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DOI

[10.1111/lam.13795](https://doi.org/10.1111/lam.13795)

Publication date

2022

Document Version

Final published version

Published in

Letters in Applied Microbiology

Citation (APA)

Alloul, A., Van Kampen, W., Cerruti, M., Wittouck, S., Pabst, M., & Weissbrodt, D. G. (2022). Exploring the role of antimicrobials in the selective growth of purple phototrophic bacteria through genome mining and agar spot assays. *Letters in Applied Microbiology*, 75(5), 1275-1285. <https://doi.org/10.1111/lam.13795>

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ORIGINAL ARTICLE

Exploring the role of antimicrobials in the selective growth of purple phototrophic bacteria through genome mining and agar spot assays

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Significance and Impact of the Study: Purple non-sulphur bacteria (PNSB) are attractive for applied microbiology and biotechnology because of their ability to grow selectively on organic carbon in photobioreactors. This capability has been attributed to traditional selection phenomena such as low oxygen levels, availability of organic acids and infrared light. In this study, a unique combination of genome mining tools and microbiological methods was used to explore whether antimicrobials contribute to selective growth. Our findings provide first insights into the antimicrobial biosynthesis potential of PNSB and provide ground for more advanced research on antimicrobial production by PNSB.

Keywords

alternative protein, animal feed, antibiotics, antimicrobial peptide, bacteriocin, probiotic, purple phototrophic bacteria.

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2022/LAMICRO-2022-0202.R1: received 8 April 2022, revised 30 June 2022 and accepted 19 July 2022

doi:10.1111/lam.13795

Abstract

Purple non-sulphur bacteria (PNSB) are an emerging group of microbes attractive for applied microbiology applications such as wastewater treatment, plant biostimulants, microbial protein, polyhydroxyalkanoates and H₂ production. These photoorganoheterotrophic microbes have the unique ability to grow selectively on organic carbon in anaerobic photobioreactors. This so-called selectivity implies that the microbial community will have a low diversity and a high abundance of a particular PNSB species. Recently, it has been shown that certain PNSB strains can produce antimicrobials, yet it remains unclear whether these contribute to competitive inhibition. This research aimed to understand which type of antimicrobial PNSB produce and identify whether these compounds contribute to their selective growth. Mining 166 publicly-available PNSB genomes using the computational tool BAGEL showed that 59% contained antimicrobial encoding regions, more specifically biosynthetic clusters of bacteriocins and non-ribosomal peptide synthetases. Inter- and intra-species inhibition was observed in agar spot assays for *Rhodobacter blasticus* EBR2 and *Rhodospseudomonas palustris* EBE1 with inhibition zones of, respectively, 5.1 and 1.5–5.7 mm. Peptidomic analysis detected a peptide fragment in the supernatant (SVLQLLR) that had a 100% percentage identity match with a known non-ribosomal peptide synthetase with antimicrobial activity.

Introduction

Purple phototrophic bacteria and particularly purple non-sulphur bacteria (PNSB) are intensively studied for microbial protein, microbial fertilizer, polyhydroxyalkanoates and H₂ production (Bayon-Vicente *et al.* 2020; Capson-Tojo *et al.* 2020; Wambacq *et al.* 2022). Most

importantly, these microorganisms harbour great metabolic versatility, allowing them to grow photo- and chemotrophically on both organic and inorganic carbon (Imhoff 2006; Alloul *et al.* 2021, 2022). For biotechnology applications, they are typically cultivated photoorganoheterotrophically in anaerobic photobioreactors, where they use light as an energy and organics as an

electron and carbon source (Capson-Tojo *et al.* 2020). These conditions allow obtaining microbial biomass with a high abundance of one dominant PNSB species and an uneven community (i.e. high microbial selectivity; Alloul *et al.* 2021). Purple sulphur bacteria, another kind of purple phototrophic bacteria, do not offer a similar selectivity advantage on organic-rich streams as PNSB do because they are only capable of limited photoorganoheterotrophy (Hunter *et al.* 2008). These purple sulphur bacteria are specialized in photoautotrophic growth by using sulphide, sulphur and H₂ as an electron donor and CO₂ as a carbon source. The potential of PNSB for selective growth is of added value for the valorization of residual organics and the production of microbial proteins with stable nutritional characteristics in the produced microbial biomass (Alloul *et al.* 2021).

Thus far, selective growth of PNSB under anaerobic photoorganoheterotrophic conditions has been attributed to a kinetic advantage (Alloul *et al.* 2019; Yu *et al.* 2021). Their maximal specific photoorganoheterotrophic growth rates in an anaerobic environment range between 0.96–7.10 day⁻¹ (Noparatnaraporn *et al.* 1987; Ponsano *et al.* 2008), therefore, potentially surpassing acidogenic fermentative microorganisms (0.3–4.0 day⁻¹; Batstone *et al.* 2002). The selective growth of PNSB has been confirmed in several reactor systems operated on various types of organic-rich effluents, but primarily on pre-fermented feedstocks like volatile fatty acids (Cerruti *et al.* 2021). In previous research, we have been able to select for PNSB with a relative abundance up to 78% and a Shannon diversity index of 0.9 in a raceway reactor (Alloul *et al.* 2021). Similar results were found in an anaerobic membrane bioreactor treating domestic wastewater, reaching abundancies up to 90% (Hülßen *et al.* 2016).

