific and theoretical bases for the interaction mechanisms between microalgae and other microorganisms. The presented results promote not only the development of effective methods for simultaneous cultivation of algae, they also encourage the increasing the efficiency of microalgal biomass growth and associated production of valuable compounds.

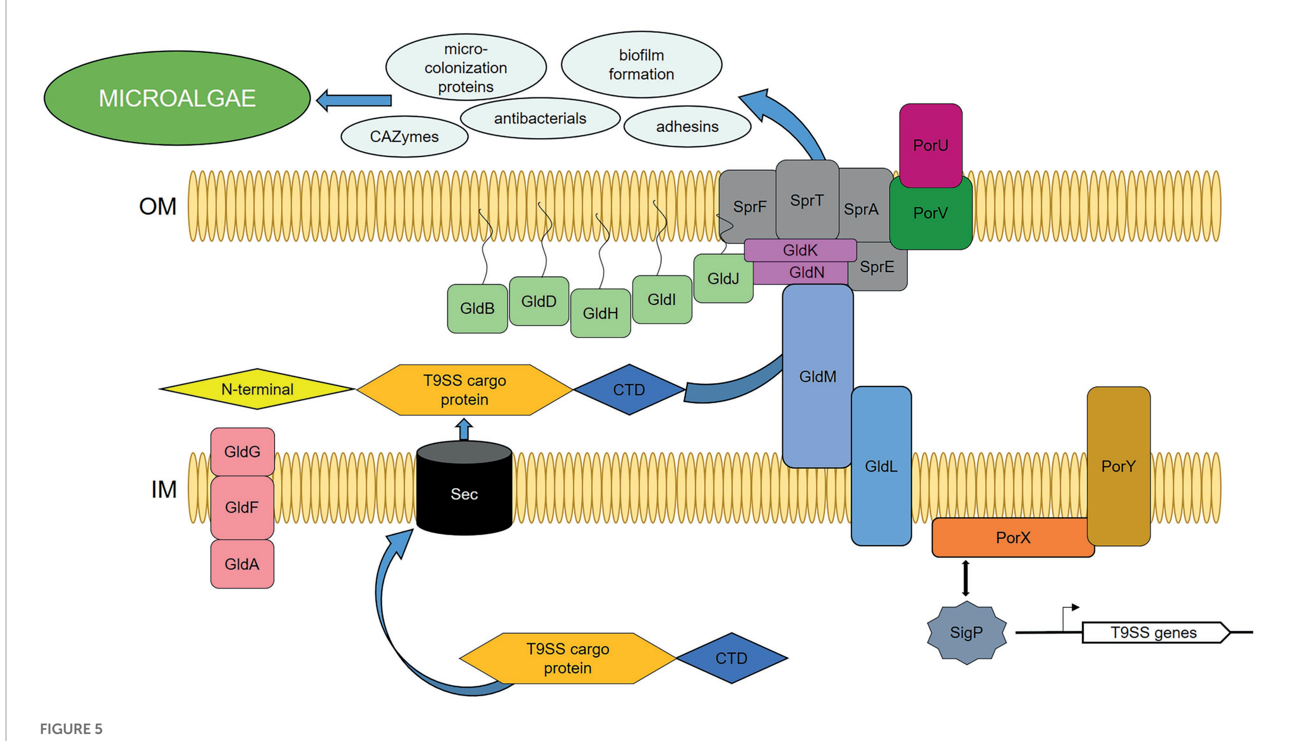

FIGURE 5
Proposed model of T9SS in *Dyadobacter* sp. HH091, serving as the secretion system of cargo-proteins. PorXY-SigP signalling system upregulates several components: T9SS category (GldK, GldL, GldM, GldN, SprA, SprE, SprF, SprT, PorU, PorV), and further Gld proteins (GldA, GldB, GldD, GldF, GldG, GldH, GldI, GldJ). C-terminal domain (CTD), N-terminal signal peptide (N-terminal), outer membrane (OM), inner membrane (IM).

Flexirubin biosynthesis conceivably involved into microalgae-bacteria interaction

Our transcriptome analysis of *Dyadobacter* sp. HH091 co-cultured with microalga *M. radians* revealed highly active genes affiliated with the cluster of flexirubin biosynthesis. This cluster includes *darA* and *darB genes*, homologs of *F. johnsoniae* UW101 (McBride et al., 2009) and *C. pinensis* (Schöner et al., 2014).

Flexirubin is a pigment consisting of a ω-(4-hydroxyphenyl)-polyene carboxylic acid chromophore, esterified with a 2,5-dialkylresorcinol (DAR), also known as novel and widespread bacterial signalling molecule (Nowak-Thompson et al., 2003; Abt et al., 2011; Schöner et al., 2014). Genes coding for the biosynthesis of these pigments are found in many bacteroidotal genomes, including *Flavobacterium psychrophilum*, *Flavobacterium johnsoniae* (McBride et al., 2009), *Leadbetterella byssophila (Abt et al.,* 2011*), Chryseobacterium artocarpi* (Venil et al., 2016), *Chryseobacterium rhizoplanae* sp. nov. (Kämpfer et al., 2015), *Flavobacterium maris* sp. nov. (Romanenko et al., 2015), and *Flavobacterium tilapiae* sp. nov. (Chen et al., 2013). Homologs of *darA*, a dialkylresorcinol condensing enzyme, and *darB*, a 3-oxoacyl-[acyl-carrier-protein] synthase III protein, were previously identified using bioinformatics tools within the genome analysis of our model organism *Dyadobacter* sp. HH091 (Astafyeva et al., 2022).

Another interesting point, is that on the plant-bacteria interaction model, flexirubin also performs as free radical scavenging antioxidant protecting from the attack of free radicals (Combes and Finet, 1997; Schöner et al., 2015). The antioxidant potential *via* hydrogen donating ability of flexirubin has been shown through the assessment using different assays such as radical scavenging activities, lipid peroxide inhibition and ferrous chelating ability (Mogadem et al., 2021). Several studies show that microalgae produce reactive oxygen species (ROS) to get an advantage in the competition for resources against other algae, be a way to prevent fouling bacteria, and act as a signalling mechanism between cells (Marshall et al., 2005). Furthermore, ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH), are thought to be produced as antibacterial agents and involved in oxidation or reduction of necessary or toxic metals (Palenik et al., 1987). Former investigation of microalga *Micrasterias* spp., demonstrated that ROS are constantly generated as by-products of general metabolic cellular pathways and can be over-produced in response to stress (Darehshouri and Lütz-Meindl, 2010; Lütz-Meindl, 2016; Felhofer et al., 2021). Our results indicate, that *Dyadobacter* sp. HH091 uses flexirubin hybrid pigments to protect itself from ROS produced by microalga, which explains this interaction, making it possible for microalgal symbiont to have a tight contact with its host.

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T9SS tangled in the symbiotic interactions of *Dyadobacter* with microalgae

The presence of different secretion systems suggests that *Dyadobacter* sp. HH091 and microalgae possess a signal exchange system allowing establishment and maintenance of a symbiosis that includes adhesion factors, microcolonization development, EPS production, and biofilm formation factors, which are important for the institution of a successful symbiosis. Previously, a comprehensive set of cell surface-associated proteins required for host cell invasion was described for other bacterial model organisms (Foster et al., 2014; Kusch and Engelmann, 2014; Hecker et al., 2018). All of these mechanisms express particular cocktails of factors that facilitate niche adaptation that include cell-host attachment, microcolonization and biofilm formation. Genes coding for the cell surface-associated proteins and secretion systems are mainly up-regulated in *Dyadobacter* sp. HH091, expecting them to be crucial for the microcolonization process because they establish interaction with the host. Cell-host interaction and adhesion factors, as well as microcolonization development, and biofilm formation succeed to a closely interaction and an exchange of growth-promoting substances between the symbiont and microalga.

Surface exposed proteins that are covalently or non-covalently bound to the cell surface and proteins are secreted into the extracellular matrix using different secretion mechanisms (Dreisbach et al., 2010; Ythier et al., 2012; Solis et al., 2014; Hecker et al., 2018). Secreted proteins accommodate the majority of virulence factors, enzymes required for nutrient acquisition or cell spreading, immune evasion proteins that can bypass the immune system or interfere with components of the complement system and many others. Overall, secretion systems are known to transport effector proteins into the cytosol of eukaryotic cells that allows the direct communication and modification of the host cells, additionally suppressing any activity of competitive microorganisms (Wooldridge, 2009). *Dyadobacter* sp. HH091 has many unique features together with the complex of different secretion systems, which are available to arbitrate secretion of proteins across the outer membrane, including T9SS, a complex translocon found only in some species of the Bacteroidota phylum (Lasica et al., 2017; Astafyeva et al., 2022).

A complex translocon of T9SS, including *gld* and *spr* genes, and *porXY-sigP* signalling system components, are proposed to serve as the secretion system of cargo-proteins. The T9SS cargo proteins have a conserved C-terminal domain (CTD) that enables them pass *via* T9SS and an N-terminal signal peptide that guides T9SS cargo proteins through the Sec system (Veith et al., 2013; Kulkarni et al., 2017). The CTD signal has been identified to be of two types, type A and type B (Kulkarni et al., 2017; Gorasia et al., 2020). Subsequent to the early *Dyadobacter* genome studies (Astafyeva et al., 2022), high activity of T9SS cargo proteins has been observed at this transcriptome analysis as well. It resulted in 48 up-regulated and 24 down-regulated genes, affiliated with T9SS cargo proteins of both types (Supplementary Table S5).

gldA, gldF and *gldG* encode components of an ATP-binding cassette (ABC) transporter that is required for motility and/or for the protein secretion (Agarwal et al., 1997; Hunnicutt et al., 2002). Genes encoding lipoproteins required for gliding (*gldB*, *gldD*, *gldH*, *gldI*, and *gldJ*) have also been identified (Hunnicutt and Mcbride, 2000; Hunnicutt and McBride, 2001; McBride and Braun, 2004; Braun and McBride, 2005). GldK, GldL, GldM, and GldN are each required for efficient motility and chitin utilization, indicating that Gld proteins may function in both gliding and chitin utilization (Braun et al., 2005). SprA is required for secretion of SprB and RemA and utilization of chitin (Nelson et al., 2007). In *F. johnsoniae*, SprA has been identified as the major translocon protein of T9SS, and it is hypothesized that SprA of *Dyadobacter* sp. HH091 can also have the same function (Lauber et al., 2018). Down-regulated gene coding for SprF is known to be essential for the secretion of SprB to the cell surface, but is not required for the secretion of extracellular chitinase (Rhodes et al., 2011). That also gives a hint that the symbiont possibly utilizes T9SS for the secretion of other proteins and not only involved in gliding motility.

Polysaccharide utilization is a crucial aspect of microalgae-bacteria interaction

T9SS is known to be tangled in the secretion of polysaccharide utilization proteins (Braun et al., 2005; Kharade and McBride, 2014). Previously, it was shown that the major chitinase (ChiA) in *F. johnsoniae* is fully secreted from the cell in soluble form by T9SS and is essential for chitin degradation (McBride and Zhu, 2013; Kharade and McBride, 2014; Larsbrink et al., 2016).

Based on genome and transcriptome analyses, presumably, *Dyadobacter* sp. HH091 has a complex of carbohydrate utilization domains for digestion of microalgae cell wall hemicelluloses, such as cellulose, xylan or mannan fibrils, and extensive matrix polysaccharides. Numerous carbohydrate-active enzymes predicted to encode GHs and esterases that could be involved in the degradation of microalgal cell wall hemicelluloses were highly active within transcriptome datasets (Supplementary Table S2). In addition, candidates like xylanases, β-xylosidases, arabinofuranosidases, and beta-glucuronidases involved in xylan digestion, β-mannosidases involved in mannan digestion, and candidate β-glycosidases and endoglucanase that could be involved in xyloglucan digestion were also identified.

Data obtained from transcriptome analysis allows to better understand the nature of the involvement of bacterial polysaccharide utilization genes into bacteria-algae liaison. In our previous study, we observed that the genome of given symbiont possesses a wide assortment of CAZymes predicted to breach algal cell wall (Astafyeva et al., 2022). Deep

investigation of transcriptome datasets unveiled the presence of these genes among DEGs. We observed that a significant number of genes (82) identified belonging to functions vital for carbohydrate transport and metabolism, including different GHs families, which are known to be involved into plant polysaccharides degradation (Kumar et al., 2017). For example, many up-regulated transcripts are affiliated with genes responsible for biosynthesis of GH5, GH13, GH25, GH30 and GH43 families enzymes, which function as effectors with roles in the degradation of plant polysaccharides (Rovenich et al., 2016; Snelders et al., 2018). These enzymes are known for acting as cellulose-degrading (Chang et al., 2016), starch-degrading (DeBoy et al., 2008), and catalysing hemicellulose and removing xyloses from xyloglucan (Glass et al., 2013; Bradley et al., 2022). Additionally, it was uncovered that genes affiliated with the synthesis of GH88 CAZyme, utilizing polysaccharide lyase activity to degrade pectins (Cantarel et al., 2009), was also up-regulated. Another highly active genes, coding for xylose isomerases, belong to CAZyme family GH43 that generally display specificity for arabinose-containing substrates. These gene combination reflects the competence of the symbiont to utilize starch and the complex of arabinan side-chains of pectin-rich cell walls as important nutrients (Ha et al., 2005; DeBoy et al., 2008).

Overall, our transcriptome analysis clearly showed, that bacteria can profit through the degradation of algal polysaccharides, while microalgae are being supplied with the repertoire of growth-promoting substances. The results of this research will serve as an efficient tool in further investigations of symbiotic microalgal–bacteria interactions. The remarkable benefit of a co-cultivation of microalgae and bacteria will have commercial and environmental positive impacts into the microalgal cultivation in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Genbank, ON237360.

Author contributions

YA and IK contributed to experimental design, lab work of metatranscriptomic, bioinformatics, and physiological analytical approaches, and writing of the research article. YA contributed to lab work of metatranscriptomic approaches and to assembly of metatranscriptomic datasets and bioinformatics approaches. MG and YA contributed to lab work of microscopic and analytical approaches. WS and IK contributed to general experimental design and writing of the research article. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1006609/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Growth measurement (OD 750nm) of *Micrasterias radians* MZCH 672 in co-culture with the strain *Dyadobacter* sp. HH091. Increased growth rate (OD 750nm) can be observed in the co-culture with HH091 compared to the antibiotic-treated *M. radians* culture.

SUPPLEMENTARY FIGURE S2
Identification and validation of flexirubin pigments. Analysis of *Dyadobacter* sp. HH091, Maribacter dokdonemsis (yellow-pigment control, no flexirubin identified, Yoon et al., 2005), and Escherichia coli DH5α (negative control) strains for the presence of flexirubin pigments. Cells were photographed before treatment (I), after exposure to 50 μL of 10 M KOH (II), and after exposure to KOH followed by exposure to 42μL 12 M HCl (III). Flexirubin-positive cells were yellow at neutral pH (I and III) and orange/red under alkaline conditions (II).

SUPPLEMENTARY TABLE S2
Differentially expressed genes (DEGs) of transcriptome dataset of *Dyadobacter* sp. HH091 co-cultured with *M. radians*.

SUPPLEMENTARY TABLE S5
Differently expressed genes (DEGs) coding for the T9SS cargo proteins.

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4 HEALTH BENEFITS OF MICROALGAE AND THEIR MICROBIOMES

Health benefits of microalgae and their microbiomes.

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Minireview

Health benefits of microalgae and their microbiomes

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Summary

Microalgae comprise a phylogenetically very diverse group of photosynthetic unicellular pro- and eukaryotic organisms growing in marine and other aquatic environments. While they are well explored for the generation of biofuels, their potential as a source of antimicrobial and prebiotic substances have recently received increasing interest. Within this framework, microalgae may offer solutions to the societal challenge we face, concerning the lack of antibiotics treating the growing level of antimicrobial resistant bacteria and fungi in clinical settings. While the vast majority of microalgae and their associated microbiota remain unstudied, they may be a fascinating and rewarding source for novel and more sustainable antimicrobials and alternative molecules and compounds. In this review, we present an overview of the current knowledge on health benefits of microalgae and their associated microbiota. Finally, we describe remaining issues and limitation, and suggest several promising research potentials that should be given attention.

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Introduction and background

Microalgae and their associated microbiota grow and survive in all climate zones and many species are well adapted to extreme temperatures and pH values. Since microalgae are photosynthetic active organisms, which can be grown under a wide variety of conditions, they are highly attractive for the biotechnological production of a wide range of different chemical compounds. They are particularly well known for their use in the production of advanced biofuels (e.g. drop-in biofuels and fourth-generation biofuels) and to some extend for the production of bioplastics (Chisti, 2007; Mata *et al.*, 2010; Hempel *et al.*, 2011; Rahman and Miller, 2017; Khan *et al.*, 2018; Onen Cinar *et al.*, 2020; Keasling *et al.*, 2021).

Recently, it has become clear that algae and their microbiota harbour a large and diverse set of genes for the biosynthesis of molecules that supress bacterial pathogens (Table 1) (Krohn-Molt *et al.*, 2013, 2017). One of the example are sterols with anti-inflammatory capacity, like diacylglycerols, triacylglycerols and phytosterols (Ostlund *et al.*, 2003; Bilbao *et al.*, 2016; Randhir

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Table 1. Key features and bioinformatical analysis of microalgae genomes and metagenomes.

Key features and bioinformatical analysis of microalgae genomes and metagenomes	*Chlamy-domonas reinhardii*	*Arthro-spira pla-tensis*	*Oscilla-toria acuminata* PCC 6304	*Gloeo-capsa* sp.	*Chryso-chromu-lina tobin*	*Chlorella variabilis* NC64A	*Cocco-myxa subellip-soidea* C-169	*Oceani-caulis* sp. HL-87 GFM and their micro-biome	*Lyngbya* sp. HA4199-MV5 and their micro-biome	*Scene-desmus quadri-cauda* and their micro-biome	*Micras-terias crux-melitensis* and their micro-biome	*Chlorella saccharo-phila* and their micro-biome	*Chlorella soro-kiniana* and their micro-biome
IMG ID	2614208541	650377906	2509276028	2503754017	3300021056	2507525016	2507525016	2588 254262	3300 034630	3300 005759	3300008886	3300 008885	3300 042370
Size (bp)	111100715	6788435	7804270	5882710	101136936	2758551	2758551	2758551	56655633	168690013	268162588	174773 623	2758551
Antibacterial activity													
Dienelactone hydrolase	6	4	3	6	40	2	2	3	52	75	255	100	60
Imidazolone-propionase	1	0	0	0	23	1	0	5	42	57	205	97	37
6-phosphogluco-nolactonase	2	1	2	1	21	3	1	0	26	36	115	54	16
Metal-dependent hydrolases, COG1235	0	3	3	0	14	0	0	1	13	23	96	34	14
Sugar lactone lactonase YvrE	0	0	1	2	24	3	3	1	43	74	202	113	30
Decanoic acid, capric acid, decylic acid (tetradecanoate)	5	4	5	6	0	3	5	5	0	0	0	0	0
Palmitoleic acid (palmitoleate)	12	8	8	10	0	13	11	8	151	0	0	0	0
gamma-Linolenic acid, γ-linolenic acid (γ-linolenate)	4	2	2	2	0	4	5	1	28	0	0	0	0
Arachidonic acid, polyunsaturated omega-6 fatty acid (arachidonate)	17	0	0	0	0	22	38	0	0	0	0	0	0
Docosahexaenoic acid (DHA)	18	1	1	0	0	40	32	6	72	0	0	0	0
Eicosapentaenoic acid (EPA) (docosapentae-noate)	13	1	1	0	0	31	49	6	47	0	0	0	0
Antiviral activity													
Phycoerythrobilin biosynthesis	2	2	4	2	0	6	4	0	2	0	0	0	0
Phycocyanobilin biosynthesis	1	2	3	2	0	4	3	0	1	0	0	0	0
Phycoviolobilin biosynthesis	1	2	3	2	0	4	3	0	1	0	0	0	0
Phycourobilin biosynthesis	1	1	2	1	0	3	2	0	2	0	0	0	0
Exopolysaccharide biosynthesis	0	3	1	6	22	1	0	2	31	54	121	55	32
D-galactose biosynthesis	1	1	2	5	18	2	1	0	21	43	199	97	29
L-arabinose biosynthesis	0	0	0	0	4	0	1	0	2	1	20	4	3
D-xylose biosynthesis	1	1	2	1	2	1	1	0	4		37	13	4

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Table 1. (Continued)

Key features and bioinformatical analysis of microalgae genomes and metagenomes	*Chlamydomonas reinhardii*	*Arthrospira platensis*	*Oscilla-toria acuminata* PCC 6304	*Gloeocapsa* sp.	*Chrysochromu-lina tobin*	*Chlorella variabilis* NC64A	*Coccomyxa subellipsoidea* C-169	*Oceani-caulis* sp. HL-87 GFM and their microbiome	*Lyngbya* sp. HA4199-MV5 and their micro-biome	*Scene-desmus quadri-cauda* and their microbiome	*Micras-terias crux-melitensis* and their microbiome	*Chlorella saccharo-phila* and their micro-biome	*Chlorella soro-kiniana* and their micro-biome
L-rhamnose biosynthesis	0	6	7	8	34	3	2	4	45	64	282	146	55
D-galacturonate biosynthesis (D-galacturonic acid)	1	1	1	1	7	0	0	1	10	12	52	32	8
Mannose biosynthesis	4	6	6	6	30	4	7	2	39	63	290	144	51
Fucose biosynthesis	0	4	4	3	35	1	1	2	38	66	264	135	53
Antioxidant activity													
Superoxide dismutase	6	0	2	2	15	4	6	1	18	22	90	46	20
Cu/Zn superoxide dismutase	0	0	0	0	6	0	0	0	6	8	23	9	4
Rhodanese-related sulfurtransferase	11	3	4	5	44	10	8	0	43	53	118	55	57
Catalase (peroxidase I)	1	0	0	0	15	0	0	1	14	28	86	44	16
Catalase	1	0	0	2	18	1	4	0	13	19	51	30	10
Mn-containing catalase (includes spore coat protein CotJC)	0	0	1	2	0	0	0	0	2	6	8	3	4
Ferritin, oxidative damage protectant	6	2	3	5	8	0	0	4	67	18	61	28	13
Glutaredoxin	8	4	3	4	41	13	8	4	50	59	188	112	38
Glutathione peroxidase	4	0	0	0	27	6	3	0	12	18	64	29	18
Cytochrome c peroxidase	0	1	0	1	4	0	0	0	6	17	68	21	15
Alkylhydroperoxidase	0	0	0	1	13	0	0	1	69	72	276	138	64
Deferrochelatase/ peroxidase	0	0	4	0	1	0	0	0	5	11	41	26	
Peroxiredoxin	9	11	12	8	54	9	11	6	54	78	314	139	74
Chlorophyll a biosynthesis	5	4	4	4	0	6	6	0	5	0	0	0	0
Carotenoid biosynthesis	0	0	2	0	0	13	13	0	0	0	0	0	0
Lutein biosynthesis	2	2	0	3	0	2	3	1	6	0	0	0	0
Zeaxanthin epoxidase	0	0	0	0	0	1	1	0	0	0	0	0	0
Violaxanthin de-epoxidase	0	0	0	0	0	1	1	0	0	0	0	0	0
astaxanthin biosynthesis	0	0	0	2	0	1	1	0	8	0	0	0	0
Anti-inflammatory and anti-cancer properties													
Phytosterol biosynthesis	5	0	0	0	0	5	7	0	1	0	0	0	0
Zymosterol biosynthesis	1	0	0	0	0	1	1	0	0	0	0	0	0
Ergosterol biosynthesis	7	0	0	0	0	7	7	0	2	0	0	0	0
Cholesterol biosynthesis	19	0	0	0	0	19	24	0	3	0	0	0	0

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Table 1. (Continued)

Key features and bioinformatical analysis of microalgae genomes and metagenomes	*Chlamy-domonas reinhardii*	*Arthro-spira pla-tensis*	*Oscilla-toria acuminata* PCC 6304	*Gloeo-capsa* sp.	*Chryso-chromu-lina tobin*	*Chlorella variabilis* NC64A	*Cocco-myxa subellip-soidea* C-169	*Oceani-caulis* sp. HL-87 GFM and their micro-biome	*Lyngbya* sp. HA4199-MV5 and their micro-biome	*Scene-desmus quadri-cauda* and their micro-biome	*Micras-terias crux-melitensis* and their micro-biome	*Chlorella saccharo-phila* and their micro-biome	*Chlorella soro-kiniana* and their micro-biome
Sulfoquinovosyl diacylglycerol biosynthesis	1	1	1	1	1	1	1	0	4	1	0	1	1
Diacylglycerol and triacylglycerol biosynthesis	10	3	2	2	40	14	14	3	43	65	254	125	48
Immune promoters and immunomodulatory activity													
1,4-alpha-glucan branching enzyme	4	2	3	3	0	2	2	0	12	0	0	0	0
Bacterial-like globin (possible phycocyanins)	11	0	1	1	0	5	2	1	15	0	0	0	0
Carotenoid cleavage dioxygenase or a related enzyme	5	1	2	2	0	5	6	1	12	0	0	0	0
Bacterial lipopolysaccharides biosynthesis (LPS)	3	5	6	10	21	0	0	2	54	28	33	13	8
Prebiotic activity													
Beta-1,3-glucan (paramylon) synthase	0	0	0	0	0	4	1	0	0	0	0	0	0
mycolyl-arabinogalactan-peptidoglycan complex biosynthesis	1	3	5	9	0	2	1	2	22	0	0	0	0
Cellulose biosynthesis	0	1	6	3	0	2	8	4	14	0	0	0	0
Algin biosynthesis (GDP-mannose biosynthesis)	4	6	6	6	0	4	7	2	39	0	0	0	0
GDP-L-fucose biosynthesis	0	2	2	1	0	0	1	0	17	0	0	0	0
dTDP-3-acetamido-α-D-fucose biosynthesis	0	0	0	0	0	1	0	0	0	0	0	0	0
agar; carrageenans biosynthesis (β-D-; α-D-; α-L-galactose)	2	1	2	5	0	3	2	0	21	0	0	0	0
GDP-L-fucose synthetase	0	2	0	1	0	0	1	0	0	0	0	0	0
Beta-galactosidase	2	0	1	2	0	2	11	0	16	0	0	0	0

Key features of antibacterial, antiviral, antioxidant activity and anti-inflammatory and anti-cancer properties as well as immune promoters and immunomodulatory activity of microalgae communities' genomes and metagenomes using IMG function search including IMG ID and total size of bp. Data shown in total number of hits for possible antibacterial activity, antiviral activity, antioxidant activity, anti-inflammatory and anti-cancer properties, and immune promoters and immunomodulatory activity.

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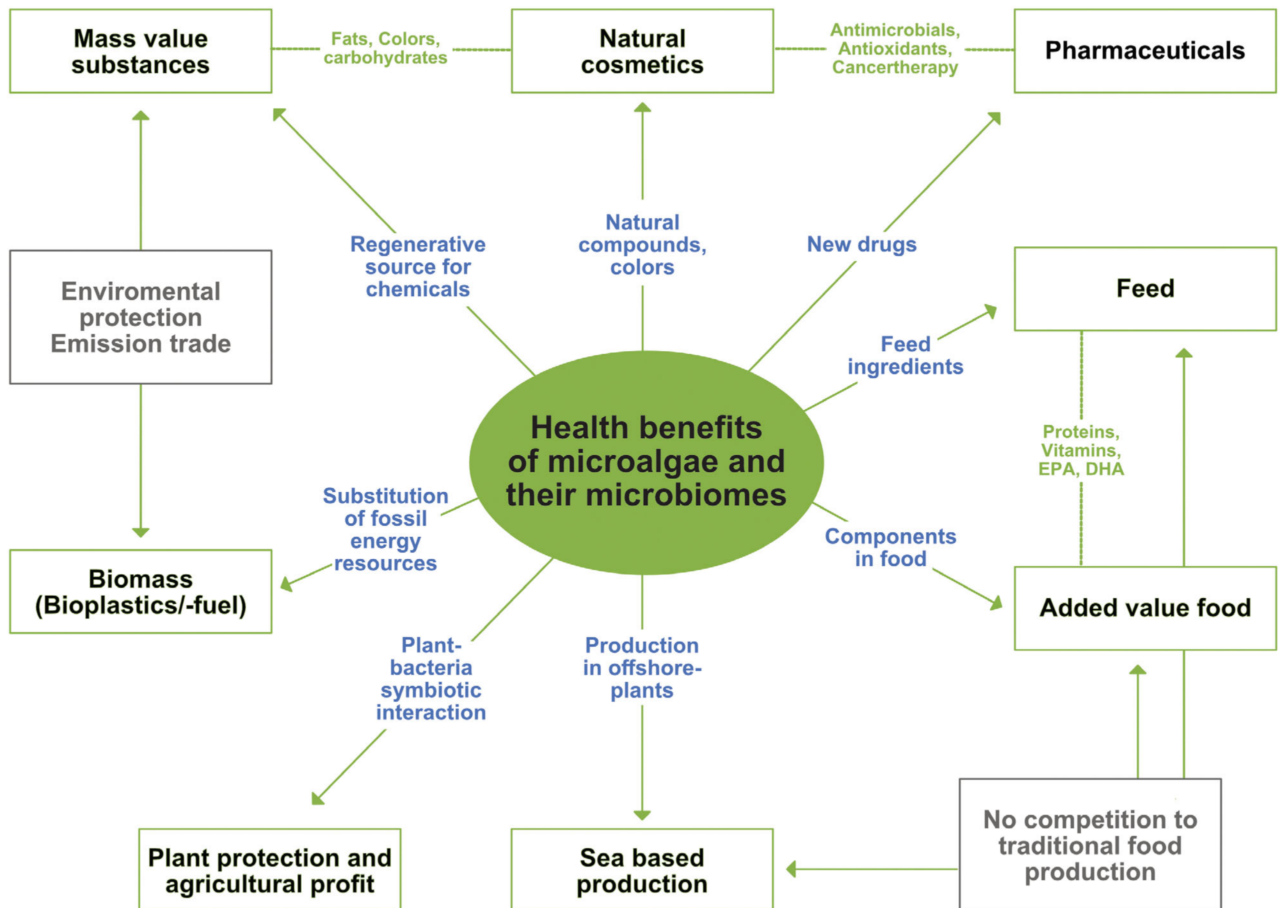

Fig. 1. Applications for microalgae including their microbiomes. Overview of potential microalgae and their communities' production and application fields; mainly reflecting clinical and human health, animal health and plant protection.

et al., 2020). In this respect, they bear a great potential to make major contributions to important societal needs linked to the treatment of infections due to human, animal and plant pathogenic microorganisms (Fig. 1). The appearance of untreatable antibiotic resistant microorganisms in clinical settings is a major concern to human health (O'Neill, 2016; WHO, 2019). Thus, there is a need to develop novel antimicrobials that are distinct in their mode of action from those already known and on the market. The awareness in the scientific community about the largely unexplored potential in microalgae has led to increased interest during the last decade, as evidenced by an exponential increase in the number of publications and patents on the subject of microalgae and health. However, compared to the large number of microalgal species, our knowledge remains sparse, and further research requires a focused and more systematic approach to better explore this promising resource with a special emphasis on human, animal and plant health and well-being. Here, we summarize current knowledge on the benefits of microalgae in health management. We further point out current limitations hindering their exploitation and address technologies that could provide a basis for a more systematic exploitation of their potential.

Antibacterial activity

Quorum sensing and quorum quenching as drives for antibiofilm strategies

Quorum sensing (QS) and Quorum Quenching (QQ) play an important role in the expression of virulence factors and antimicrobial resistance, and are involved in the formation of bacterial biofilms (Ahlgren *et al.*, 2011; Waters and Goldberg, 2019). The latter are of major concern in clinical and industrial settings, as they are very difficult to control and treat and cause severe problems in patients and industries. Both QQ and various mechanisms of QS interference have been outlined and discussed as possible strategies to prevent and treat microbial biofilm formation (Singh *et al.*, 2000; Ahlgren *et al.*, 2011; Fetzner, 2015; Waters and Goldberg, 2019).

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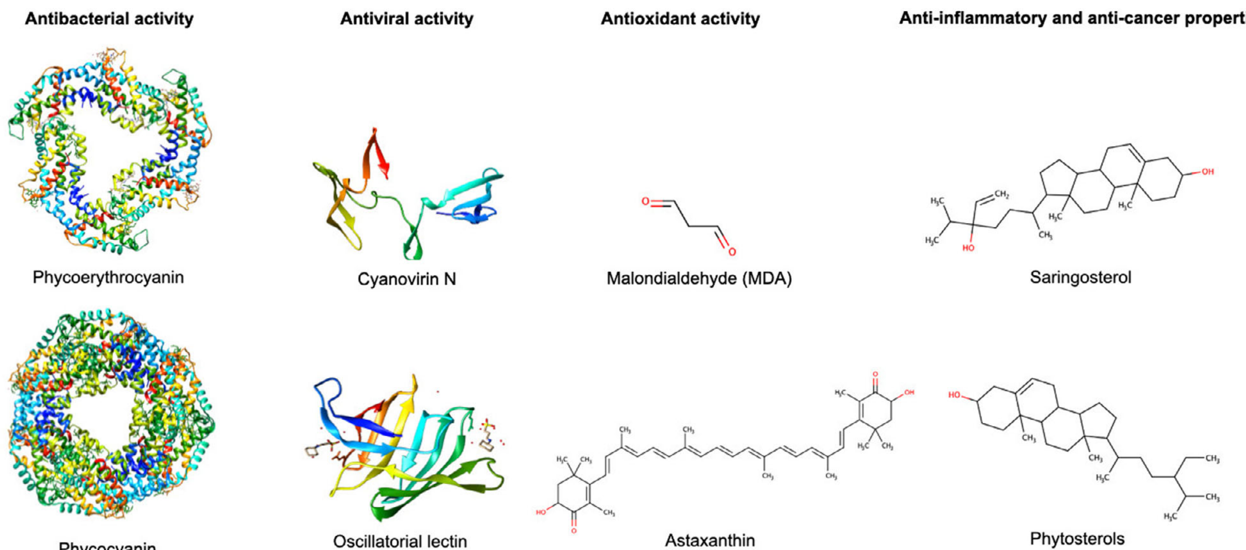

Fig. 2. Molecular structures. Selected biologically active compounds derived from microalgae and their associated microbiota. Antibacterial activity: Phycocyanin (Wang *et al*., 2001), Phycoerythrocyanin (Schmidt *et al*., 2006), Antiviral activity: Cyanovirin N (Yang *et al*., 1999), Oscillatorial lectin (Koharudin *et al*., 2011), Antioxidant activity: Malondialdehyde (MDA), Astaxanthin (https://pubchem.ncbi.nlm.nih.gov/) Anti-inflammatory and anti-cancer properties: Saringosterol, Phytosterols (https://pubchem.ncbi.nlm.nih.gov/). Marvin was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin version 21.17.0, ChemAxon (https://www.chemaxon.com). UCSF Chimera was used for molecular modelling and for analysis of molecular structures, developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (https://www.cgl.ucsf.edu/chimera/docs/credits.html).

Microalgae and their associated bacterial microbiota may be valuable tools to further verify this concept (Table 1).

Microalgae microbiomes offer both QQ enzymes and a broad variety of QS molecules that have been shown to interfere with pathogens (Ghanei-Motlagh *et al*., 2021a, 2021b). In this framework, the screening of 19 strains of microalgae, reported that one microbial community of *Chlorella saccharophila* and one of *Chlorella vulgaris* both degraded *N*-acyl homoserine lactones (AHLs), resulting in the inhibition of violacein production in the reporter strain (Natrah *et al*., 2011). When using a *E. coli* (JB523)-strain sensitive to N-(3-oxohexanoyl)-L-homoserine lactone, the *C. saccharophila* associated microbiome was found to significantly suppress bacterial QS and further to inhibit AHL-regulated bioluminescence in the pathogen *Vibrio harveyi* (Natrah *et al*., 2011). AHL degradation can occur indirectly, such as in cultures of *Tetraselmis suecica* and *Chaetoceros muelleri* that were associated with AHL-degrading bacteria belonging to the genera *Bacillus* and *Pseudomonas* (Pande *et al*., 2015). These bacterial isolates were found to degrade AHL molecules, and *Bacillus* sp. was reported to suppress the quorum sensing system of *Vibrio campbelli* and thus protected the larvae of the giant river prawn (*Macrobrachium rosenbergii*) from infection, improving survival from 42 to 67% during an infection challenge.

Another route of interference with quorum sensing systems is through the secretion of molecular mimics. In general, in Gram-negative bacteria, many important changes in gene expression and behaviour are regulated in a population density-dependent fashion by N-acyl homoserine lactone (AHL) signal molecules. Plants are able to secrete substances, which mimic bacterial N-acyl homoserine lactones. These mechanisms affect population density-dependent behaviours in associated bacteria. For example, ethyl acetate, extracted AHL mimics from *Chlamydomonas reinhardii*, was found to affect the expression of 34 proteins, 25 of which were also affected by AHL (Teplitski *et al*., 2004).

The analysis of algae and microalga microbiomes available at IMG/MER (https://img.jgi.doe.gov) revealed numerous QQ genes. The study of metagenomes of *Scenedesmus quadricauda*, *Chlorella saccharophila*, *Chlorella sorokiniana* and *Micrasterias crux-melitensis* unveiled dienelactone hydrolases, imidazolonepropionases, 6-phosphogluconolactonases and metal-dependent hydrolases, associated with QQ, which are potential candidates for overexpression experiments and biotechnological studies (Table 1).

Phycobiliproteins have antimicrobial effects

Multiple compounds from microalgae and their affiliated microbiota have been reported to have antimicrobial properties, sometimes as a secondary benefit distinct from their primary function. This is the case for

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phycobiliproteins, a family of water-soluble light-harvesting pigments, that play a central role in the photosynthesis of cyanobacteria and the red algae *Rhodophyta*. Phycobiliproteins are divided into four groups: phycocyanin, phycoerythrin, phycoerythrocyanin and allophycocyanin, of which phycocyanin is the most common in the environment (Li *et al*., 2019, Pagels *et al*., 2019, Fig. 2). Overall, in our bioinformatic analyses, it was found that publicly available genomes of *Chlorella variabilis* NC64A and *Coccomyxa subellipsoidea* C-169 (IMG ID 2507525016) contain phycoerythrobilin, phycocyanobilin, phycoviolobilin and phycourobilin biosynthesis genes (Table 1). For example, screening of 19 microalgae supernatants containing the whole microbial community identified multiple active phycobiliproteins, including phycocyanin and phycoerythrin, which exhibited significant antifungal property and growth inhibition of both Gram-positive and -negative bacteria (Najdenski *et al*., 2013). A phycobiliprotein extract from *Arthrospira platensis* was found to have antifungal effect against the plant pathogen *Botrytis cinerea* when applied at doses from 0.3 to 4.8 mg ml^{-1}, both reducing the fungal growth as well as protecting tomato fruits from infection (Righini *et al*., 2020). Interestingly, environmental conditions have been found to affect the production of phycobiliproteins, both in terms of quantity and of the distribution of phycobiliprotein produced, with factors such as the pH, quality of light and nutrient source all having a significant effect (Pagels *et al*., 2019). Khattar *et al*. (2015) reported optimized culture conditions for *Anabaena fertilissima* that involved a slightly alkaline pH as well as supplement of nitrite and illumination with blue light, resulting in a 1.6-fold increase in the total production of phycobiliproteins (from 383 to 627 μg mg^{-1}; $P < 0.05$) and a 4.5-fold in the production of phycoerythrin (from slightly more than 100 to almost 500 μg mg^{-1}; $P < 0.05$). Overall, as every microalgae will require its own optimization to achieve its maximum potential, it is one of the main issues for practical application of these products.

Fatty acids play a key role as antimicrobials

Fatty acids, including those from microalgae, have strong antimicrobial effects. Notably, extracts from cyanobacteria have been shown to be inhibitory against *Streptococcus pyogenes* and *Staphylococcus aureus*, while fatty acids from the cyanobacterium *Synechocystis* sp. were inhibitory against *Bacillus cereus*, *Escherichia coli* and the yeast *Candida albicans* (Najdenski *et al*., 2013). Ruffel et al. tested 29 different types of purified fatty acids from different species of microalgae using disk-diffusion assay. The results show, that 3 of 29 fatty acids were inhibitory against *E. coli*, while 15 were inhibitory against *S. aureus* (Ruffell *et al*., 2016). The effective dose ranged from 250 to 2000 μg per disk and polyunsaturated fatty acids (PUFA) were found to be more significantly more likely to display antimicrobial activity compared to monounsaturated or saturated acids (11 of 13, compared to two of seven and two of nine respectively).

Testing of extracts from *Chlorococcum* strain HS-IO1 and *Dunaliella primolecta* showed that α-linolenic acid from these algae had antimicrobial properties against methicillin-resistant *Staphylococcus aureus* (MRSA) (Ohta *et al*., 1995). Similarly, the fatty acid fraction of the acidophilic *Coccomyxa onubensis* has been shown to have antimicrobial activity against multiple Gram-positive and -negative bacterial pathogens. Although these authors did not test the individual fatty acids involved in this activity, the most common fatty acids in the extracts included palmitic acid and oleic acid alongside the PUFA linoleic acid and linolenic acid (Navarro *et al*., 2017). In *Chlorella* spp., a mixture of fatty acids, termed ‘chlorellin’ is known to have antimicrobial properties, and, for example, ethanol and isopropanol-extracts from *Chlorella* spp. were shown to have inhibitory capacity equivalent to ampicillin and oxacillin against *Staphylococcus* spp., although this author did not investigate the effect of the individual fatty acids (Acurio *et al*., 2018). Taken together, these results suggest that various microalgal fatty acids can exert antimicrobial activity, although PUFA appeared more likely to do so. Unfortunately, drawing firm conclusions is hindered by the fact that many authors treat all fatty acids together rather than attempt to separate the various species of fatty acids to test them separately.