Microbes are known to produce a plethora of antimicrobial compounds to enhance their competitiveness in engineered and natural environments. These antimicrobial compounds either inhibit the biosynthesis of the bacterial cell wall, protein synthesis or nucleic acid metabolism or disrupt the cellular membrane as membrane-active compounds (Maróti *et al.* 2011). Interestingly, antimicrobial production has also been detected in several PNSB strains such as *Rhodobacter sphaeroides* SS15, *Afifella marina* STW181, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* LMG 18881 (Chumpol *et al.* 2019; Alloul *et al.* 2021). In these research papers, PNSB were mainly explored in an aquaculture context for probiotic applications due to their ability to inhibit shrimp *Vibrio* pathogens. Research on the actual active compound secreted by PNSB is, however, limited. Only one study has reported the ability of *Rb. capsulatus* ATCC 17016 to produce bacteriocins (Lee *et al.* 2009). It remains, nonetheless, unclear which specific antimicrobials are produced by PNSB, how

widespread this biosynthetic trait is and whether this can enhance their competitiveness in engineered and natural microbial communities.

This paper aims to explore which type of antimicrobial compounds are produced by PNSB to induce microbial inhibition, how widespread this genetic ability is, and whether such compounds might contribute to their selective growth. To achieve these goals, PNSB genomes were mined to determine their genomic potential for antimicrobial biosynthesis and agar spot assays were conducted. Competition experiments were finally performed in liquid co-cultures to explore the potential contribution of antimicrobials on microbial selectivity.

Results and discussion

Mining of PNSB genomes uncovers potential for antimicrobial peptides production

The 166 publicly-available PNSB genomes were mined to uncover their potential to produce secondary metabolites (including antimicrobials) using antiSMASH and BAGEL. A high frequency was observed through antiSMASH for three classes of antimicrobial compounds or enzymes, namely bacteriocins, non-ribosomal peptide synthetases and type 1 polyketide synthases (Figure 1). As expected, a high frequency of terpenes (94%), a carotenoid precursor required for phototrophic growth and homoserine lactone clusters, signalling molecules used in quorum sensing, were detected (Schuster *et al.* 2013). Bacteriocins, a group of ribosomally synthesized peptides and proteins with antimicrobial properties (Gálvez *et al.* 2007), were detected in 44% of the PNSB genomes. Compared to conventional antibiotics, bacteriocins often target bacteria closely related to the producing microorganism (Shelburne *et al.* 2007). All bacteriocins are bactericidal but the mechanism of action differs, with inhibition of cell wall synthesis or pore formation in the cellular membrane being the most common (Cotter *et al.* 2005). Next to bacteriocins, non-ribosomal peptide synthetases were also detected in 36% of the PNSB genomes. These compounds are enzymes that construct a wide range of naturally active compounds, like antibiotics and immunosuppressive agents (Challis *et al.* 2000).

Once the genomic potential for secondary metabolite production was uncovered through antiSMASH, the PNSB genomes were explored in detail using BAGEL (Figure 2). This bioinformatics tool mines the genome for ribosomally synthesized and post-translationally modified peptides (RiPPs). According to the algorithm of BAGEL, the highest frequency of RiPPs in PNSB are sactipeptides, also known as sactibiotics due to the antimicrobial nature of these compounds (Himes *et al.* 2016),