Contrary to the percentage of lipids in microalgal cells which is considered roughly comparable between microalgal species, fatty acids are highly variable both in terms of their relative concentration and the repartition of the various species of fatty acids (Hu, 2013). Several environmental factors have been reported to influence the fatty acid profile of phytoplankton, for example, higher temperatures are associated with an increased in the proportion of saturated fatty acids, whereas decreased light levels were associated with an increased in polyunsaturated fatty acids (plausibly due to an increase in the presence of thylakoids to improve photosynthetic activity) (Guedes *et al*., 2010; Li *et al*., 2011). Interestingly, comparison of the fatty acid profiles of diatoms and dinoflagellates by Peltomaa *et al*. (2019) suggested that fatty acid contents were higher in freshwater species than marine ones. However, genetic and phylogeny appear to be the main factor dictating the fatty acid profile of microalgae. For example, screening of 1145 species, representing six major groups of both marine and freshwater phytoplankton species, showed that phylogeny was the main factor influencing the fatty

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acid profile of microalgae (Galloway and Winder, 2015) accounting for about 36 to 44% of the total variation in fatty acid profiles. Similar results were reported by Jónasdóttir (2019) based on the screening of the fatty acid profiles of 160 species representing seven phyla of marine phytoplankton.

For example, Cavonius *et al*. (2014) reported that fatty acid represented 12% of the dry mass of *Tetraselmis galbana*, but only 4–5% in *Phaeodactylum tricornutum*. Similarly, screening of 2076 strains of microalgae by Lang *et al*. (2011) showed that fatty acids were particularly low in *Chlorophyta* and *Streptophyta*. Comparison of the fatty acid profiles of six commonly cultivated strains of microalgae showed that *C. vulgaris* had a comparatively high concentration of 16:2 fatty acid. In addition, both *C. vulgaris* and *Tetradesmus obliquus* had comparatively high levels of 18:1 fatty acids as well as 18:3, linolenic acid (Chacón-Lee and González-Mariño, 2010). This is in accordance with the reports of Cepas *et al*. (2021) who, after screening the fatty acid profiles of several strains of cyanobacteria also reported that *C. vulgaris* and *T. obliquus* were particularly rich in linolenic acid.

Other microalgal molecules with antibacterial effects

EPS from several algal cultures were inhibitory against multiple bacterial and fungal isolates when tested using both the agar diffusion and the minimal inhibitory concentration (MIC) (Najdenski *et al*., 2013). Of these, the most effective were EPS from *Gloeocapsa* sp. with MIC values ranging from 0.125 for *S. aureus* to 1.0 mg ml^{-1} for *S. pyogenes* (Najdenski *et al*., 2013). Similarly, crude extracts from *H. pluvialis* demonstrated inhibitory effects against several bacterial pathogens using disk-diffusion assays, resulting in inhibition zone between 6.1 and 10.2 mm (Rather *et al*., 2021).

Extraction of *A. platensis* compounds using different solvents revealed that methanolic extracts had the highest antimicrobial activity against bacterial pathogens. Whereby, MIC of were 128 and 256 μg ml^{-1} against *S. aureus* and *E. coli*, respectively, although the compounds involved were not further characterized (Kaushik and Chauhan, 2008). Extraction of a variety of antimicrobial compounds from *Cosmarium* sp. showed that all had some potential as antimicrobials, although the methanol, hexane and aqueous extracts were not effective against the Gram-positive bacteria tested (Challouf *et al*., 2012).

Other microalgae cultures including their microbiomes have been found to display antimicrobial properties, but without the responsible compounds being further characterized. This is the case for eight freshwater microalgae (belonging to the genera *Oscillatoria*, *Lyngbya*, *Oedogonium* and *Spirogyra*) whose ethanolic and methanolic fractions, tested at concentrations ranging from 0.16 to 0.66 mg ml^{-1} using the disk-diffusion method, demonstrated some inhibitory properties against some Gram-negative and one Gram-positive pathogenic bacteria with zone of inhibitions ranging from 7 to 12 mm, for *O. sancta* extracted using ethanol at a dose of 0.35 mg ml^{-1} and *S. decimina* extracted using a methanol solvent and applied at 0.20 mg ml^{-1} respectively (Prakash *et al*., 2011).

For example, methanolic extracts from the cyanobacterium *A. platensis* at 100 ng ml^{-1} have clear inhibitory effects on the biofilm formation of several bacteria, including pathogens. Biofilm formation of *Vibrio parahaemolyticus* was inhibited by 90%, of *Vibrio alginolyticus* by 88%; of *Aeromonas hydrophila by* 74%; and by 61 to 84%, in *S. aureus* (LewisOscar *et al*., 2017).

In addition, for the green alga *Chlamydomonas reinhardtii*, Vishwakarma and Sirisha (2020) reported that extracted sulfated polysaccharides displayed activity against the biofilms of *Salmonella enterica* and *V. harveyi*, distorting the biofilms and reducing their formation by about 50% when applied at concentrations of 0.5 mg ml^{-1} against *S. enterica* and 8 mg ml^{-1} against *V. harveyi*. Ghaidaa *et al*. (2020) reported similar findings with *C. reinhardtii* reducing the formation of biofilms of several bacterial species by about 50%. Several human pathogens were also more susceptible to these compounds since *S. aureus* biofilms were reduced by up to 90%.

Antiviral activity

In cyanobacteria, a variety of antiviral and antimicrobial molecules has been described over the years, as recently reviewed by Mazur-Marzec *et al*. (2021) and Khalifa *et al*. (2021), including cyanovirin N, isolated from *Nostoc ellipsosporum*, which is known to interfere with human immunodeficiency virus (HIV's) binding onto CD+ T-cells. The antiviral compound cyanovirin-N binds the viral spike protein gp120 that is required for HIV interactions with receptors on the host cells and has been shown to have antiviral activity against HIV (Dey *et al*., 2000; Singh *et al*., 2005). Cyanovirin-N has also demonstrated inhibitory action on other enveloped viruses such as herpes virus and measles virus as well as feline immunodeficiency virus (FIV) at concentrations as low as 10 nM (Dey *et al*., 2000). More recently, *in silico* docking simulations have suggested that cyanovirin-N could form stable covalent bonds with the homotrimeric transmembrane spike glycoprotein of severe acute respiratory syndrome coronavirus-2 (Lokhande *et al*., 2020).

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Among these, lectins are ubiquitous proteins, whose carbohydrate domains have been shown to interact with the surface of cells and viruses. For example, a lectin recently isolated from the cyanobacterium *Oscillatoria acuminate* was found to be inhibitory against herpes simplex virus type-1 at doses at low as 20 ng ml^{-1} with highest inhibition at a dose of 2 mg ml^{-1} (Saad *et al*., 2020). Because pre-treating the cells prior to viral infection was not found to be protective, it is most likely that this lectin interacts and interferes with viral receptors. Figure 2 shows as an example the structures of cyanovirin N and oscillatorial lectin.

A further interesting fact is that many microalgae communities are able to produce polysaccharides, which are well known to exert a broad spectrum of biological activities, especially antiviral properties (Chaisuwan *et al.* 2021). These polysaccharides were found among numerous algal microbiomes, including *S. quadricauda* (IMG ID 3300005759), *C. saccharophila* (IMG ID 3300008885), *C. sorokiniana* (IMG ID 3300042370) and *M. crux-melitensis* (IMG ID 3300008886) (Table 1).

Antioxidant activity

It is well established that multiple compounds from microalgae have antioxidant activity. The best known algal antioxidant is the keto-carotenoid pigment astaxanthin, in particular extracted from the green algae *Haematococcus pluvialis* (Plaza *et al*., 2009, Fig. 2). Astaxanthin can neutralize singlet oxygen and scavenge free radicals, resulting in a powerful antioxidant effect, approximately 10 times stronger than β-carotene and 100 times greater than that of α-tocopherol (Shimidzu *et al*., 1996; Lorenz and Cysewski, 2000). *H. pluvialis* exists under different morphotypes, influenced by environmental conditions, including high temperature, intense light, in particular UV-light, alongside other stressors such as salinity, drought or nutrient scarcity. Under the effect of these environmental stressors, it switches from a flagellated free-living cell to a coccoid cyst called an aplanospore (Lim *et al*., 2018; Molino *et al*., 2018). This is accompanied by a degeneration of the cell's chloroplasts, hence the name 'red phase' given to this phase of the life cycle, a thickening of the outer cell wall and secretion of two layers of extracellular matrix as well as the accumulation of large quantities of astaxanthin in oil droplets within the aplanospore (Wayama *et al*., 2013). During this process, the relative volume of astaxanthin changes from 0.2% of the total free-living cell to 52% in the aplanospores.

The antioxidant capacity of astaxanthin has recently been shown in juvenile Asian tiger shrimp (*Penaeus monodon*). Supplementation of 80 mg astaxanthin per kg of diet was associated with a significant increase in the total antioxidant status and superoxide dismutase (SOD) in the haemolymph of the shrimp (Pan *et al*., 2003). This was connected to an improved recovery following exposure to various stressors and a decrease in both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values in the haemolymph, suggesting a hepatoprotective effect of astaxanthin. Comparable results were reported after feeding diet supplemented with 8 g kg^{-1} of astaxanthin to yellow catfish (*Pelteobagrus fulvidraco*), which resulted in an increase in SOD and HSP70 activity, and a reduction in both ALT and AST, as well as an increased survival following stress and infectious challenge with *Proteus mirabilis* (Liu *et al*., 2016).

Another example of compounds with antioxidant activity is malondialdehyde (MDA, Fig. 2). Feeding of lambs with *A. platensis* (incorporated at 0.1 g kg^{-1} of food) resulted in a decrease of the animals´ serum concentrations of MDA (from 99 to 16 nmol ml^{-1} in the animals receiving the control and supplemented feed respectively). At the same time, an increase in both vitamin A (from 690 to 710 ng ml^{-1}) and glutathione (from 90 to 140 ng ml^{-1}) was recorded in the animals' sera (EL-Sabagh *et al*., 2014). The same authors further reported reduction in the sera's alanine and aspartate aminotransferase, which is consistent with reduced oxidative stress to the liver. Diet supplemented with *A. platensis* (at doses ranging from 25 to 100 g kg^{-1} of feed) fed to the fish *O. mykiss* was correlated with an increase in the serum antioxidant activity (at doses ranging from 50 to 100 g kg^{-1} of feed) alongside an increased expression of the superoxide dismutase and catalase genes in the liver of the fish, when given at doses of 75 or 100 g kg^{-1} of feed (Teimouri *et al*., 2019). Feeding of the fruit fly *Drosophila melanogaster* with *Chlorella sorokiniana* (incorporated at doses of 2 or 4 mg ml^{-1}) resulted in an increased expression of *SOD1*, a superoxide dismutase encoding gene, as well as resistance against H_2O_2-induced oxidative stress (Qiu *et al*., 2020). Phycobiliproteins have also demonstrated potent antioxidant capacities and promoted the elimination of reactive oxygen species and increasing the concentration of antioxidative enzymes (Li *et al*., 2019). Future analyses could be more investigated for bioinformatical analysis of algae and their microbiomes. So far, data sets published at IMG/MER revealed genes coding for SOD, catalases and rhodanese-related sulfurtransferases (Table 1). Furthermore, the studying of algal genomes (*C. variabilis* NC64A and *C. subellipsoidea* C-169) demonstrated the presence of genes coding for the biosynthesis of known antioxidants, such as chlorophyll, carotenoid, lutein and astaxanthin. These genomes are available under the accession number IMG ID 2507525016 (Table 1).

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Anti-inflammatory and anti-cancer properties

Sterols represent a subgroup of steroid molecules and are widespread in the cell membranes of eukaryotic organisms. Figure 2 shows the molecular structures of saringosterol and phytosterols. Various sterols have been linked to diverse health benefits, including anti-inflammatory and anti-cancer properties. Phytosterols from *H. pluvialis* have been shown to have cytotoxic effects on human IMR-32 neuroblastoma cells, with a dose of 100 or 200 μM inhibiting neuronal activity by about 60% (Bilbao *et al*., 2016). Sanjeewa *et al*. (2016) have also reported anti-inflammatory effect of the hexanoic fraction of extracts from *Nannochloropsis oculata*, suppressing nitric oxide production in LPS activated macrophages when applied at doses of 6.25, 12.5 or 25 μg ml^{-1}. This fraction also showed anti-proliferative and pro-apoptotic effects, when applied at doses of 25 μg ml^{-1}, in several human cancer cell lines.

Sterols are mostly employed in cardiovascular health, because of their ability to hinder cholesterol adsorption in the intestine (Ostlund *et al*., 2003). The saringosterol from the kelp species *Lessonia nigrescens* has been shown to have antimicrobial activity on *Mycobacterium tuberculosis* with MIC values equivalent to rifampin at 0.25 μg ml^{-1} (Wächter *et al*., 2001). Interestingly, it has been estimated that the current source of phytosterols will be unable to meet demands by 2030 (Randhir *et al*., 2020). The presence of a high variety of sterols is well established in eukaryotic algae and, more controversially, also in cyanobacteria (Volkman, 2003), although the subject has received very little considerations and much remains to be investigated in this field (Randhir *et al*., 2020).

Immune promoters and immunomodulatory activity

A large number of plants and microorganisms are known to possess an immunostimulatory activity (Riccio and Lauritano, 2020), although the mechanisms through which microalgae cultures exert this immunostimulatory effect, often remain to be clarified. However, microalgal products have been known to induce the expression of various immune genes. Moreover, Xu *et al*. (2014) have reported an increase in digestive enzyme and an improvement in the growth performance of gibel carp (*Carassius auratus gibelio*) fed dried powder of *Chlorella* sp. (incorporated at doses as low as 4 g kg^{-1} of food). Similarly, Adel *et al*. (2016) reported a significant increase in protease activity as well as the population of lactic acid bacteria in the intestine of sturgeons (*Huso huso*) fed *A. platensis* at dose of 50 or 100 g kg^{-1} of food. These results suggested that an improvement in the digestive health of the fish, possibly linked to a prebiotic effect of the algae, may have contributed to the improved immune parameters reported in the studies. For example, polysaccharides from *Chlorella vulgaris* have been shown to promote the transcription of nitric oxide, prostaglandin E2, TNF-α, IL-6 and IL-10, as well as promote cell proliferation in the murine macrophage cell line RAW264.7 (Tabarsa *et al*., 2015). Feeding of *Nile tilapia* (*Oreochromis niloticus*) with feed supplemented with 50 mg kg^{-1} of either β-Carotene or phycocyanin-supplemented feed resulted in a significant elevation of the activity of multiple blood immune parameters (phagocytic and lysozyme activity, immunoglobulin M levels), while expression of the genes coding for the interferon gamma and interleukin 1β was upregulated (Hassaan *et al*., 2021). Addition of dry powder from *C. vulgaris* to the diet of the Koi carp *Cyprinus carpio* at doses ranging from 50 to 100 g kg^{-1} of feed resulted in the proliferation of red and white blood cells, while inclusion of the algae at doses ranging from 20 to 100 g kg^{-1} of feed increased lysozyme activity (Khani *et al*., 2017).

Reports on supplementing feed for rainbow trout (*Oncorhynchus mykiss*) with β-carotene rich extracts from the marine phytoplankton *Dunaliella salina* at doses of 100 to 200 mg kg^{-1} resulted in an increase in the phagocytic rate and the serum complement and lysozyme activity in the fish (Amar *et al*., 2004). In shrimp, it has been reported that feed supplementation with 3 g kg^{-1} of *Arthrospira platensis* resulted in an improvement of the phagocytic activity of haemocytes from the banana shrimp *Penaeus merguiensis*, as well as resistance to infection by *Vibrio harveyi* (Lee *et al*., 2003). In mammals, diet supplementation with extracts from *A. platensis* was found to increase the levels of IgG1 in the serum and IgA in the intestine, alongside the antibody produced in the supernatants of lymphoid cell cultures from the spleens and mesenteric lymph nodes of *A. platensis*-fed mice (Hayashi *et al*., 1998). Interestingly, the effect was class-specific as IgE levels were unaffected by this feed supplement. Comparable results were obtained by dogs where supplementation of the diet with 0.2% of spray-dried *A. platensis* resulted in an increase in the levels of serum antibodies and faecal IgA with the following vaccination with a commercial anti-rabies vaccines (Satyaraj *et al*., 2021). Investigations using *Dunaliella tertiolecta* found that extracts and purified sterols (at concentrations of 0.4 mg ml^{-1} and 0.8 mg ml^{-1} diluted 1 in 3) from this microalgae had anti-inflammatory effects in sheep, reducing proliferation of peripheral blood mononuclear cells as well as the production of interleukin-6, which was the opposite to what Tabarsa *et al*. (2015) reported in *C. vulgaris*, while promoting secretion of IL-10 (Caroprese *et al*., 2012). Administration of 50 mL a day of warm-water extracts from *A. platensis* to sheep was reported to induce secretion

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interleukin 12 subunit beta (IL12 p40) by peripheral blood mononuclear cells as well as the secretion of interferon-gamma and the cytotoxic activity of activated NK cells (Hirahashi *et al*., 2002). However, diet supplemented with increasing doses of *A. platensis* (ranging from 5 to 20 g kg^{-1}) resulted a dose-dependent increase in harvested macrophages, with a higher percentage of macrophages phagocytosing sheep red blood cells (SRBC) and a higher average number of SRBC in each macrophage (Al-Batshan *et al*., 2001). The authors of this study also reported a significant increase in nitric-oxide production in macrophage stimulated with bacterial LPS.

Prebiotic activity

The indigenous microbiota of microalgae represent an early and important barrier to infection. Local bacteria can inhibit bacterial infections either directly through the secretion of antimicrobial or antiviral compounds, or by competing with them for nutrient and attachment sites, a phenomenon known as competitive exclusion (Irianto and Austin, 2002; Ghanei-Motlagh *et al*., 2021a, 2021b). Consequently, research has been performed on the possibility to defend against infection either through the direct ingestion of beneficial microorganisms (probiotic treatments) or the ingestion of substances that promote the growth of beneficial bacteria (prebiotic treatments). Several microalgae have been shown to have prebiotic activity, for example, *C. vulgaris* and *A. platensis* are known to increase the viability and survival of multiple beneficial bacteria such as lactobacilli and bifidobacteria when incorporated at doses ranging from 0.25 to 1.00% (Beheshtipour *et al*., 2012). Moreover, co-culture with *C. vulgaris* or *Nannochloropsis oculata* has been shown to improve the antimicrobial activity of *Sulfitobacter* spp. or *Roseobacter* sp., respectively, against *Vibrio anguillarum* (Sharifah and Eguchi, 2011, 2012). More recently, it has been reported that supplementation of dogs' diets with 2 g kg^{-1} spray-dried *A. platensis* improved the stability of the gut microbiota in dogs during periods of physical exercises (Satyaraj *et al*., 2021). It has been suggested that some algae have the opposite effect, such as sequestering valuable nutrients and reducing their availability to bacteria.

Discussion and limitations

Compound production and toxic compounds

The production of compounds from microalgae microbiomes is complicated by the fact that their production is often strongly influenced by the culture conditions of the algae and that these conditions are not always known for all algal strains (Abu-Ghannam and Rajauria, 2013; Fatma, 2009). This is further complicated by different culture conditions that may affect various beneficial factors in different ways. For example, cultivation in Zarrouk medium improved the production of β-carotene and the antioxidant properties of several strains of *Arthrospira* spp. (several dozen times for some strains) while a medium deprived of some mineral ingredients, RM6, allowed for an improved production of phycobiliproteins (Tarko *et al*., 2012). A strong seasonal effect has also been reported, although this may simply be a side-effect of changes in light and temperature conditions (Abu-Ghannam and Rajauria, 2013). Changes in light intensity to intensities inducing light stress have been shown to increase production of triacylglycerol by 250% and sterols by 1200% in *H. pluvialis* (Bilbao *et al*., 2016).

Optimal culture conditions for the algae will often be different from the conditions for the optimal production of the compounds of interest, as is the case for the production of carotene or antimicrobial fatty acids (Ruffell *et al*., 2016; Molino *et al*., 2018; Kaha *et al*., 2021). As one could expect, protective secondary metabolites are often produced in response to stressors which will impair algal growth (Little *et al*., 2021). However, there is no universal rule correlating harsher culture conditions with the accumulation of beneficial compounds. For example, Ru *et al*. (2020) have reported that poor growth conditions lead to an increase in the starch content of *C. vulgaris* (Ru *et al*., 2020). Even within the same species, there can be considerable differences between strains, and it will be necessary to confirm that the strain does produce the compound of interest in high quantities under the expected culture conditions (Tarko *et al*., 2012).

Several microalgae are known to secrete phycotoxins. In particular dinoflagellates are known as a major source of toxins in the marine environment (Wang, 2008). These toxins, including the alkaloid saxitoxin that is considered the most toxic among them, have been associated with neurotoxicity. Dinoflagellates toxins are normally present at relatively low levels in the environment, although this level increases during algal blooms involving these species and have been correlated with mortality events in aquatic life, for example, *Alexandrium tamarense* has been associated with mass mortalities in fish, birds and aquatic mammals in the Saint Lawrence Estuary in Canada (Starr *et al*., 2017). These toxins are known to bioaccumulate along the food chain with predatory carnivorous fish harbouring higher levels of the toxins, and diseases in humans are often associated with the consumption of contaminated seafood. In the case of ciguatera, caused by ciguatoxin and maitotoxin produced by microalgae of the genus *Gambierdiscus*, cases are more generally associated with the consumption of contaminated reef-dwelling fish. The presence of

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these toxins is likely a major reason why dinoflagellates have received less attention as a source of health products (Friedman *et al*., 2017).

Cyanobacteria are known to secrete a large number of various toxins with hepatotoxic effects as well as known neurotoxins such as kalkitoxin and saxitoxin, and more than 100 species of cyanobacteria have been shown to secrete toxins (Singh *et al*., 2005; Sacilotto Detoni *et al*., 2016; Zerrifi *et al*., 2021). Extracts from the marine cyanobacterium *Trichodesmium erythraeum* from microalgal blooms on the Brazilian coastline were found to contain microcystins, cylindrospermopsins and saxitoxins, and showed toxic antimitotic activity against larvae of the green sea urchin (*Lytechinus variegatus*) but not against mice (Proença *et al*., 2009). Saxitoxins from *T. erythraeum* were also connected to mortality events in farmed pearl oysters (Negri *et al*., 2004). Some of these cytotoxic activities may prove beneficial, for example, in the development and antitumor therapeutants; however, they will make commercial adoption of microalgae more complex (Parra-Riofrío *et al*., 2020).

Sustainability – effect of environmental conditions and costs

From a sustainability perspective, microalgae production allows to capture CO_2 with higher average bioenergetic yield on sunlight than higher plants: 10% vs. 5% respectively (Williams and Laurens, 2010). Such higher yield allows to reduce the use of land for cultivation. While closed photobioreactors enable higher productivity and finer control of growth conditions than open pond systems, they also display higher energy consumptions (Brentner *et al*., 2011; Valdovinos-García *et al*., 2021). Recent Life Cycle Assessments (LCA) studies of microalgae cultivation in closed reactors (Pérez-lópez *et al*., 2014a; 2014b; Porcelli *et al*., 2020) showed that in pilot scale astaxanthin production from *Haematococcus pluvialis* and in the production of *Tetraselmis suecica* and *Phaeodactylum tricornutum*, the main contributor to most environmental impact categories were by far the electricity consumption during the cultivation stage. In these cultivation systems, electricity is mainly consumed for mixing and pumping large water volumes and for lighting the reactor when necessary. Regarding outdoor cultivation, the need to thermoregulate the system is one of the main drivers for electricity consumption (Pérez-López *et al*., 2017; Smetana *et al*., 2017; Schade and Meier, 2019; Duran Quintero *et al*., 2021). A common insight from these studies is that the environmental performance of cultivating a specific strain of microalgae is highly dependent on the location, cultivation period and suitable thermal range of the strain (Duran Quintero *et al*., 2021).

Microalgae are becoming increasingly interesting for the extraction of high value compounds, rather than as feedstock for the refining of low value products such as biofuels. Concerning this extraction, Pérez-lópez *et al*. (2014a, 2014b) showed that the extraction stage (methanol and KOH solutions) for the production of PUFAs, α-tocopherol, chlorophyll, β-carotenoid and polyphenols by *Tetraselmis suecica* was the second most important contributor to most environmental impact categories, but remained far behind the cultivation stage. Supercritical CO_2 fluid extraction was used for astaxanthin extraction Pérez-lópez *et al*. (2014a, 2014b) and accounted for less than 10% of the considered impact categories. Overall, even if the extraction method depends on the targeted bioactive compound, cultivation will remain the main environmental hotspot for new microalgal strains. Crucial to limit the environmental impacts of a microalgal production is the valorization of coproducts within an integrated biorefinery approach (Da Silva *et al*., 2014; 't Lam et al., 2018). Microalgae residual biomass can serve as substrate for biogas production via anaerobic digestion and the residual digestate can substitute the production of fertilizers (Collet *et al*., 2011; Pérez-lópez *et al*., 2014a, 2014b). Depending on the nutrient profile of the biomass, it could also be used to substitute animal feed (Draganovic, 2013). Due to the high diversity of the assumptions, parameters and production technologies, for strains that have not been cultivated yet, it is worth noting that their behaviour, optimal growth conditions and productivity in given reactors and locations are currently difficult to anticipate and therefore highly uncertain (Mata *et al*., 2010; Barra *et al*., 2014).

The potential need to induce bioactive molecule production by specific cultivation conditions, such as high-intensity lighting for astaxanthin production from *Haematococcus pluvialis*, could greatly affect the final environmental impacts (Pérez-lópez *et al*., 2014a; 2014b; Onorato and Rösch, 2020). Ultimately, the overall sustainability of producing microalgae-based bioactive molecules depends on the final efficiency and quality of the produced compounds. Finding synergistic products would therefore be of great interest. Indeed, the compound's capacity to substitute alternatives, tackle key issues such as fish farming health management and the needed doses will highly affect its environmental performance (Liu *et al*., 2016; Lieke *et al*., 2020).

Opportunities for future research

Currently, the main knowledge gap is linked to the correspondingly low numbers of algal species studied, as only a small percentage of species have been investigated compared to the large number of microalgal species that

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exist (Connelly, 2014), as illustrated by the number of times the same genus names are repeated in the present review. This is especially true when taking into account the strain differences previously mentioned. Therefore, one of the most urgent tasks is to increase our knowledge by investigating new microalgal species, and focusing on other than well-known genera (Yarnold *et al*., 2019).

Optimal culture conditions are not always known for all algal strains, and neither are the optimal conditions for the production of the compounds of interest. This means that additional research is needed for each individual strain to optimize the farming protocol for both biomass and the production of the compounds of interest (Ruffell *et al*., 2016).

Another approach is to identify or create mutants that overexpress the compounds of interest. Genetic engineering of microalgae is shown to be possible, although it is more technically difficult than for other organisms (Qin *et al*., 2012; Spicer and Purton, 2017; Charoonnart *et al*., 2018).

Consequently, alternative and potentially more promising pathways could be integrated in multi-omics approaches to stimulate production within microalgae and their microbiota, and transfer and expression of biosynthetic pathways in suitable heterologous host species and strains. (Meta)genomics, (meta)transcriptomics, (meta)proteomics and MS-based metabolomics approaches can thereby help unravelling biosynthetic pathway activity constraints in microalgae and their microbiomes and identifying beneficial chemical compounds, that is, elicitors, that can be used to stimulate the production of compounds of interest (Maghembe *et al*., 2020). Such approaches in combination with advanced bioinformatics assessment of (meta)genomic contents, for example, using tools like antiSMASH and MiBIG (Kautsar *et al*., 2020; Blin *et al*., 2021) can also help identifying the boundaries if biosynthetic gene clusters (BGCs) that encode the biosynthetic machinery for such compounds (Table 1). Combining this knowledge with advanced long-insert cloning technologies and transfer to a panel of heterologous expression hosts can yield production of the compound of interest in a system that is better accessible to genetic modification for the purpose of optimizing production and further compound engineering (Nah *et al*., 2017; Ke and Yoshikuni, 2020). Such techniques may also be helpful in reducing the presence of detrimental compounds, for example, toxic compounds, produced by these algae.

Conclusions

Microalgae in combination with their associated microbiota are very promising as health management tools: not only have several species shown potential, harbouring antimicrobial, immune-stimulating and antioxidant substances but also because of their variety and the relatively small numbers that have been investigated, it is likely that many useful compounds remain to be discovered. In this context, much research remains to be performed to identify new compounds. In addition, we have to clarify their mechanisms of action and make their application practical, by optimizing production methods and reducing their costs of production as well as multi-omics approaches.

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Conflict of interest

None declared.

Authors' contributions

IK, SML, GH, YA, PJ, JLN, MP, AW and WRS contributed to general concept and design and writing of the article. All authors contributed to manuscript revision, read and approved the submitted version.

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5 DISCUSSION

5.1 Deep RNA sequencing identified novel clues of microalgae and bacteria interactions

To understand the mechanisms of interaction between microalgae and other microorganisms, it is essential to thoroughly understand their metabolic pathways in a multispecies system, including symbiotic and competitive interactions. This requires a solid scientific and theoretical foundation. This study's findings promote the development of effective methods for the simultaneous cultivation of algae and encourage the increasing efficiency of microalgal biomass growth and the associated production of valuable compounds.

My doctoral research aimed to investigate the specialised distribution of assignments at the algae-bacterial phycosphere. Evaluating the top 250 gene hits resulted in identifying genes supporting the distribution of assignments at the microbial consortium. Identified genes were related to general metabolic activities, carbohydrate degradation, biofilm formation, transport mechanisms, and secretion systems. Former reports on the interactions within the microalga-bacteria consortia have also provided a blueprint for constructing mutually beneficial synthetic ecosystems in which the general metabolic activities played a significant role (45–47).

The broad-spectrum complex relationships through competition and synergy in algae-bacterial phycosphere biofilms were studied to investigate the hypothesis that dominant bacterial members possess the role of superior competitors. Members of the microbiome of *S. quadricauda* participate in the consortium niche in a competitive way that is reflected in a heat map with the correspondence to genes affiliated with potential antibiotic substances, endonucleases and exonucleases, permeases, proteases, heme synthesis, and quorum quenching, which are known as essential factors required for biofilm formation, virulence, and competition (48–51). The analysis of the microbiome of *S. quadricauda* revealed different proteins supposed to be beneficial during the competition for space and nutrients on surfaces in biofilms. Bacterial dominance can be attributed to the ability of these organisms to form microcolonies rapidly and their ability to produce extracellular antibacterial compounds (52). The *S. quadricauda* microbiome is composed of single-species populations or mixed populations with varying levels of interaction, depending on the exponential or stationary growth phase. *Porphyrobacter*, *Dyadobacter*, and *Variovorax* were found to be the dominant producers of numerous antibacterial proteins, which can eliminate other microorganisms or exhibit solid inhibitory activity against them.

The signalling molecules related to quorum sensing and quorum quenching activity were affiliated with the α-Proteobacteriota and Bacteroidota. Among metatranscriptome data sets, proteins predicted as quorum quenching included dienelactone hydrolase, imidazolonepropionase, 6-phosphogluconolactonase, gluconolactonase, oxidoreductases, and metal-dependent hydrolases of the b-lactamase superfamily, related to quorum quenching activity. Highly

transcribed genes were observed, mainly at the stationary growth phase, that fulfil the competitive needs of bacteria to comprise one of the dominant heterotrophic bacterial groups in aquaculture. Dienelactone hydrolase, a quorum quenching enzyme that degrades or modifies N-Acyl homoserine lactones (AHLs) (53, 54), was established for *Dyadobacter* and *Porphyrobacter*. Gluconolactonases, reported as quorum quenching enzymes (55), were mapped to *Dyadobacter*. Another enzyme class, oxidoreductase, shown to catalyse the oxidation or reduction of the acyl side chain (56, 57), originated in *Variovorax* and *Dyadobacter*. The analysis also revealed several phenotypes beneficial for bacterial surface colonisation, including motility, exopolysaccharide production, biofilm formation, and toxin production, often regulated by quorum sensing (8, 14, 15). Simultaneously, several members of the *S. quadricauda* microbiome appeared to be the leading suppliers of vitamins to microalga. Genes involved in thiamine, cobalamin, biotin, and riboflavin synthesis were established for α-Proteobacteriota and Cytophagaceae, which confirms the strong evidence that alga-associated bacteria are responsible for the supply of the essential vitamins to alga (18). Thus, this study shows that this interaction involves the strong collaboration between members of the alga-bacterial phycosphere with the support of nutritional components and the synergetic exchange of biosynthetic compounds.

To facilitate the proliferation and survival inside eukaryotic hosts, many pathogenic bacteria are known to use secretion systems by the secretion of protein effectors or protein-DNA complexes (58). The presence of the T6SS in the genome of *Dyadobacter* suggests the distribution of tasks among members of studied consortia, providing fitness and colonisation advantages, which are not restricted to virulence. T9SS machinery components, established for *Dyadobacter* within the Bacteroidota phylum, represent the assembly of the gliding motility apparatus and possible external release of proteins with various functions, including cell surface exposition, attachment, and other virulence factors (56, 57). The widespread occurrence of gliding motility genes was previously revealed among the members of the same phylum, the gliding bacterium *Flavobacterium johnsoniae*, and the nonmotile oral pathogen *Porphyromonas gingivalis*. *F. johnsoniae* uses T9SS as the gliding motility apparatus, and for chitinase secretion, *P. gingivalis* secretes through this system gingipain protease virulence factors. The same route likely secretes other polysaccharide-digesting enzymes produced by various members of the phylum. The mechanisms underlying the processes of gliding motility and cell surface machinery to utilise polysaccharides remain unclear (59, 60). Nevertheless, it is known that T9SS machinery secretes most of CAZymes, such as glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and accessory proteins (61, 62).

In this study, I identified a significant number of genes of high importance for root colonisation, biofilm formation, invasion (63, 64), virulence, and pathogenicity (65–67). This study describes genes affiliated with overall plant-bacteria interaction pathways, including ROS tolerance, LRR proteins, and invasion-associated proteins (19). Numerous genes, known for ROS tolerance,

were highly transcribed at the stationary phase, which explains the necessity of bacteria to protect themselves from massive amounts of reactive oxygen species released by microalgae, previously suggested to expose them to pathogens (68). Furthermore, it is supposed that dominating microorganisms can use LRR and invasion proteins as signalling and detecting components to establish the interaction with the possible innate immune system of the *S. quadricauda*.
This study gave a detailed insight into the mutualistic collaboration of microalgae and bacteria, including the involvement of competitive interplay between bacteria. Further, it was followed by unravelling the complex nutrient exchange and mutual support in different aspects of the cross-kingdom synergistic network and signalling between the bacteria and microalgae (19, 44).

5.2 Extensive investigation of the interplay between the microalga *Micrasterias radians* and its symbiont *Dyadobacter* sp. HH091

5.2.1 Flexirubin biosynthesis conceivably involved in microalgae-bacteria liaison

The most comprehensive and fundamental understanding of microbial metabolic pathways in a multispecies system and symbiotic and competitive interactions is required to provide scientific and theoretical bases for the interaction mechanisms within microalgae-bacteria liaison. The presented results indicate the requirement for more advanced techniques for algae cultivation. These results highlight the importance of improving the efficiency of microalgal biomass growth and the synthesis of valuable compounds.
The transcriptome analysis of *Dyadobacter* sp. HH091 co-cultured with microalga *M. radians* revealed highly active genes affiliated with the cluster of flexirubin biosynthesis. This cluster includes *darA* and *darB* genes, homologs of *F. johnsoniae* UW101 (69) and *C. pinensis* (70).
Flexirubin is a pigment consisting of a ω-(4-hydroxyphenyl)-polyene carboxylic acid chromophore, esterified with a 2,5-dialkylresorcinol (DAR), also known as a novel and widespread bacterial signalling molecule (70–72). Genes coding for the biosynthesis of these pigments are found in many bacteroidotal genomes, including *Flavobacterium psychrophilum, Flavobacterium johnsoniae* (69), *Leadbetterella byssophila* (72), *Chryseobacterium artocarpi* (73), *Chryseobacterium rhizoplanae* sp. nov. (74), *Flavobacterium maris* sp. nov. (75), and *Flavobacterium tilapiae* sp. nov. (76). Homologs of *darA*, a dialkylresorcinol condensing enzyme, and *darB*, a 3-oxoacyl-[acyl-carrier-protein] synthase III protein, were previously identified using bioinformatics tools within the genome analysis of our model organism *Dyadobacter* sp. HH091 (19).
An additional noteworthy aspect is that in the plant-bacteria interaction model, flexirubin also performs as a free radical scavenging antioxidant that protects from the attack of free radicals

(70, 77, 78). The antioxidant potential *via* hydrogen donating ability of flexirubin has been shown through the assessment using different assays such as radical scavenging activities, lipid peroxide inhibition and ferrous chelating ability (79). Several studies show that microalgae produce ROS to get an advantage in the competition for resources against other algae, be a way to prevent fouling bacteria and act as a signalling mechanism between cells (80). Additionally, ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH), are known as antibacterial agents involved in the oxidation or reduction of necessary or toxic metals (81). Former investigation of microalga *Micrasterias* spp. demonstrated that ROS are constantly generated as by-products of general metabolic cellular pathways and can be over-produced in response to stress (82–84). Our results indicate that *Dyadobacter* sp. HH091 uses flexirubin hybrid pigments to protect itself from ROS produced by microalga, which explains this interaction, making it possible for microalgal symbiont to have tight contact with its host.

5.2.2 T9SS tangled in the symbiotic interactions of *Dyadobacter* with microalgae

The presence of different secretion systems suggests that *Dyadobacter* sp. HH091 and microalgae possess a signal exchange system that establishes and maintains a symbiosis that includes adhesion factors, microcolonisation development, extracellular polymeric substances production, and biofilm formation factors, which are essential for successful symbiosis. Previously, a comprehensive set of cell surface-associated proteins required for host cell invasion was described for other bacterial model organisms (85–87). These mechanisms express certain factors facilitating niche adaptation, including cell-host attachment, microcolonisation and biofilm formation. Genes coding for the cell surface-associated proteins and secretion systems are mainly up-regulated in *Dyadobacter* sp. HH091, expecting them to be crucial for the microcolonisation process because they establish interaction with the host. Cell-host interaction, adhesion factors, microcolonisation development, and biofilm formation succeed in close interaction and exchange of growth-promoting substances between the symbiont and microalga. Surface-exposed proteins that are covalently or non-covalently bound to the cell surface and proteins are secreted into the extracellular matrix using different secretion mechanisms (87–90). Secreted proteins accommodate the majority of virulence factors, enzymes required for nutrient acquisition or cell spreading, immune evasion proteins that bypass the immune system or interfere with components of the complement system and many others. Overall, secretion systems are known to transport effector proteins into the cytosol of eukaryotic cells, allowing the direct communication and modification of the host cells and suppressing any activity of competitive microorganisms (91). *Dyadobacter* sp. HH091 has many unique features together with the complex of different secretion systems, which are available to arbitrate secretion of

proteins across the outer membrane, including T9SS, a complex translocon found only in some species of the Bacteroidota phylum (19, 44, 92).

A complex translocon of T9SS, including *gld* and *spr* genes and *porXY-sigP* signalling system components, are proposed to serve as the secretion system of cargo proteins. The T9SS cargo proteins have a conserved C-terminal domain (CTD) that enables them to pass *via* T9SS and an N-terminal signal peptide that guides T9SS cargo proteins through the Sec system (93, 94). The CTD signal has been identified as type A and B (94, 95). High activity of T9SS cargo proteins has been observed in this transcriptome analysis, which was also shown in the former studies on the *Dyadobacter* genome (19). It resulted in 48 up-regulated and 24 down-regulated genes affiliated with T9SS cargo proteins of both types (44).

gldA, gldF and *gldG* encode components of an ATP-binding cassette (ABC) transporter required for motility and/or protein secretion (96, 97). Genes encoding lipoproteins necessary for gliding (*gldB*, *gldD*, *gldH*, *gldI*, and *gldJ*) have also been identified (98–102). GldK, GldL, GldM, and GldN are each required for efficient motility and chitin utilisation, indicating that Gld proteins may function in gliding and chitin utilisation (101). SprA is necessary for the secretion of SprB and RemA and the utilisation of chitin (103). In *F. johnsoniae*, SprA has been identified as the significant translocon protein of T9SS, and it is hypothesised that SprA of *Dyadobacter* sp. HH091 can also have the same function (104). The down-regulated gene coding for SprF is essential for the secretion of SprB to the cell surface but is not required for the secretion of extracellular chitinase (105). That also hints that the symbiont possibly utilises T9SS for the secretion of other proteins and is not only involved in gliding motility.

5.2.3 Polysaccharide utilisation is a crucial aspect of microalgae-bacteria interaction

T9SS is known to be tangled in the secretion of polysaccharide utilisation proteins (106, 107). Previously, it was shown that the significant chitinase (ChiA) in *F. johnsoniae* is fully secreted from the cell in soluble form by T9SS and is essential for chitin degradation (59, 107, 108).

Based on genome and transcriptome analyses, presumably, *Dyadobacter* sp. HH091 has a complex of carbohydrate utilisation domains for the digestion of microalgae cell walls, hemicelluloses, such as cellulose, xylan or mannan fibrils, and extensive matrix polysaccharides. Numerous carbohydrate-active enzymes predicted to encode GHs and esterases that could be involved in the degradation of microalgal cell wall hemicelluloses were highly active within transcriptome datasets (19, 44). In addition, candidates like xylanases, β-xylosidases, arabinofuranosidases, and beta-glucuronidases involved in xylan digestion, β-mannosidases involved in mannan digestion, and candidate β-glycosidases and endoglucanase that could be involved in xyloglucan digestion were also identified.

Data obtained from transcriptome analysis allows us to understand better the nature of bacterial polysaccharide utilisation genes' involvement in bacteria-algae liaison. In the previous study, I observed that the genome of a given symbiont possesses a wide assortment of CAZymes predicted to breach algal cell walls (19). A deep investigation of transcriptome datasets unveiled the presence of these genes among differentially expressed genes (DEGs). A significant number of genes (82) among these DEGs belong to functions vital for carbohydrate transport and metabolism, including different GHs families, which are known to be involved in plant polysaccharides degradation (109). For example, many up-regulated transcripts are affiliated with genes responsible for the biosynthesis of GH5, GH13, GH25, GH30 and GH43 family enzymes, which function as effectors with roles in the degradation of plant polysaccharides (110, 111). These enzymes are known for acting as cellulose-degrading (112), starch-degrading (113), catalysing hemicellulose and removing xyloses from xyloglucan (114, 115). Additionally, it was uncovered that genes affiliated with the synthesis of GH88 CAZyme, utilising polysaccharide lyase activity to degrade pectins (61), were also up-regulated. Other highly active genes coding for xylose isomerases belong to the CAZyme family GH43 that generally display specificity for arabinose-containing substrates. This gene combination reflects the competence of the symbiont to utilise starch and the complex of arabinan sidechains of pectin-rich cell walls as essential nutrients (113, 116).

In this transcriptome analysis, I demonstrated that bacteria could profit through the degradation of algal polysaccharides, while microalgae are supplied with a repertoire of growth-promoting substances. The results of this research will serve as an efficient tool in further investigations of symbiotic microalgae-bacteria interactions. The co-cultivation of microalgae and bacteria has significant benefits for both commercial and environmental purposes in microalgal cultivation.

Furthermore, I gave a concise clarification regarding microalgae and its related microorganisms, emphasising their immense potential in promoting health benefits. In addition, I highlighted the challenges that are hindering the widespread adoption and advancement of associated technologies.

5.3 Potential benefits of microalgae and affiliated microbiomes

From a sustainability perspective, microalgae production allows capturing CO_2 with a higher average bioenergetic yield on sunlight than higher plants: 10% vs 5%, respectively (117). In addition, such higher yield allows reducing in the use of land for cultivation.

Microalgae are becoming increasingly interesting for extracting high-value compounds rather than as feedstock for refining low-value products such as biofuels. Concerning this extraction, Perez-Lopez et al. (118, 119) showed that the extraction stage (methanol and KOH solutions) for producing PUFAs, a-tocopherol, chlorophyll, b-carotenoid, and polyphenols by *Tetraselmis*

suecica was the second most important contributor to most environmental impact categories but remained far behind the cultivation stage. Regardless of the extraction method for a specific bioactive compound, the cultivation of new microalgal strains will continue to be the leading environmental concern. To minimise the adverse effects of microalgal production on the environment, it is important to utilise co-products through an integrated biorefinery approach. (120, 121). Microalgae residual biomass can serve as a substrate for biogas production via anaerobic digestion, and the residual digestate can substitute for the production of fertilisers (118, 119, 122). If the nutrient profile of the biomass meets the requirements, it is a viable option as an alternative to animal feed (123). Due to the high diversity of the assumptions, parameters, and production technologies for strains that have not been cultivated yet, it is worth noting that their behaviour, optimal growth conditions and productivity in given reactors and locations are currently challenging to anticipate and, therefore highly uncertain (23, 124).

The potential need to induce bioactive molecule production by specific cultivation conditions, such as high-intensity lighting for astaxanthin production from *Haematococcus pluvialis*, could significantly affect the final environmental impacts (118, 119, 125). Ultimately, the overall sustainability of producing microalgae-based bioactive molecules depends on the final efficiency and quality of the produced compounds. Finding synergistic products would therefore be of great interest. Indeed, the compound's capacity to substitute alternatives and tackle critical issues such as fish farming health management will highly affect its environmental performance (126, 127).

Currently, the significant knowledge gap is linked to the correspondingly low numbers of algal species studied, as only a small percentage of species have been investigated compared to the large number of microalgal species that exist (128), as illustrated by the number of times the same genus names are repeated in the present review. Therefore, one of the most urgent tasks is to increase our knowledge by investigating new microalgal species and focusing on other than well-known genera (129).

Optimal culture conditions are only sometimes known for all algal strains nor optimal for producing the compounds of interest. This means that additional research is needed for each strain to optimise the farming protocol for biomass and the production of the compounds of interest (130). Another approach is identifying or creating mutants that overexpress the compounds of interest. Though genetic engineering of microalgae is possible, it is more technically challenging than for other organisms (131–133).

Consequently, alternative and potentially more promising pathways could be integrated into multi-omics approaches to stimulate production within microalgae and their microbiota and transfer and expression of biosynthetic pathways in suitable heterologous host species and strains. (Meta)genomics, (meta)transcriptomics, (meta)proteomics, and MS-based metabolomics approaches can help unravel biosynthetic pathway activity constraints in microalgae and their microbiomes and identify beneficial chemical compounds.

6 CONCLUSION

Throughout my doctoral research, I thoroughly studied the unique interaction between microalgae and the associated microbiota. According to the study's transcriptome analysis, the *Scenedesmus quadricauda* microbiota consists of less than ten distinct microbial species that are metabolically active. Furthermore, I presented compelling evidence linking the dominant species with the *Variovorax*, *Porphyrobacter*, and *Dyadobacter* genera. Based on experimental investigations and transcriptome analysis, it is evident that *Dyadobacter* plays a critical role in promoting the growth and overall fitness of algae in the phycosphere within multispecies interactions. While presumably under light conditions, the alga provides the energy source to the bacteria, *Dyadobacter* produces and releases mainly a large variety of polysaccharides modifying enzymes. This is coherent with the high-level expression of the T9SS in alga cocultures. The transcriptome data further implied that quorum quenching proteins and biosynthesis of vitamins B_1, B_2, B_5, B_6, and B_9 were expressed by *Dyadobacter* at high levels compared to *Variovorax* and *Porphyrobacter*. Notably, *Dyadobacter* produced a significant number of leucine-rich repeat proteins and enzymes involved in bacterial reactive oxygen species tolerance. Complementary to this, *Variovorax* expressed the biosynthesis genes of vitamins B_2, B_5, B_6, B_7, B_9, B_{12}, and *Porphyrobacter* specialised in producing vitamins B_2 and B_6. This work significantly enlarges our knowledge of algae-bacteria interaction and demonstrates physiological investigations of microalgae and associated bacteria using microscopy observations, photosynthetic activity measurements, and flow cytometry.

Based on this detailed insight into the mutualistic collaboration of microalga and its microbiome, I established an artificial plant-bacteria system of the microalga *Micrasterias radians* MZCH 672 and the bacterial isolate *Dyadobacter* sp. HH091. The bacteria, affiliated with the phylum Bacteroidota, strongly stimulated microalga growth when added to axenic algal cultures. For further advances, I studied the isolate HH091 and its interaction with the microalga *M. radians* using transcriptome and extensive genome analyses. The genome of HH091 contains predicted polysaccharides utilising gene clusters co-working with the type IX secretion system (T9SS) and conceivably involved in the algae-bacteria liaison. Here, I focused on characterising the mechanism of T9SS, implementing the attachment and invasion of microalga by *Dyadobacter* sp. HH091. Omics analysis exposed T9SS genes: *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprF*, *sprT*, *porU,* and *porV*. Besides, *gld* genes not considered as the T9SS components but required for gliding motility and protein secretion (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ)* were also identified in this analysis. In this research, I proposed the first model of the T9SS apparatus of *Dyadobacter*. By using the combination of fluorescence labelling of *Dyadobacter* sp. HH091, I examined the bacterial colonisation and penetration into the cell wall of the algal host *M. radians* MZCH 672.

Additionally, I studied the potential of microalgae in combination with their associated microbiota as promising health management tools. This study demonstrated that various species of microalgae contain substances that possess antimicrobial, immune-stimulating, and antioxidant properties. Considering a variety of those species and a relatively few substances that have been investigated, it is likely that many beneficial compounds remain to be discovered. In this context, much research remains to identify new compounds. Furthermore, it is crucial to clarify their mechanisms of action and make their application practical by optimising production methods and reducing their production costs and multi-omics approaches.

7 OTHER PUBLICATIONS

In addition to the present study, I contributed to the following studies:

Astafyeva, Y., Alawi, M., Indenbirken, D., Danso, D., Grundhoff, A., Hanelt, D., Streit, W. R., & Krohn, I. (2020). Draft Genome Sequence of the Green Alga *Scenedesmus acuminatus* SAG 38.81. Microbiology Resource Announcements, 9(24). https://doi.org/10.1128/MRA.01278-19

Peters, M. K., **Astafyeva, Y.**, Han, Y., Macdonald, J. F. H., Indenbirken, D., Nakel, J., Virdi, S., Westhoff, G., Streit, W. R., & Krohn, I. (2023). Novel marine metalloprotease-new approaches for inhibition of biofilm formation of *Stenotrophomonas maltophilia*. Applied microbiology and biotechnology. Advance online publication. https://doi.org/10.1007/s00253-023-12781-0

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9 APPENDIX

9.1 Microalgae and Bacteria Interaction—Evidence for Division of Diligence in the Alga Microbiota

All supplemental tables can be accessed online via
https://doi.org/10.1128/spectrum.00633-22

Supplemental FIGURES and TABLES

Supplemental FIGURE S1: Genome annotation and phylogenetic tree of *Dyadobacter* sp. HH091. A) circular graphical display of the distribution of the genome annotation of *Dyadobacter* sp. HH091. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. B) The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (see Subsystems). C) The phylogenetic tree highlighting the position of *Dyadobacter* sp. HH091 relative to the type strains of other species within Bacteroidota. The phylogenetic tree was generated using the codon tree method within PATRIC, which used cross-genus families (PGFams) as homology groups [73]. 100 PGFams were found among these selected genomes using Codon Tree analysis, and the aligned proteins and coding DNA from single-copy genes were used for RAxML analysis. *Dyadobacter* sp. HH091 was included as an outgroup. FigTree was used for the tree visualization [74].

Supplemental FIGURE S2: FACS analyses of *Scenedesmus quadricauda* MZCH 10104 in co-culture with the strain Dyadobacter sp. HH091. A) algae + bacteria at the starting point of experiment, 5.56% of bacteria (I),13.2% of lysed algal cells (II), 59.4% of algae (III); B) algae + bacteria over a time period of 13 days, 3.49% of bacteria (I), 18.8% of lysed algal cells (II) and, 70.8% of healthy microalgae (III); C) axenic culture of algae without HH091, 3.24% of bacteria (I), 27.4% of lysed algal cells (II) and 57.7% of algae cells (III). FACs analyses demonstrated the improved fitness of *S. quadricauda* co-cultured with HH091.

Supplemental TABLE S1: Overall numbers of sequences and contigs generated for Genome analyses of *Dyadobacter* sp. HH091.

Supplemental TABLE S2: Predicted *Dyadobacter* sp. HH091 glycosyl hydrolases.

Supplemental TABLE S3: Predicted *Dyadobacter* sp. HH091 glycosyl hydrolases involved in microalgae polysaccharides digestion.

Supplemental TABLE S4: Predicted *Dyadobacter* sp. HH091 polysaccharide lyases.

Supplemental TABLE S5: Predicted *Dyadobacter* sp. HH091 carbohydrate esterases involved in polysaccharide utilization.

Supplemental TABLE S6: Overall numbers of sequences and contigs generated for the transcriptome datasets.

Supplemental TABLE S7: Protein family comparison of possible competitive and plant-bacteria interaction pathways across the dominant members of microbiome (*Porphyrobacter, Variovorax* and *Dyadobacter*) of *S. quadricauda* MZCH 10104. Numbers of proteins are marked with the following colors: ■ 0, □ 1, □ 2, and ■ 3+.

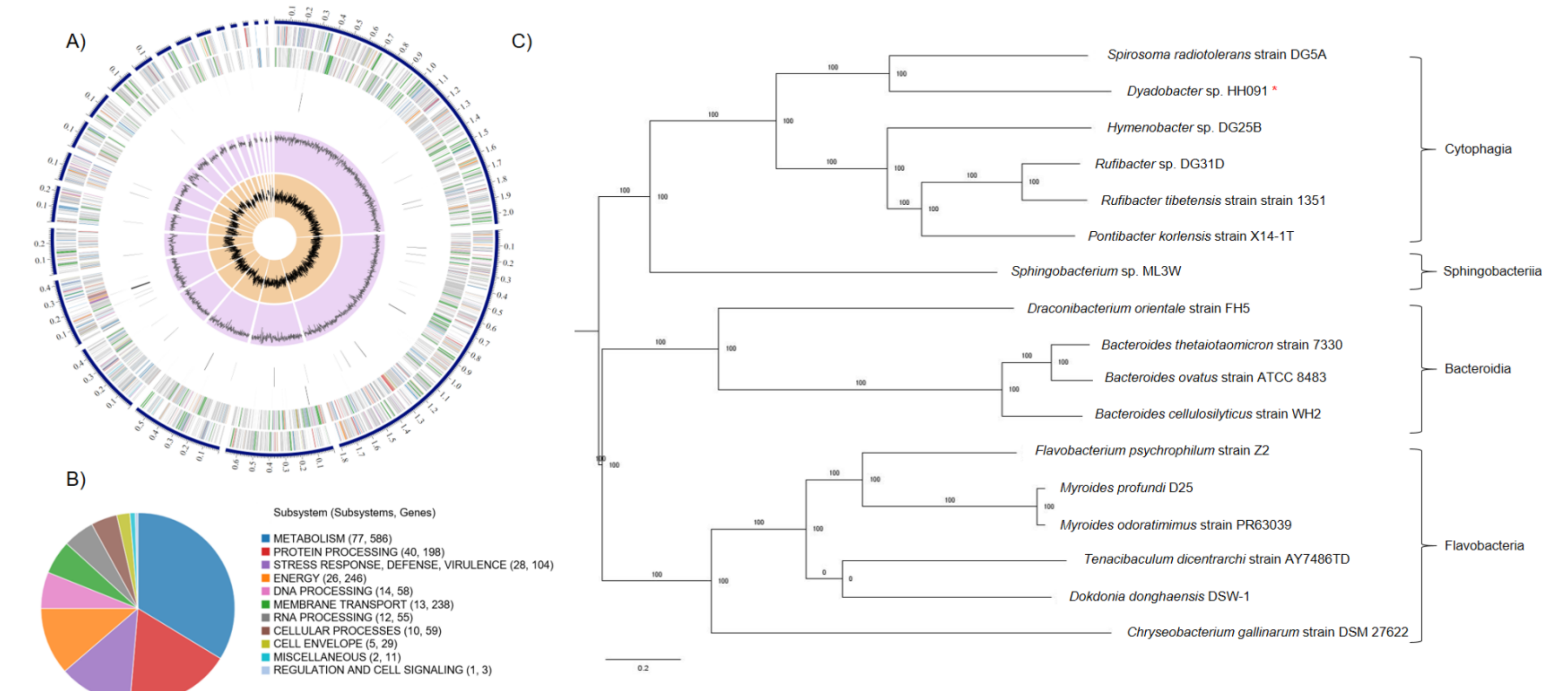


FIGURE S1: Genome annotation and phylogenetic tree of *Dyadobacter* sp. HH091. A) circular graphical display of the distribution of the genome annotation of *Dyadobacter* sp. HH091. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. B) The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (see Subsystems). C) The phylogenetic tree highlighting the position of *Dyadobacter* sp. HH091 relative to the type strains of other species within Bacteroidota. The phylogenetic tree was generated using the codon tree method within PATRIC, which used cross-genus families (PGFams) as homology groups [73]. 100 PGFams were found among these selected genomes using Codon Tree analysis, and the aligned proteins and coding DNA from single-copy genes were used for RAxML analysis. *Dyadobacter* sp. HH091 was included as an outgroup. FigTree was used for the tree visualization [74].

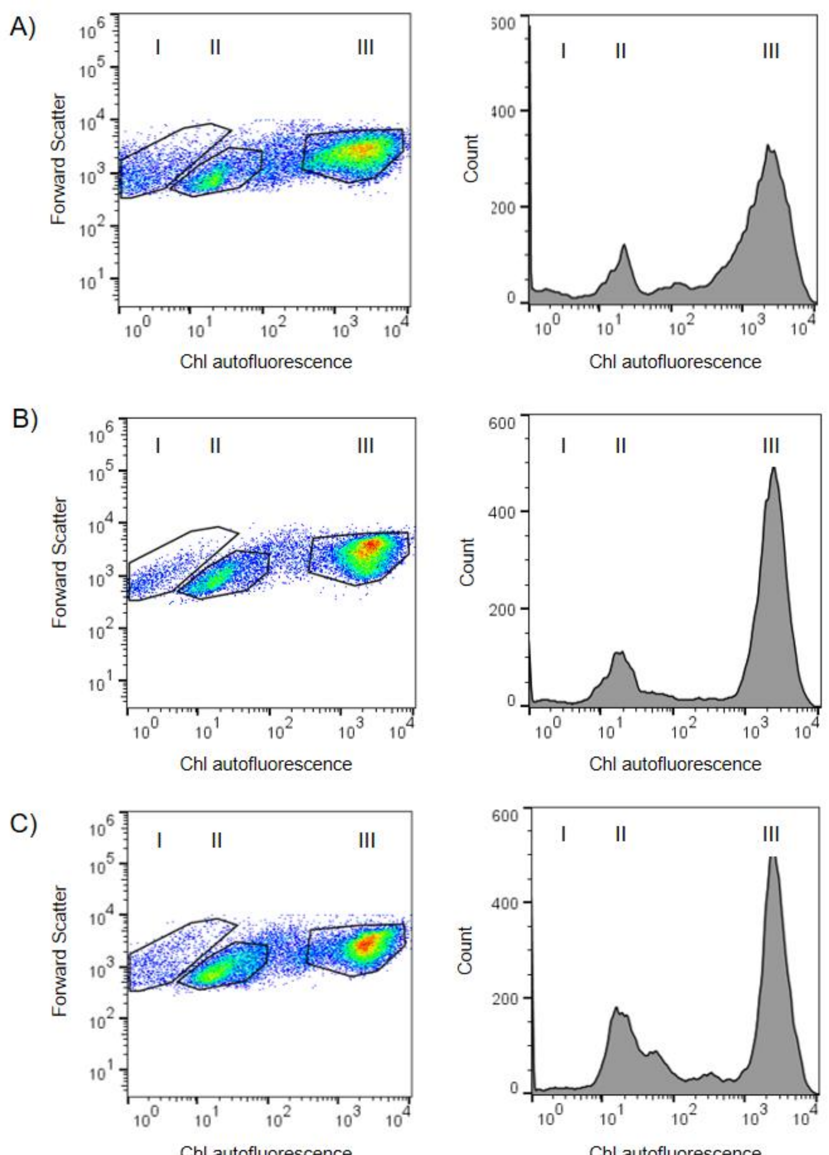


FIGURE S2: FACS analyses of *Scenedesmus quadricauda* MZCH 10104 in co-culture with the strain *Dyadobacter* sp. HH091. A) algae + bacteria at the starting point of experiment, 5.56% of bacteria (I),13.2% of lysed algal cells (II), 59.4% of algae (III); B) algae + bacteria over a time period of 13 days, 3.49% of bacteria (I), 18.8% of lysed algal cells (II) and, 70.8% of healthy microalgae (III); C) axenic culture of algae without HH091, 3.24% of bacteria (I), 27.4% of lysed algal cells (II) and 57.7% of algae cells (III). FACs analyses demonstrated the improved fitness of *S. quadricauda* co-cultured with HH091.

Supplemental TABLE S1: Overall numbers of sequences and contigs generated for Genome analyses of *Dyadobacter* sp. HH091.

	***Dyadobacter* sp. HH091**
Contigs-assembly (Spades)	
No.	80
Total length (bp)	7,862,706
No. ≥ 1000 bp	66
N50 size (bp)	607,803
Largest (bp)	1,195,963
GC (%)	43.81
IMG ID genome statistic	
IMG ID	222279
Number of bases	7,837,776
GC count [%]	43.87
Number of protein coding genes	6,565
% of assembled protein coding genes	99.05

Supplemental TABLE S2: Predicted *Dyadobacter* sp. HH091 glycosyl hydrolases[1].

Pfam	Glycosyl hydrolase family	Number of genes
pfam00232	Glycosyl hydrolase family 1	2
pfam00703	Glycosyl hydrolases family 2 (beta-glucuronidase)	4
pfam00933	Glycosyl hydrolase family 3	2
pfam00759	Glycosyl hydrolase family 9	2
pfam00331	Glycosyl hydrolase family 10 (xylanase)	1
pfam00723	Glycosyl hydrolases family 15	1
pfam00722	Glycosyl hydrolases family 16	3
pfam00704	Glycosyl hydrolases family 18	1
pfam00728	Glycosyl hydrolase family 20	6
pfam01183	Glycosyl hydrolases family 25	1
pfam01183	Glycosyl hydrolases family 25 (1,4-beta-N-acetylmuramidase)	1
pfam02156	Glycosyl hydrolase family 26 (mannan endo-1,4-beta-mannosidase)	2
pfam00295	Glycosyl hydrolases family 28	6
pfam02055	Glycosyl hydrolase family 30	1
pfam17189	Glycosyl hydrolase family 30	2
pfam01055	Glycosyl hydrolases family 31	3
pfam08244	Glycosyl hydrolases family 32	1
pfam17167	Glycosyl hydrolase family 36	1
pfam01229	Glycosyl hydrolases family 39	1
pfam02449	Glycosyl hydrolase family 42 (Beta-galactosidase)	4
pfam08532	Glycosyl hydrolase family 42M (Beta-galactosidase trimerisation domain)	1
pfam04616	Glycosyl hydrolases family 43 (beta-galactosidase, beta-xylosidase, alpha-N-arabinofuranosidase)	8
pfam12891	Glycoside hydrolase family 44	1
pfam03632	Glycosyl hydrolase family 65	1
pfam07477	Glycosyl hydrolase family 67	1
pfam02446	Glycosyl hydrolase family 77 (4-alpha-glucanotransferase)	1
pfam07470	Glycosyl Hydrolase Family 88	3

pfam07971	Glycosyl hydrolase family 92	2
pfam17132	Glycosyl hydrolase family 106 (alpha-L-rhamnosidase)	1
pfam07944	Glycosyl hydrolase family 127 (Beta-L-arabinofuranosidase)	3
pfam04041	Glycosyl hydrolase family 130 (beta-1,4-mannooligosaccharide phosphorylase)	2
Total		69

[1] Predicted functions were assigned by routines used for updating the Carbohydrate Active Enzymes database (http://www.cazy.org/).

Supplemental TABLE S3: Predicted *Dyadobacter* sp. HH091 glycosyl hydrolases involved in microalgae polysaccharides digestion[1].

Gene ID	Predicted function [1]	IMG Product Name [2]	Homologs [3]	Predicted localization [4]	Molecular mass (kDa) [5]	Enzymatic and other domains [6]
2842104189	Candidate cellulase	Aryl-phospho-beta-D-glucosidase BglC	*Chitinophaga niabensis* (71% identity)	Unknown	43.3	GH5
2842105209	Candidate cellulase	Endoglucanase	*Chitinophagaceae bacterium* BR5-29 (70% identity)	Unknown, non-Cytoplasmic	40.1	GH5
2842104157	related to cellulase	Endoglucanase	*Cytophagaceae bacterium* SJW1-29 (58% identity)	Unknown, non-Cytoplasmic	65.2	GH9
2842108135	related to chitinase	chitinase	*Chitinophagaceae bacterium* PMP191F (58% identity)	Cytoplasmic	43.2	GH18
2842105133	related to beta-N-acetylhexosaminidase	hypothetical protein	*Chitinophaga* sp. BN140078 (52% identity)	Unknown	98.6	GH20
2842107906	related to beta-N-acetylhexosaminidase	hexosaminidase	*Flavobacteriales bacterium* (50% identity)	Outer membrane (Beta-N-acetylhexosaminidase, Chitobiase)	95.5	GH20-CHB_HEX
2842108966	related to beta-N-acetylhexosaminidase	hexosaminidase	*Cytophagaceae bacterium* (56% identity)	Periplasmic	61.0	GH20
2842106454	related to beta-N-acetylhexosaminidase	hypothetical protein	*Runella* sp. YX9 (63% identity)	Periplasmic	78.9	GH20
2842106655	related to beta-N-acetylhexosaminidase	hexosaminidase	*Cytophagaceae bacterium* SJW1-29 (63% identity)	Outer membrane	97.1	GH20-CHB_HEX
2842109900	related to beta-N-acetylhexosaminidase	hypothetical protein	*Chitinophaga* sp. XS-30 (62% identity)	Periplasmic	84.7	GH20
2842105702	candidate beta-glucanase	beta-glucanase	*Dyadobacter soli* (70% identity)	Extracellular	32.17	GH16
2842104231	Candidate beta-xylosidase	beta-xylosidase	*Cytophagaceae bacterium* SAT51 (78.60% identity)	Unknown, non-Cytoplasmic	36.79	GH43
2842108192	Related to alpha-D-xyloside xylohydrolase	alpha-D-xyloside xylohydrolase	*Chitinophagaceae bacterium* PMP191F (68% identity)	Unknown	81.41	GH31

[1] Predicted functions were assigned by routines used for updating the Carbohydrate Active Enzymes database (http://www.cazy.org/) using the following criteria: typically, 70% or greater amino acid identity with a protein domain with a biochemically determined function at the time of analysis resulted in “candidate” status; 30% to 70% amino acid identity with a protein domain with a known function resulted in “related to” status; and less than 30% amino acid identity with a protein domain with a known function resulted in “distantly related to” status. Because the threshold of similarity that correlates with a change of substrate specificity is variable from one glycoside hydrolase family to another, the criteria were tightened or loosened appropriately for several families. All analyses were conducted domain by domain to avoid problems arising from the modular structure of many of the proteins.
[2] The Integrated Microbial Genomes (IMG) Product Name.
[3] Homologs were identified by a BlastP search with the Swiss-Prot database.
[4] Localization was predicted using the default settings of PSORTb (Gardy et al., 2005). Predicted lipoproteins were identified using LipoP.
[5] Predicted molecular mass of the primary product of translation, including any predicted signal peptide.
[6] CHB_HEX, carbohydrate binding domain represents the N-terminal domain in chitobiases and beta-hexosaminidases. It is composed of a beta sandwich structure that is similar in structure to the cellulose binding domain of cellulase from *Cellulomonas fimi*. (Tews et al., 1996). GH, glycoside hydrolase as assigned by CAZY (the numbers indicate families).

Supplemental TABLE S4: Predicted *Dyadobacter* sp. HH091 polysaccharide lyases[1].

Gene ID	Predicted function [1]	IMG Product Name [2]	Homologs [3]	Predicted localization [4]	Molecular mass (kDa) [5]	Enzymatic and other domains [6]
2842104046	Candidate pectin or pectate lyases	polygalacturonase	Chitinophaga niabensis (71% identity)	Unknown, Cytoplasmic	73105.54	PL, PG
2842105135	Related to pectin or pectate lyases	polygalacturonase	Chitinophagaceae bacterium (63% identity)	Extracellular	55711.69	PL, PG, GH28
2842105517	Related to pectin or pectate lyases	polygalacturonase	*Flavobacterium reichenbachii* (39% identity)	Extracellular	52862.19	PL, PG, GH28
2842107938	Related to pectin or pectate lyases	polygalacturonase	*Bacteroides cellulosilyticus* (42% identity)	Cytoplasmic	44247.94	PL, PG, GH28
2842108446	Candidate pectin or pectate lyases	polygalacturonase	*Cytophagaceae bacterium* SJW1-29 (74% identity)	Extracellular	49580.00	PL, PG, GH28
2842105518	Related to pectin or pectate lyases	polygalacturonase	*Chitinophagaceae bacterium* BR5-29 (38% identity)	Extracellular	55889.57	Pl, PG, GH28
2842109095	Related to pectin or pectate lyases	hypothetical protein	*Pedobacter* sp. OK628 (32% identity)	Extracellular	67179.60	PL, GH28
2842108200	Exo-poly-alpha-D-galacturonosidase	DNA sulfur modification protein DndE	*Cytophagales bacterium* (60% identity)	Extracellular	85717.11	PL, GH28, GDSL_2
2842108975	Related to Pectin lyase-like	hypothetical protein	*Alcanivorax* sp. (42% identity)	Unknown, Non-Cytoplasmic	51861.62	PL

[1] Predicted functions as assigned by routines used for updating the Carbohydrate-Active Enzymes database (http://www.cazy.org/CAZY/) using the criteria indicated in Table 1.
[2] Localization predicted using the default settings of PSORTb (Gardy, J. L., et al. 2005. Bioinformatics 21:617-623).
[3] Predicted molecular mass of primary product of translation, including any predicted signal peptide.
[4] Modular structure is indicated by abbreviations: GH, glycoside hydrolase as assigned by CAZY (the numbers indicate families) (http://www.cazy.org/CAZY/). PL: polysaccharide lyase (number indicates family) as assigned by CAZY. GDSL-like Lipase, (GDSL esterases and lipases are hydrolytic enzymes with multifunctional properties [1]. This new subclass of lipolytic enzymes possesses a distinct GDSL sequence motif different from the GxSxG motif found in many lipases [2]. Members include; Aeromonas hydrophila lipase, Vibrio mimicus arylesterase, Vibrio parahaemolyticus thermolabile haemolysin, rabbit phospholipase (AdRab-B), and Brassica napus anter-specific proline-rich protein.)

Supplemental TABLE S4: Predicted *Dyadobacter* sp. HH091 polysaccharide lyases[1].

Gene ID	Predicted function [1]	IMG Product Name [2]	Homologs [3]	Predicted localization [4]	Molecular mass (kDa) [5]	Enzymatic and other domains [6]
2842104046	Candidate pectin or pectate lyases	polygalacturonase	Chitinophaga niabensis (71% identity)	Unknown, Cytoplasmic	73105.54	PL, PG
2842105135	Related to pectin or pectate lyases	polygalacturonase	Chitinophagaceae bacterium (63% identity)	Extracellular	55711.69	PL, PG, GH28
2842105517	Related to pectin or pectate lyases	polygalacturonase	*Flavobacterium reichenbachii* (39% identity)	Extracellular	52862.19	PL, PG, GH28
2842107938	Related to pectin or pectate lyases	polygalacturonase	*Bacteroides cellulosilyticus* (42% identity)	Cytoplasmic	44247.94	PL, PG, GH28
2842108446	Candidate pectin or pectate lyases	polygalacturonase	*Cytophagaceae bacterium* SJW1-29 (74% identity)	Extracellular	49580.00	PL, PG, GH28
2842105518	Related to pectin or pectate lyases	polygalacturonase	*Chitinophagaceae bacterium* BR5-29 (38% identity)	Extracellular	55889.57	Pl, PG, GH28
2842109095	Related to pectin or pectate lyases	hypothetical protein	*Pedobacter* sp. OK628 (32% identity)	Extracellular	67179.60	PL, GH28
2842108200	Exo-poly-alpha-D-galacturonosidase	DNA sulfur modification protein DndE	*Cytophagales bacterium* (60% identity)	Extracellular	85717.11	PL, GH28, GDSL_2
2842108975	Related to Pectin lyase-like	hypothetical protein	*Alcanivorax* sp. (42% identity)	Unknown, Non-Cytoplasmic	51861.62	PL

[1] Predicted functions as assigned by routines used for updating the Carbohydrate-Active Enzymes database (http://www.cazy.org/CAZY/) using the criteria indicated in Table 1.
[2] Localization predicted using the default settings of PSORTb (Gardy, J. L., et al. 2005. Bioinformatics 21:617-623).
[3] Predicted molecular mass of primary product of translation, including any predicted signal peptide.
[4] Modular structure is indicated by abbreviations: GH, glycoside hydrolase as assigned by CAZY (the numbers indicate families) (http://www.cazy.org/CAZY/). PL: polysaccharide lyase (number indicates family) as assigned by CAZY. GDSL-like Lipase, (GDSL esterases and lipases are hydrolytic enzymes with multifunctional properties [1]. This new subclass of lipolytic enzymes possesses a distinct GDSL sequence motif different from the GxSxG motif found in many lipases [2]. Members include; Aeromonas hydrophila lipase, Vibrio mimicus arylesterase, Vibrio parahaemolyticus thermolabile haemolysin, rabbit phospholipase (AdRab-B), and Brassica napus anter-specific proline-rich protein.)

Supplemental TABLE S5: Predicted *Dyadobacter* sp. HH091 carbohydrate esterases involved in polysaccharide utilization[1].

Gene ID	Predicted function [1]	IMG Product Name [2]	Homologs [3]	Predicted localization [4]	Molecular mass (kDa) [5]
2842104000	Related to polysaccharide deacetylase	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family)	*Flavobacterium álbum* (54% identity)	Cytoplasmic	25806.77
2842105287	Related to polysaccharide deacetylase	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family)	*Flavobacteriales bacterium* (46.88% identity)	Cytoplasmic	28782.40
2842105600	Related to polysaccharide deacetylase	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family)	*Flavobacterium kingsejongi* (66.30% identity)	Unknown (This protein may have multiple localization sites.)	31934.59
2842106253	Candidate polysaccharide deacetylase	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family)	*Cytophagaceae bacterium* SCN 52-12 (61.96% identity)	Unknown	44623.98
2842108758	Related to polysaccharide deacetylase	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family)	*Cytophagaceae bacterium* SJW1-29 (48.85% identity)	Cytoplasmic	32059.73
2842106071	Candidate polysaccharide deacetylase	Hypothetical protein	*Siphonobacter* sp. BAB-5404 (62% identity)	Cytoplasmic	30585.36
2842104846	Candidate acetylxylan esterase	cephalosporin-C deacetylase-like acetyl esterase	*Cytophagaceae bacterium* SJW1-29 (73.49%)	Unknown	48188.31
2842107040	Candidate acetylxylan esterase	hypothetical protein	*Cytophagaceae bacterium* CAR-16 (64.36%)	Cytoplasmic	42412.16

[1] Predicted functions were assigned by routines used for updating the Carbohydrate Active Enzymes database (http://www.cazy.org/) using the following criteria: typically, 70% or greater amino acid identity with a protein domain with a biochemically determined function at the time of analysis resulted in “candidate” status; 30% to 70% amino acid identity with a protein domain with a known function resulted in “related to” status; and less than 30% amino acid identity with a protein domain with a known function resulted in “distantly related to” status. All analyses were conducted domain by domain to avoid problems arising from the modular structure of many of the proteins.
[2] The Integrated Microbial Genomes (IMG) Product Name
[3] Homologs were identified by a BlastP search with the Swiss-Prot database.
[4] Localization was predicted using the default settings of PSORTb (Gardy et al., 2005).
[5] Predicted molecular mass of the primary product of translation, including any predicted signal peptide.

Supplemental TABLE S6: Overall numbers of sequences and contigs generated for the transcriptome datasets.

	Bacteria-exponential phase	Bacteria-stationery phase	Eukaryotic-exponential phase	Eukaryotic-stationery phase
Reads Illumina (filtered)				
Total no.	79,956,806	98,243,390	73,776,383	76,211,700
Average length (bp)	73	74	168	177
Duplicates (%)	79.0	75.9	90.1	86.0
Fails (%)	45	36	18	18
GC (%)	51	56	56	57
Contigs-assembly (CoMW)				
No.	13,259	22,651	39,255	51,222
Total length (bp)	14,577,971	27,842,014	57,814,484	80,131,706
No. ≥ 1000 bp	4,427	8,553	22,528	30,347
N50 size (bp)	1,211	1,453	1,829	1,979
Largest (bp)	16,877	20,249	11,273	16,587
GC (%)	55.67	57.75	56.95	57.24

Supplemental TABLE S7: Protein family comparison of possible competitive and plant-bacteria interaction pathways across the dominant members of microbiome (*Porphyrobacter, Variovorax* and *Dyadobacter*) of *S. quadricauda* MZCH 10104. Numbers of proteins are marked with the following colors: ■ 0, □ 1, ■ 2, and ■ 3+.

Porphyrobacter cryptus DSM 12079
Porphyrobacter sp. AAP60
Porphyrobacter sp. AAP82
Porphyrobacter sp. HL-46
Dyadobacter alkalitolerans DSM 23607
Dyadobacter beijingensis strain CGMCC 1.6375
Dyadobacter crusticola DSM 16708
Dyadobacter sp. HH091 *
Dyadobacter tibetensis Y620-1
Variovorax paradoxus B4
Variovorax paradoxus EPS
Variovorax paradoxus S110
Variovorax sp. 770b2
Variovorax sp. CF313
Variovorax sp. URHB0020

Secretion systems

T1SS

T1SS membrane fusion protein, HlyD family

T1SS ATPase

T1SS ATPase, LssB family LapB

T2SS, T4SS

T2SS, T4SS ATP hydrolase TadA/VirB11/CpaF, TadA subfamily

T2SS, T4SS ATPase TadZ/CpaE, associated with Flp pilus assembly

T2SS, T4SS protein TadC, associated with Flp pilus assembly

T2SS, T4SS protein TadC, associated with Flp pilus assembly

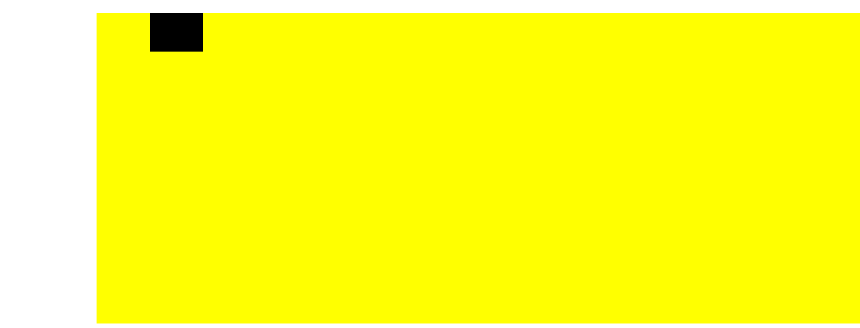
T2SS, T4SS secretin RcpA/CpaC, associated with Flp pilus assembly

T4SS component, VirD4

T5SS

Protein translocase subunit SecA

Protein translocase subunit SecE
Protein translocase subunit SecY
Protein translocase subunit SecDF
Protein translocase membrane subunit SecG
Inner membrane protein translocase and chaperone YidC
Signal recognition particle receptor FtsY
Signal recognition particle protein Ffh
Protein translocase subunit YajC



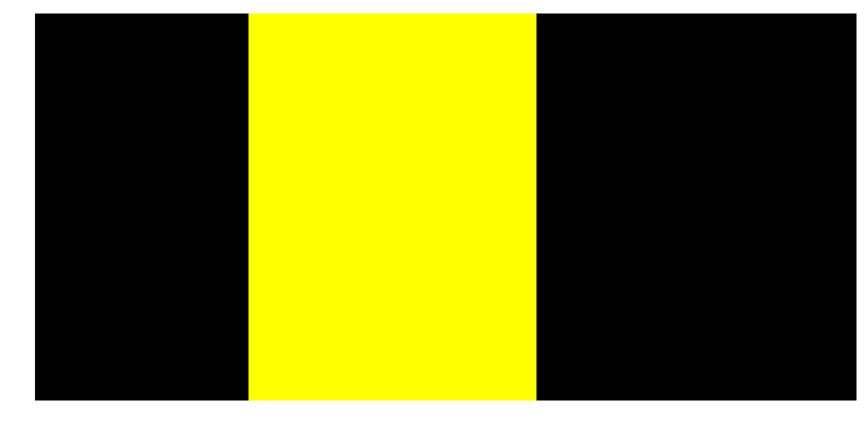
T6SS

T6SS protein serine/threonine phosphatase, PppA
T6SS component, Hcp
T6SS component, TssF
T6SS component, TssC
T6SS component, TssG
T6SS lysozyme-like component, TssE
T6SS AAA+ chaperone, ClpV (TssH)
T6SS outer membrane component, TssL
T6SS Serine/threonine protein kinase, PpkA
T6SS secretion lipoprotein, TssJ
T6SS component, TssK
T6SS forkhead associated domain protein, ImpI/VasC
T6SS associated component, TagF
T6SS component, TssB
T6SS component, TssM
T6SS component, TssA
T6SS associated component, TagJ
T6SS-associated peptidoglycan hydrolase, TagX
T6SS peptidoglycan-binding component, TagN
T6SS baseplate protein J-like component
T6SS PAAR-repeat protein
T6SS component FIG00414986

T9SS

gliding motility-associated ABC transporter ATP-binding protein, GldA
gliding motility-related protein, GldB
gliding motility-related protein, GldC
gliding motility-related protein, GldD
gliding motility-related protein, GldE

gliding motility-associated ABC transporter permease protein, GldF
gliding motility-associated ABC transporter substrate-binding protein, GldG
gliding motility-related protein, GldH
gliding motility-related protein, GldL
gliding motility-related protein, GldM
gliding motility-related protein, GldN and/or GldO
gliding motility-related protein, SprA
gliding motility-related protein, SprB
gliding motility-related protein, SprE
gliding motility-related protein, SprF



Response and transcriptional regulators

Two-component transcriptional response regulator, LuxR family
Acyl-homoserine lactone-binding transcriptional activator, LuxR family
Two-component transcriptional response regulator, LuxR family, but with unusual receiver domain
Transcriptional regulator, LuxR family
Two-component transcriptional response regulator, NarL/FixJ family
RNA polymerase sigma-70 factor
Sigma-70 factor

Polysaccharides degradation/modification

GH

beta-galactosidase, GH2, GH1, GH35, GH39, GH42 (EC 3.2.1.23)
alpha-L-arabinofuranosidase, GH2, GH39, GH62 (EC 3.2.1.55)
beta-mannosidase, GH2 (EC 3.2.1.25)
beta-glucosidase, GH2, GH1, GH30, GH39 (EC 3.2.1.21)
beta-glycosyl hydrolase, GH3
mannan endo-1,4-beta-mannosidase, GH5, GH26 (EC:3.2.1.78)
beta-1,4-glucanase (cellulase), GH9, GH6, GH7, GH8, GH45 (EC 3.2.1.4)
endo-1,4-beta-xylanase, GH10, GH8, GH30 (EC 3.2.1.8)
xylanase, GH11 (EC:3.2.1.-)
alpha-amylase, GH13 (EC 3.2.1.1)
malto-oligosyltrehalose synthase , GH13 (EC 5.4.99.15)
Chitinase, GH18, GH19 (EC 3.2.1.14)
N-acetyl-beta-hexosaminidase, GH20, GH84 (EC 3.2.1.52)
beta-N-acetylglucosaminidase, GH20 (EC 3.2.1.52)

lyzozyme M1 (1,4-beta-N-acetylmuramidase), GH25, GH18 (EC 3.2.1.17)
alpha-galactosidase, GH31, GH4, GH27 (EC 3.2.1.22)
cyclic beta-1,2-glucan synthase, GH32, GH16 (EC 2.4.1.-)
UDP-N-acetylgalactosaminyltransferase, GH32, GH16 (EC 2.4.1.-)
levanase, GH32 (EC 3.2.1.65)
beta-glucanase precursor, GH32, GH6, GH7 (EC 3.2.1.73)
alpha-1,3-N-acetylgalactosamine transferase, GH32, GH16 (EC 2.4.1.-)
N-acetylglucosaminyltransferase, GH32, GH16 (EC 2.4.1.-)
alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase, GH32, GH16 (EC 2.4.1.-)
Endo-beta-1,3-1,4 glucanase, GH32, GH7, GH17, GH26 (EC 3.2.1.73)
xylose isomerase, GH43 (EC 5.3.1.5)
alpha-L-arabinofuranosidase, GH43, GH39, GH62 (EC 3.2.1.55)
arabinan endo-1,5-alpha-L-arabinosidase, GH43
xylan 1,4-beta-xylosidase, GH43, GH1, GH30, GH39 (EC 3.2.1.37)
beta-agarase, GH43 (EC 3.2.1.81)
dextrin alpha-1,6-maltotetraose-hydrolase, GH48 (EC 3.2.1.196)
alpha-L-rhamnosidase, GH78 (EC 3.2.1.40)
alpha-L-fucosidase, GH95, GH29 (EC 3.2.1.51)
UDP-glucose 6-dehydrogenase, GH4 (EC 1.1.1.22)
chitinase, GH18
mannan endo-1,4-beta-mannosidase, GH26
polygalacturonase , pectin lyase, GH28 (EC 3.2.1.15)
glycosyl hydrolase, GH30
alpha-glucosidase , GH31, GH4, GH63, GH76 (EC 3.2.1.20)
sialidase, GH33 (EC 3.2.1.18)
alpha-galactosidase, GH36
maltose phosphorylase, GH65
xylan alpha-1,2-glucuronosidase, GH67
alpha-1,6-mannanase, GH76
glycosyl hydrolase, GH88
alpha-1,2-mannosidase, GH92
glycoside hydrolase family, GH127
4-O-beta-D-mannosyl-D-glucose phosphorylase, GH130 (EC 2.4.1.281)
maltodextrin glucosidase, GH4, GH76 (EC 3.2.1.20)
xyloglucan-specific endo-beta-1,4-glucanase, GH44 (EC 3.2.1.151)
glucoamylase, GH15 (EC 3.2.1.3)
glucan 1,4-alpha-glucosidase, GH15 (EC 3.2.1.3)

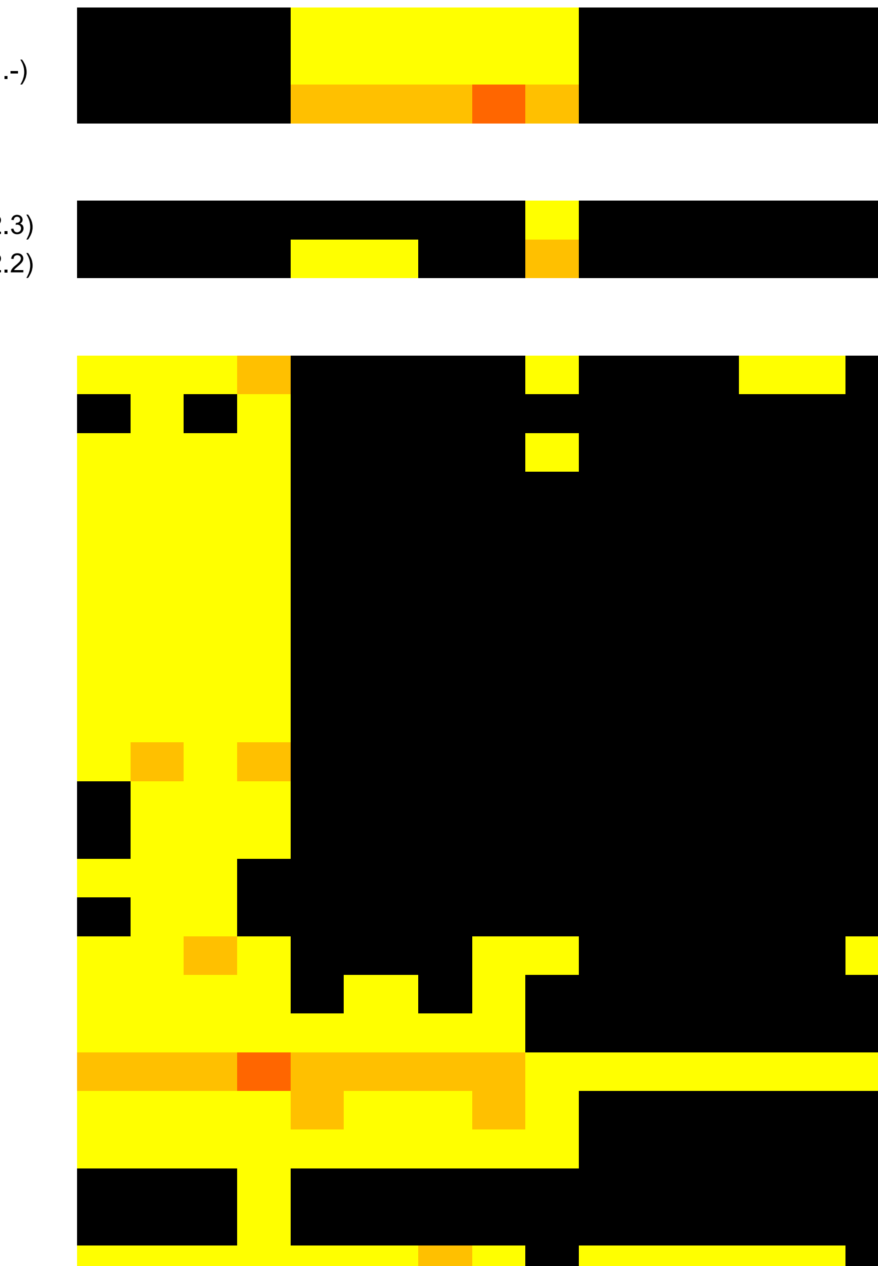
Enzyme	EC
trehalase, GH15, GH37	(EC 3.2.1.28)
beta-porphyranase, GH16	(EC 3.2.1.178)
alpha-galactosidase, GH31	(EC 3.2.1.22)
alpha-xylosidase, GH31	(EC 3.2.1.177)
1,4-alpha-glucan branching enzyme, GH13	(EC 2.4.1.18)
4-alpha-glucanotransferase (amylomaltase), GH57, GH77, GH13	(EC 2.4.1.25)
xylan alpha-1,2-glucuronosidase, GH67	(EC 3.2.1.131)
alpha-N-acetylglucosaminidase, GH89	(EC 3.2.1.50)
Maltose phosphorylase, GH65	(EC 2.4.1.8)
4-O-beta-D-mannosyl-D-glucose phosphorylase, GH130	(EC 2.4.1.281)
GT	
glycosyltransferase, GT1	
N-acetylglucosaminyltransferase, GT1, GT2, GT4, GT5	(EC 2.4.1.-)
b-glycosyltransferase, GT2	
glycosyltransferase YkoT, GT2	
a-glycosyltransferase-related protein, GT4	
alpha-1,3-N-acetylgalactosamine transferase PglA, GT4, GT5	(EC 2.4.1.-)
alpha-1,2-fucosyltransferase, GT11	
trehalose-6-phosphate phosphatase, GT20	(EC 3.1.3.15)
UDP-N-acetylglucosamine:L-malate glycosyltransferase, GT41	
alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase, GT1, GT2, GT4, GT5	
lipid carrier : UDP-N-acetylgalactosaminyltransferase, GT1, GT2, GT5	(EC 2.4.1.-)
cyclic beta-1,2-glucan synthase, GT2, GT4, GT5	(EC 2.4.1.-)
Glycogen phosphorylase, GT35	(EC 2.4.1.1)
Penicillin-insensitive transglycosylase, GT4	(EC 2.4.2.-)
Similar to glycogen synthase, GT5	(EC 2.4.1.21)
Glycogen synthase, ADP-glucose transglucosylase, GT5	(EC 2.4.1.21)
Lipid-A-disaccharide synthase, GT19	(EC 2.4.1.182)
Similar to glycogen synthase, GT3	(EC 2.4.1.21)
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase, GT28	(EC 2.4.1.227)
Biofilm PGA synthesis N-glycosyltransferase PgaC, GT2	(EC 2.4.-.-)
1,2-diacylglycerol 3-glucosyltransferase, GT28	(EC 2.4.1.157)
Cellulose synthase, GT2	(EC 2.4.1.12)
Cyclic beta-1,2-glucan synthase, GT84	(EC 2.4.1.-)
Dolichol-phosphate mannosyltransferase, GT2	(EC 2.4.1.83)

Multimodular transpeptidase-transglycosylase, GT51
Membrane carboxypeptidase (penicillin-binding protein), GT51

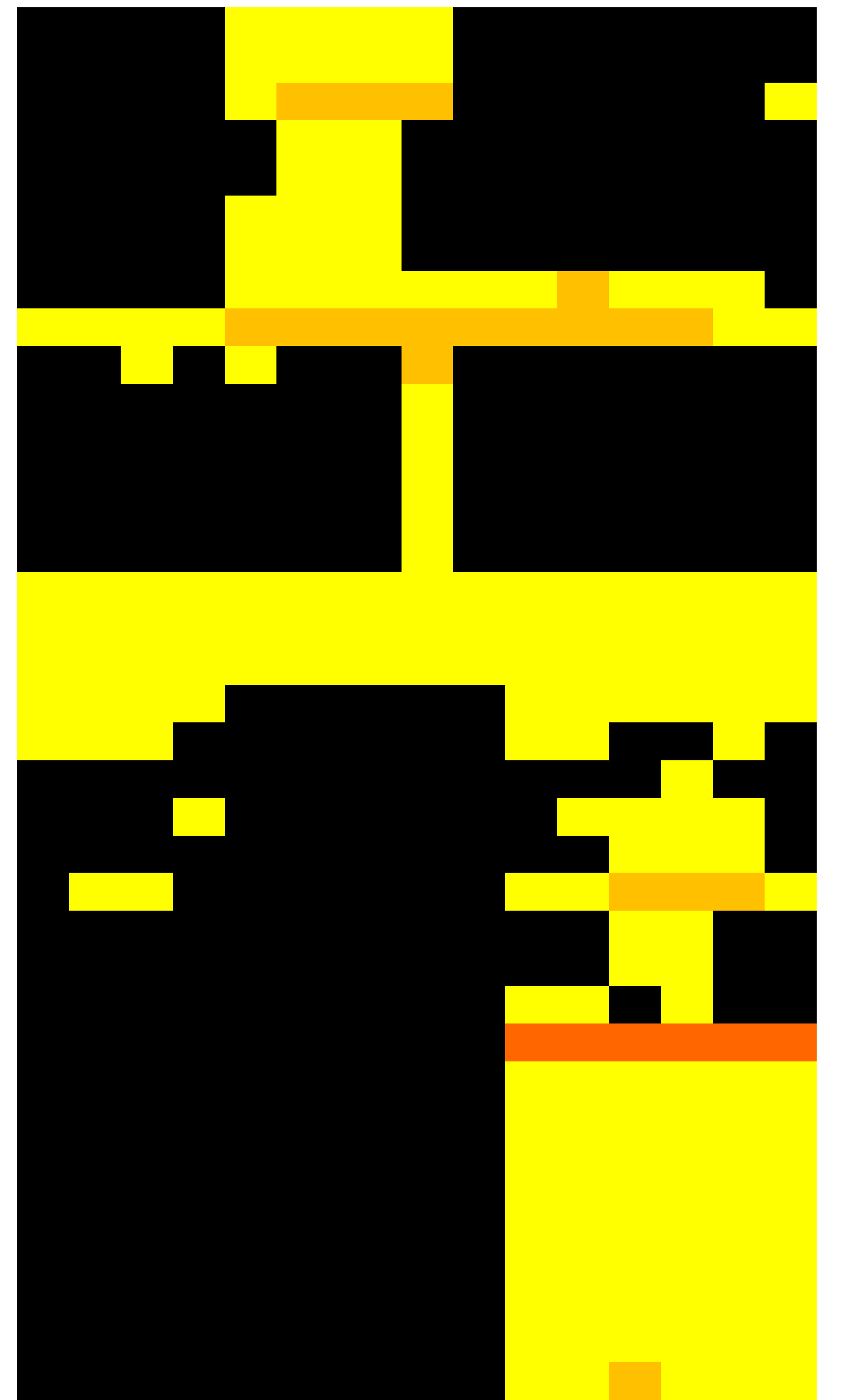
CE
carbohydrate esterase, CE1
indoleacetamide hydrolase, CE4 (EC 3.5.1.-)
polysaccharide deacetylase, CE4

PL
alginate lyase precursor, PL7 (EC 4.2.2.3)
Pectate lyase, PL9 (EC 4.2.2.2)

Peptidases
Peptidase M20
Peptidase S9, alanyl dipeptidyl peptidase
Peptidase M19, Zn-dependent dipeptidase
Peptidase M28, leucine aminopeptidase-related protein
Peptidase M13
Peptidase M14, zinc-dependent carboxypeptidase
Peptidase M16
Peptidase B M17
Peptidase S24
Peptidase M24, Xaa-Pro dipeptidase
Peptidase C82, L,D-transpeptidase
Pepdidase M14, zinc carboxypeptidase domain protein
Peptidase S10, carboxypeptidase-related protein
Peptidase M23/M37
Peptidase S9, dipeptidyl anminopeptidase
Peptidase S9, prolyl oligopeptidase family protein
Peptidase M28, aminopeptidase CC_2544
Peptidase M16, zinc protease
Peptidase S26, signal peptidase I
Peptidase S9, dipeptidyl peptidase IV
Peptidase S49, signal peptide peptidase SppA (protease 4)
Peptidase M48
Peptidase M23, membrane proteins related to metalloendopeptidases
Peptidase T2, isoaspartyl aminopeptidase



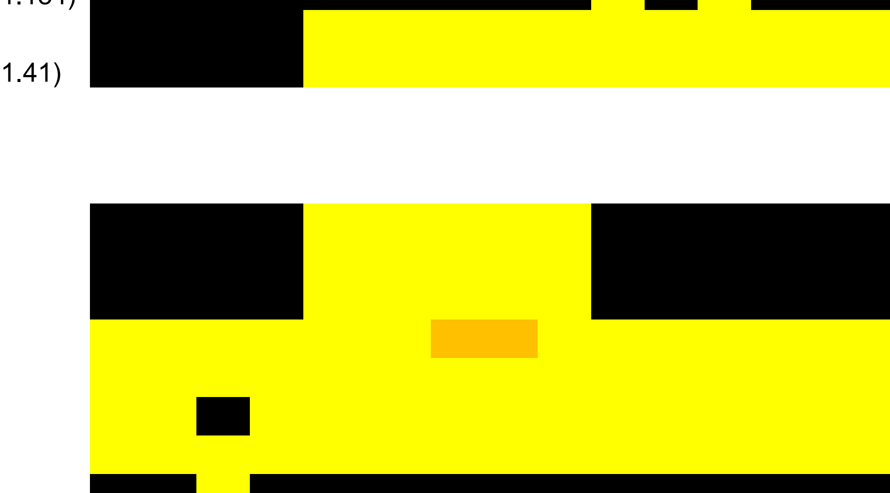
Peptidase M13, metallopeptidase
Peptidase M17, cytosol aminopeptidase PepA
Peptidase S9, prolyl endopeptidase
Peptidase M1, Zn-dependent aminopeptidase
Peptidase M14, carboxypeptidase A precursor
Peptidase M23/M37
Peptidase M28, aminopeptidase Y
Peptidase M49, dipeptidyl-peptidase III
Peptidase M10A/M12B, matrixin/adamalysin
Peptidase C39
Peptidase M28, glutamate carboxypeptidase II
Peptidase M61
Peptidase M28, aminopeptidase
Peptidase M1, aminopeptidase
Peptidase M28, putative aminopeptidase
Peptidase M24B, Xaa-Pro aminopeptidase
Peptidase M28, aminopeptidase
Peptidase M23B
Peptidase M1, putative aminopeptidase
Peptidase M13
Peptidase S41, C-terminal processing peptidase, tail-specific protease
Peptidase M28
peptidase M19, renal dipeptidase
Peptidase M1
Peptidase M15, D-alanyl-D-alanine dipeptidase
Peptidase M20B, tripeptide aminopeptidase
Peptidase M16
Peptidase Y M28, aminopeptidase Y
Peptidase M1, membrane alanine aminopeptidase N
Peptidase M3, oligoendopeptidase F-like protein
Peptidase M28, putative aminopeptidase
Peptidase A8, lipoprotein signal peptidase
Peptidase M16
Peptidase S9, X-Pro dipeptidyl-peptidase
Peptidase S8/S53, subtilisin/kexin/sedolisin
Peptidase M14, N-terminal Zinc-dependent carboxypeptidase
Peptidase M1, aminopeptidase N-like protein


Peptidase S33, proline iminopeptidase
Peptidase M23
Peptidase S15
Peptidase M19, putative periplasmic dipeptidase
Peptidase S9, dipeptidyl aminopeptidase
Peptidase M1, Zn-dependent aminopeptidase
Peptidase M19, renal dipeptidase
Peptidase S58, D-aminopeptidase
Peptidase M24, methionine aminopeptidase
Peptidase M3, dipeptidyl carboxypeptidase Dcp
Peptidase M50
Peptidase M28
Peptidase M1, aminopeptidase
Peptidase S41
Peptidase S9
Peptidase M23/M37
Peptidase S66, muramoyltetrapeptide carboxypeptidase
Peptidase M56, peptidoglycan D,D-transpeptidase MrdA
Peptidase M1, membrane alanine aminopeptidase N
Peptidase A24, type IV prepilin peptidase TadV/CpaA
Peptidase M50
Peptidase M20
Peptidase M14
Peptidase M20/M25/M40
Peptidase M20, peptidase
Peptidase M20, metal-dependent amidase/aminoacylase/carboxypeptidase
Peptidase M48, Ste24p
Peptidase M20, glutamate carboxypeptidase
Peptidase M48, uncharacterized integral membrane endopeptidase Bmul_2226
Peptidase M48, Ste24p precursor
Peptidase T1, ATP-dependent protease HslVU (ClpYQ), peptidase subunit
Peptidase M3, oligopeptidase A
Peptidase M20D, amidohydrolase
Peptidase A24, leader peptidase (Prepilin peptidase)
Peptidase C40, murein-DD-endopeptidase
Peptidase S1 and S6, chymotrypsin/Hap
Peptidase C15, pyrrolidone-carboxylate peptidase

Peptidase M23, membrane proteins related to metalloendopeptidases
Peptidase M48, Ste24p
Peptidase M55, D-aminopeptidase dipeptide-binding protein DppA
Peptidase C14, caspase catalytic subunit p20
Peptidase C82, L,D-transpeptidase
Peptidase T3, gamma-glutamyltranspeptidase
Peptidase S33, proline iminopeptidase
Peptidase S01A, lysyl endopeptidase
Peptidase M24
Peptidase C39, bacteriocin resistance protein
Peptidase S33, proline iminopeptidase
Peptidase C39, ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain
Peptidase S11, D-alanyl-D-alanine carboxypeptidase

Endonucleases and exonucleases

Single-stranded-DNA-specific exonuclease RecJ
Endonuclease III
HNH endonuclease
mRNA 3-end processing exonuclease
endonuclease
probable DNA repair exonuclease
Ribonuclease J (endonuclease and 5' exonuclease)
Type II restriction endonuclease
Type III restriction system endonuclease
Endonuclease/exonuclease/phosphatase
Endonuclease V
Exonuclease SbcC
Exonuclease SbcD
IncQ plasmid conjugative transfer DNA nicking endonuclease TraR
3'-5' exonuclease
Endonuclease containing a URI domain
DNA repair exonuclease family protein YhaO
CRISPR-associated RecB family exonuclease Cas4
tRNA 3 endonuclease
CRISPR-associated endonuclease Cas9
Endonuclease NmeDIP



Heme synthesis

Uroporphyrinogen-III synthase, HemD
Coproporphyrinogen III oxidase, aerobic (EC 1.3.3.3), CPOX, HemF
Coproporphyrinogen III oxidase, oxygen-independent
Porphobilinogen synthase, HemB
Glutamyl-tRNA synthetase,GltX
Protoporphyrinogen IX oxidase, novel form, HemJ
Ferrochelatase, protoheme ferro-lyase, HemH
Heme A synthase, cytochrome oxidase biogenesis protein, Cox15-CtaA
Glutamate-1-semialdehyde 2,1-aminomutase, HemL
Glutamyl-tRNA reductase, HemA

QQ

6-phosphogluconolactonase
dienelactone hydrolase
gluconolactonase
metal-dependent hydrolase
NUDIX hydrolase
acyl-homoserine lactone acylase PvdQ (EC 3.5.1.-), quorum-quenching
Zn-dependent hydrolase of the beta-lactamase fold-like protein

QS

N-acyl-L-homoserine lactone synthase, LuxI family (EC 2.3.1.184)
Dialkylrecorsinol condensing enzyme, DarA
3-oxoacyl-[acyl-carrier-protein] synthase III, DarB (EC 2.3.1.41)

Vitamins biosynthesis

Thiamine biosynthesis, B1

2-iminoacetate synthase, ThiH
Hydroxyethylthiazole kinase
Thiamin pyrophosphokinase
Thiamine monophosphate synthase
Thiamine-monophosphate kinase
Thiazole synthase, ThiGH
Phosphomethylpyrimidine synthase, ThiC
Sulfur carrier protein, ThiS

Riboflavin/FAD biosynthesis, B2
GTP cyclohydrolase II
6,7-dimethyl-8-ribityllumazine synthase, riboflavin synthase beta chain
Pyrimidine reductase, riboflavin biosynthesis
FMN adenylyltransferase / Riboflavin kinase
Riboflavin synthase eubacterial/eukaryotic

Pantothenate/CoA biosynthesis, B5
Aspartate 1-decarboxylase
Ketopantoate reductase
Dephospho-CoA kinase
Ketopantoate hydroxymethyltransferase
Panthothenate synthetase
Pantothenate kinase type III, CoaX-like
Phosphopantetheine adenylyltransferase
Pyridoxal 5'-phosphate, B6
Pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5)
Pyridoxine 5'-phosphate synthase (EC 2.6.99.2)
4-hydroxythreonine-4-phosphate dehydrogenase (EC 1.1.1.262)
Phosphoserine aminotransferase (EC 2.6.1.52)
Biotin biosynthesis, B7
Adenosylmethionine-8-amino-7-oxononanoate aminotransferase, BioA
Dethiobiotin synthetase, BioD
7-keto-8-aminopelargonate synthetase or related enzyme, BioF
malonyl-CoA O-methyltransferase, BioC
3-oxoacyl-[acyl-carrier-protein] synthase I, FabB
3-oxoacyl-[acyl-carrier-protein] synthase II, FabF
3-oxoacyl-[acyl-carrier protein] reductase, FabG
beta-hydroxyacyl-(acyl-carrier-protein) dehydratase, FabZ
enoyl-[acyl-carrier protein] reductase I, FabI
Biotin synthase, BioB
Biotin-protein ligase. BirA2
8-amino-7-oxononanoate synthase
Folate biosynthesis, B9
2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase
Dihydrofolate reductase
Dihydroneopterin aldolase

Dihydropteridine reductase
FMN reductase (NADPH)

Cobalamine/B12 biosynthesis

Cobalamin synthase, CobS (EC 2.7.8.26)
alpha ribazole-5'-P phosphatase in cobalamin synthesis, CobC
nicotinate-nucleotide dimethylbenzimidazole-P phophoribosyl transferase, CobT
adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase, CobU
synthesis of vitamin B12 adenosyl cobalamide precursor, CobD
cob(I)alamin and cobinamide adenolsyltransferase, BtuR, CobA, CobO
Aerobic cobaltochelatase, CobN (EC 6.6.1.2)
cobyrinic acid a,c-diamide synthase, CbiA, CobB (EC 6.3.5.9,11)
synthesis of vitamin B12 adenosyl cobalamide precursor, CbiP
cobyrinic acid a,c-diamide synthase, CobB
precorrin-2 dehydrogenase / sirohydrochlorin ferrochelatase, CysG
Aerobic cobaltochelatase, CobS (EC 6.6.1.2)
Aerobic cobaltochelatase, CobT (EC 6.6.1.2)
sirohydrochlorin cobaltochelatase, CbiK
precorrin-8X/cobalt-precorrin-8 methylmutase, CbiC, CobH
cobalt-precorrin-7 (C5)-methyltransferase, CbiE
cobalt-precorrin-6B (C15)-methyltransferase, CbiT, CobL
cobalt-precorrin-5B (C1)-methyltransferase, CbiD
cobalt-precorrin 5A hydrolase, CbiG
precorrin-4/cobalt-precorrin-4 C11-methyltransferase, CbiF
cobalt-precorrin-3 C(17)-methyltransferase, CbiH
precorrin-2/cobalt-factor-2 C20-methyltransferase, CbiL

Histidine biosynthesis

Hydrolase, HAD superfamily
ATP phosphoribosyltransferase , HisGs
ATP phosphoribosyltransferase regulatory subunit, HisG
Phosphoribosyl-ATP pyrophosphatase, HisI2
ATP phosphoribosyltransferase, HisGl
Phosphoribosyl-AMP cyclohydrolase, HisI1
Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase, HisA
Ribose-phosphate pyrophosphokinase, PrsA
Imidazole glycerol phosphate synthase amidotransferase, HisH

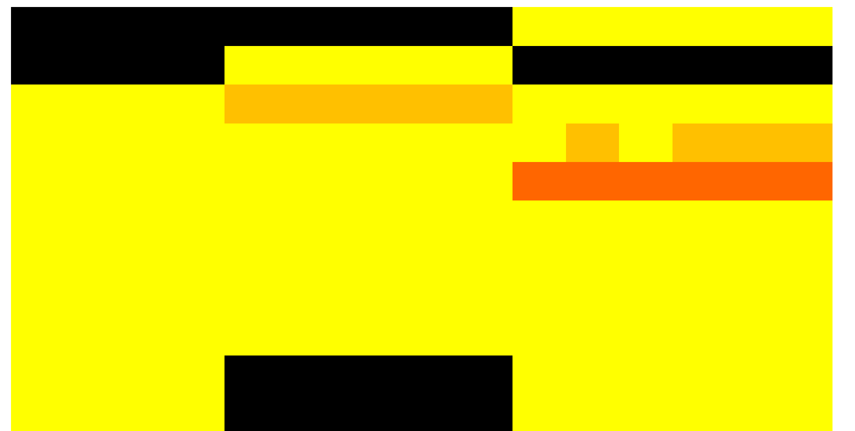
Histidinol dehydrogenase, HisD
Histidinol-phosphatase, HisB1

Bacteriochlorophyll synthesis and phototropic activity

2-desacetyl-2-hydroxyethyl bacteriochlorophyllide A dehydrogenase, BchC
2-vinyl bacteriochlorophyllide hydratase, BchF
Bacteriochlorophyll synthase
Protein BchJ, involved in reduction of C-8 vinyl of divinyl protochlorophyllide
Chlorophyllide reductase subunit, BchX
Chlorophyllide reductase subunit, BchY
Chlorophyllide reductase subunit, BchZ
Light-harvesting LHI, alpha subunit
Photosynthetic reaction center H subunit
Photosynthetic reaction center L subunit
Photosynthetic reaction center M subunit
Photosynthetic complex assembly protein RPC_1320
Protein BchJ, involved in reduction of C-8 vinyl of divinyl protochlorophyllide
Light-independent protochlorophyllide reductase iron-sulfur ATP-binding protein, ChlL
Light-independent protochlorophyllide reductase subunit B
Light-independent protochlorophyllide reductase subunit N
Divinyl protochlorophyllide a 8-vinyl-reductase
Mg-protoporphyrin IX monomethyl ester oxidative cyclase (anaerobic)
Mg protoporphyrin IX monomethyl ester oxidative cyclase (aerobic)

Phenylalanine, tyrosine and tryptophane biosynthesis

2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha, AroGA
2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta, AroG1
3-dehydroquinate synthase, AroB
3-dehydroquinate dehydratase, AroD
Shikimate 5-dehydrogenase I alpha, AroE
Shikimate kinase I, AroK
3-phosphoshikimate 1-carboxyvinyltransferase, AroA
Chorismate synthase, AroC
Chorismate mutase, PheA
Prephenate dehydratase, PheA2
Cyclohexadienyl dehydrogenase, TyrC



Prephenate and/or arogenate dehydrogenase, TyrA
Para-aminobenzoate synthase, TrpE
Anthranilate phosphoribosyltransferase, TrpD
Phosphoribosylanthranilate isomerase, TrpF
Indole-3-glycerol phosphate synthase, TrpC
Tryptophan synthase alpha chain, TrpA
Tryptophan synthase beta chain, TrpB

Cysteine and methionine biosynthesis

serine acetyltransferase (EC 2.3.1.30)
cysteine synthase (EC 2.5.1.47)
cystathionine gamma-lyase (EC 4.4.1.1)
cystathionine beta-synthase (EC 4.2.1.22)
S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)
SAM-dependent methyltransferase
S-adenosylmethionine synthetase (EC 2.5.1.6)
homoserine O-acetyltransferase (EC 2.3.1.31)
homoserine dehydrogenase (EC 1.1.1.3)
O-succinylhomoserine sulfhydrylase (EC 2.5.1.48)
aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)
aspartokinase (EC 2.7.2.4)
5-methyltetrahydrofolate--homocysteine methyltransferase (EC 2.1.1.13)
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)

Indole-3-acetic acid biosynthesis

indolepyruvate ferredoxin oxidoreductase
indoleacetamide hydrolase , IamA, IamB (EC 3.5.1.-)
nitrilase, NitA (EC 3.5.5.1,7)
cobalt-containing nitrile hydratase (EC 4.2.1.84)
tryptophan 2-monooxygenase, IaaM

ROS tolerance

Superoxide dismutase [Cu-Zn] precursor
Superoxide dismutase [Fe]
Superoxide dismutase [Mn]
Rhodanese-related sulfurtransferase
Catalase-peroxidase

NTP pyrophosphohydrolases including oxidative damage repair enzymes		
Cell wall-associated hydrolases (invasion-associated proteins)		
Leucine-rich repeat proteins	pfam13855, COG4886	

9.2 Interplay between the microalga *Micrasterias radians* and its symbiont *Dyadobacter* sp. HH091

All supplemental tables can be accessed online via
https://doi.org/10.3389/fmicb.2022.1006609

Supplemental TABLE S1: Overall numbers of sequences generated for the transcriptome datasets.

Reads Illumina (filtered)	
Total no.	43,164,382
Average length (bp)	151
Duplicates (%)	65.8
GC (%)	46

TABLE S3: Predicted gene clusters for flexirubin biosynthesis in *Dyadobacter* sp. HH091. Domain guided annotation is based on conserved domains detected by STRING analysis of *Dyadobacter* sp. HH091 primary sequences against the genome of *Flavobacterium* spp (IMG 2511231122, IMG 644736369).

Gene ID	Gene Product Name	Pfam/Families	Gene	Organism	Identity (%)	AA Length
2842105285	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	pfam07977-FabA; COG0764===3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase	FCOL_11760, FabA/FabZ	*Flavobacterium columnare* (strain ATCC 49512 / CIP 103533 / TG 44/87)	36	119
2842105287	peptidoglycan/xylan/chitin deacetylase (PgdA/CDA1 family), flxP	pfam01522 - Polysacc_deac_1; COG0726 - Peptidoglycan/xylan/chitin deacetylase, PgdA/CDA1 family	Fjoh_1086, flxP	*Flavobacterium johnsoniae* UW101	31	256
2842105288	hypothetical protein	pfam13723 – Ketoacyl-synt_2	Fjoh_1087	*Flavobacterium johnsoniae* UW101	31	321
2842105289	3-oxoacyl-[acyl-carrier-protein] synthase-1	pfam02801===Ketoacyl-synt_C<<>>pfam00109===ketoacyl-synt; COG0304===3-oxoacyl-(acyl-carrier-protein) synthase	Fjoh_1088	*Flavobacterium johnsoniae* UW101	52	393
2842105291	acyl carrier protein	pfam00550===PP-binding; COG0236===Acyl carrier protein	Fjoh_1089	*Flavobacterium johnsoniae* UW101	57	85
2842105292	hypothetical protein		Fjoh_1090	*Flavobacterium johnsoniae* UW101	31	204
2842105293	3-oxoacyl-[acyl-carrier-protein] synthase-1	pfam00109===ketoacyl-synt<<>>pfam02801===Ketoacyl-synt_C; COG0304===3-oxoacyl-(acyl-carrier-protein) synthase	Fjoh_1093	*Flavobacterium johnsoniae* UW101	39	358
2842105294	acyl-CoA thioester hydrolase	pfam13279===4HBT_2; COG0824===Acyl-CoA thioesterase FadM	Fjoh_1094	*Flavobacterium johnsoniae* UW101	40	139
2842105295	predicted hotdog family 3-hydroxylacyl-ACP dehydratase	COG4706===Predicted 3-hydroxylacyl-ACP dehydratase, HotDog domain	Fjoh_1101	*Flavobacterium johnsoniae* UW101	28	138

2842105296	predicted LPLAT superfamily acyltransferase	pfam03279===Lip_A_acyltrans; COG4261===Predicted acyltransferase, LPLAT superfamily	Fjoh_1104	*Flavobacterium johnsoniae* UW101	39	288
2842105297	acyl carrier protein	pfam00550===PP-binding; COG0236===Acyl carrier protein	Fjoh_1105	*Flavobacterium johnsoniae* UW101	47	88
2842105299	methionine-R-sulfoxide reductase	pfam01641===SelR; COG0229===Peptide methionine sulfoxide reductase MsrB	Fjoh_0270	*Flavobacterium johnsoniae* UW101	36	141
2842105302	3-oxoacyl-[acyl-carrier-protein] synthase-1	pfam00109===ketoacyl-synt<<>>pfam02801===Ketoacyl-synt_C; COG0304===3-oxoacyl-(acyl-carrier-protein) synthase	Fjoh_1106	*Flavobacterium johnsoniae* UW101	64	406
2842105303	3-oxoacyl-[acyl-carrier protein] reductase (short-chain dehydrogenase); flxB	pfam13561===adh_short_C2; COG1028===NAD(P)-dependent dehydrogenase, short-chain alcohol dehydrogenase family	Fjoh_1107, flxB	*Flavobacterium johnsoniae* UW101	65	243
2842105304	histidine ammonia-lyase; flxA	pfam00221===Lyase_aromatic; COG2986===Histidine ammonia-lyase	Fjoh_1109, flxA	*Flavobacterium johnsoniae* UW101	52	509
2842105305	1-acyl-sn-glycerol-3-phosphate acyltransferase; flxU	pfam03176===MMPL<<>>pfam13847===Methyltransf_31<<>>pfam01553===Acyltransferase; COG0204===1-acyl-sn-glycerol-3-phosphate acyltransferase<<>>COG4258===Predicted exporter	Fjoh_1078, flxU	*Flavobacterium johnsoniae* UW101	28	1277
2842105306	all-trans-retinol 13,14-reductase; flxV	pfam13450===NAD_binding_8; COG1233===Phytoene dehydrogenase-related protein	Fjoh_1077, flxV	*Flavobacterium johnsoniae* UW101	44	509
2842105307	flavin-dependent dehydrogenase; flxK	pfam01494===FAD_binding_3; COG0644===Dehydrogenase (flavoprotein)	Fjoh_1110, flxK	*Flavobacterium johnsoniae* UW101	47	410
2842105308	3-oxoacyl-(acyl-carrier-protein) synthase	pfam13723===Ketoacyl-synt_2; COG0304===3-oxoacyl-(acyl-carrier-protein) synthase	Fjoh_1087	*Flavobacterium johnsoniae* UW101	34	355

2842105340	3-oxoacyl-(acyl-carrier-protein) synthase	pfam02801===Ketoacyl-synt_C<<>>pfam00109===ketoacyl-synt; COG0304===3-oxoacyl-(acyl-carrier-protein) synthase	Fjoh_1088	*Flavobacterium johnsoniae* UW101	45	399
2842105341	acyl carrier protein	pfam00550===PP-binding; COG0236===Acyl carrier protein	Fjoh_1089	*Flavobacterium johnsoniae* UW101	48	88
2842105342	ABC-2 type transport system permease protein; darJ	pfam12698===ABC2_membrane_3; COG0842===ABC-type multidrug transport system, permease component	Fjoh_1095, darJ	*Flavobacterium johnsoniae* UW101	32	425
2842105343	ABC-2 type transport system ATP-binding protein; darI	pfam00005===ABC_tran; COG1131===ABC-type multidrug transport system, ATPase component	Fjoh_1096, darI	*Flavobacterium johnsoniae* UW101	52	249
2842105344	hypothetical protein (BtrH); darH	pfam16169===DUF4872<<>>pfam14399===BtrH_N	Fjoh_1097, darH	*Flavobacterium johnsoniae* UW101	48	343
2842105345	hypothetical protein		Fjoh_1098	*Flavobacterium johnsoniae* UW101	53	132
2842105346	3-oxoacyl-[acyl-carrier-protein] synthase-3; darB	pfam08541===ACP_syn_III_C; COG0332===3-oxoacyl-[acyl-carrier-protein] synthase III	darB	*Flavobacterium johnsoniae* UW101	50	380
2842105347	hypothetical protein; darA		darA	*Flavobacterium johnsoniae* UW101	39	303
2842108407	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase/3-hydroxyacyl-[acyl-carrier-protein] dehydratase; FabZ	pfam03331 - LpxC pfam07977 – FabA; COG0774 - UDP-3-O-acyl-N-acetylglucosamine deacetylase COG0764 - 3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase	fabZ	*Flavobacterium johnsoniae* UW101		

TABLE S4: Predicted components of the type IX protein secretion system (T9SS) in *Dyadobacter* sp. HH091. Domain guided annotation is based on conserved domains detected by STRING analysis of *Dyadobacter* sp. HH091 primary sequences against the genome of *Flavobacterium* spp (IMG 644736369, IMG 640753027, IMG 2731957834).

Gene ID	Gene Product Name	Pfam/Families	Gene	Organism	Identity (%)	AA Length
2842106854	GldA; Gliding motility-associated ABC transporter ATP-binding protein	TIGR03522; pfam00005 - ABC_tran	Fjoh_1516, GldA	*Flavobacterium johnsoniae* UW101	62	235
2842108806	GldB; gliding motility-associated protein	TIGR03514	Fjoh_1793, GldB	*Flavobacterium johnsoniae* UW101	27	347
2842104678	GldC; gliding motility-associated protein	TIGR03515; pfam19937 - GldC-like	Fjoh_1794, GldC	*Flavobacterium johnsoniae* UW101	42	113
2842109028	GldD; gliding motility-associated protein	TIGR03512	Fjoh_1540, GldD	*Flavobacterium johnsoniae* UW101	36	198
2842106522	GldE; gliding motility-associated protein	TIGR03520; pfam00571 – CBS; pfam03471 - CorC_HlyC	Fjoh_1539, GldE	*Flavobacterium johnsoniae* UW101	36	443
2842106486	GldF; Gliding motility-associated ABC transporter permease protein	pfam12679 - ABC2_membrane_2	Fjoh_2722, GldF	*Flavobacterium johnsoniae* UW101	49	242
2842109240	GldG; gliding-associated putative ABC transporter substrate-binding component	TIGR03521; pfam09822 - ABC_transp_aux	Fjoh_2721, GldG	*Flavobacterium johnsoniae* UW101	35	552
2842110280	GldH; gliding motility-associated lipoprotein	TIGR03511; pfam14109 - GldH_lipo	Fjoh_0890, GldH	*Flavobacterium johnsoniae* UW101	25	161
2842108793	GldI; gliding motility-associated protein	pfam00254 - FKBP_C	Fjoh_2369, GldI	*Flavobacterium johnsoniae* UW101	24	256
2842109190	GldJ; gliding motility-associated protein	TIGR03530; pfam03781 - FGE-sulfatase	Fjoh_1557, GldJ	*Flavobacterium johnsoniae* UW101	42	417
2842108285	GldK; gliding motility-associated lipoprotein	TIGR03529; pfam03781 - FGE-sulfatase	Fjoh_1853, GldK	*Flavobacterium johnsoniae* UW101	35.2	357
2842108286	GldL; gliding motility-associated protein	TIGR03513	Fjoh_1854, GldL	*Flavobacterium johnsoniae* UW101	35.2	270
2842108287	GldM; gliding motility-associated protein	TIGR03517; pfam12080 - GldM_C; pfam12081 - GldM_N	Fjoh_1855; GldM	*Flavobacterium johnsoniae* UW101	23	530
2842108288	GldN; gliding motility associated protein	TIGR03523; pfam19841 – GldN	Fjoh_1856; GldN	*Flavobacterium johnsoniae* UW101	41	342
2842109196	type IX secretion system PorP/SprF family membrane protein	TIGR03519; pfam11751 - PorP_SprF	FP0017; PorP/SprF	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	23	342
2842110175	type IX secretion system PorP/SprF family membrane protein	TIGR03519; pfam11751 - PorP_SprF	FP0017; PorP/SprF	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	19	336

2842108613	SprB	TIGR04131; pfam13585 - CHU_C	FP0016, sprB	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	35	683
2842109195	SprB	TIGR04131; pfam13585 - CHU_C; pfam18911 - PKD_4	FP0016, sprB	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	34	961
2842106852	RemA	TIGR04183; pfam19081; Ig-like domain CHU_C associated; pfam18962 Secretion system C-terminal sorting domain	RCH33_2786; remA	*Flavobacterium daejeonense* RCH33		1369
2842105604	SprE; tetratricopeptide (TPR) repeat protein	COG0457 - Tetratricopeptide (TPR) repeat; pfam13174 - TPR_6	Fjoh_1051; sprE	*Flavobacterium johnsoniae* UW101	23	726
2842106491	SprA; cell surface protein	TIGR04189; pfam14349 - SprA_N	FP2121, sprA	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	32	2408
2842106773	PorU	pfam01364 - Peptidase_C25	FP1388, porU	*Flavobacterium psychrophilum* (strain ATCC 49511 / DSM 21280 / CIP 103535 / JIP02/86)	26	1129
2842106772	PorV	pfam19572 - PorV	Fjoh_1555; porV	*Flavobacterium johnsoniae* UW101	33	385

10 ACKNOWLEDGEMENTS

„Cuando una persona desea realmente algo, el Universo entero conspira para que pueda realizar su sueño. "— Paulo Coelho, El alquimista

„And, when you want something, all the universe conspires in helping you to achieve it. "— Paulo Coelho, The Alchemist

„Wenn deine Entschlossenheit stark genug ist, wird sich das Universum verschwören, um deinen Traum Wirklichkeit werden zu lassen. "— Paulo Coelho, Der Alchimist

„Когда человек действительно хочет чего-то, вся Вселенная вступает в сговор, чтобы помочь этому человеку осуществить свою мечту. "— Пауло Коэльо, Алхимик

Yekaterina Astafyeva

Hamburg 2023