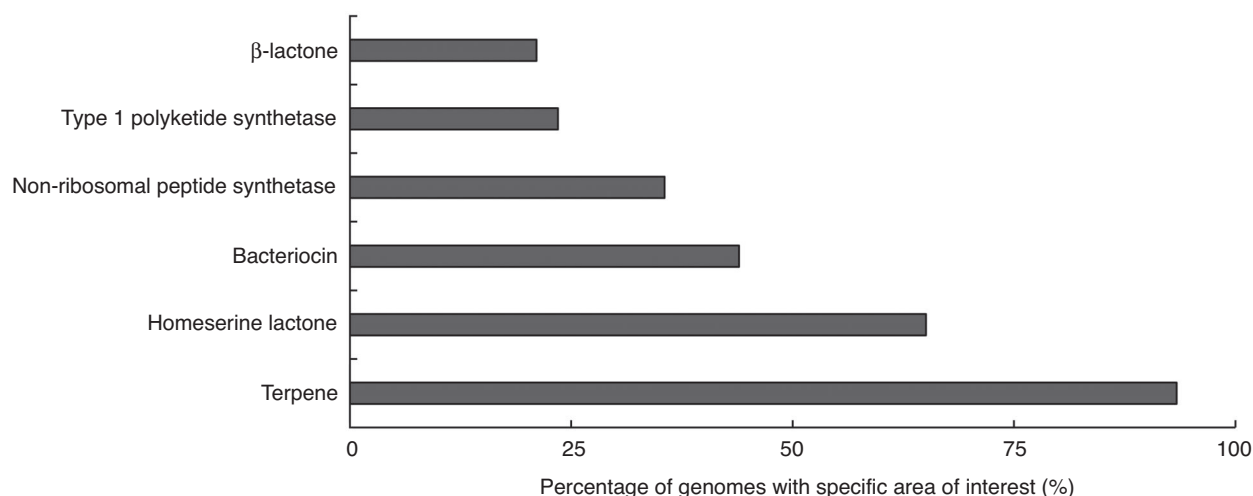


Figure 1 Areas of interest for secondary metabolites with an occurrence over 20% mined in purple non-sulphur bacteria genomes through anti-SMASH. The dominant antimicrobial encoding regions were related to bacteriocins, non-ribosomal peptide synthetases and type 1 polyketide synthetases.

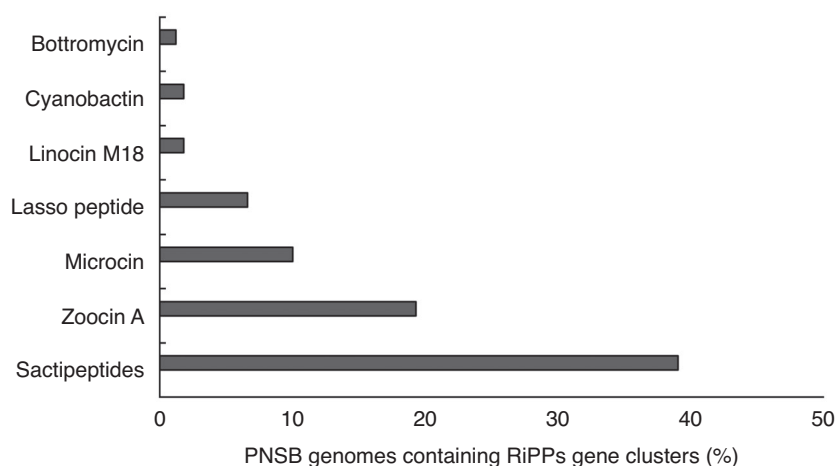


Figure 2 Analysis of 166 publicly-available purple non-sulphur bacteria genomes using the BACTERIOCIN GENE mining tool (BAGEL) showing ribosomally synthesized and post-translationally modified peptides (RiPPs) identified based on biochemical structures and bacteriocins identified through a core peptide hit.

followed by microcins, a collective term for antimicrobial peptides smaller than 10 kDa and lasso peptides, characterized by their knot-like structure (Hegemann *et al.* 2013). *In vivo* research determining the antimicrobials produced by PNSB is limited. Chumpol *et al.* (2019) have done a preliminary attempt to unravel the structure of the antimicrobials produced by *Rb. sphaeroides*. Chumpol *et al.* (2019) have suggested that the compound is a cationic molecule containing NH_2 groups. Lee *et al.* (2009) have also studied the PNSB strain *Rb. capsulatus* ATCC 17016 for bacteriocin production and found that the molecular weight was 14 kDa.

Overall, 59% of the publicly-available PNSB genomes contained antimicrobial encoding regions. On the genus level, differences in the presence of antimicrobial-related gene clusters were detected (Table 1). In particular, strains belonging to the genus *Rhodospirillum rubrum* showed the highest number of areas of interest (82%). This variation in antimicrobial gene clusters can partially be attributed to the high prevalence of known bacteriocin zoocin A in 66% of *Rhodospirillum rubrum* genomes. This indicates that there is a strong conserved relation between the *Rhodospirillum rubrum* genus and the number of antimicrobial gene clusters (i.e. 1.97 antimicrobial gene clusters per genome; Table 1). The

Table 1 Percentage of strains within one genus having gene clusters with potential antimicrobial activity identified by the bioinformatics tool BACTERIOGIN GENE mining tool (BAGEL; column 1). Number of gene clusters with potential conserved antimicrobial activity per genome sorted per genus (column 2). Only genera with a large enough sample size ($n > 14$) were used, namely *Rhodoferrax* ($n = 39$), *Rhodopseudomonas* ($n = 24$), *Rhodobacter* ($n = 49$) and *Rhodovulum* ($n = 14$)

Genus	Genomes containing gene clusters (%)	Gene clusters per genome (–)
<i>Rhodoferrax</i>	82	1.97
<i>Rhodopseudomonas</i>	58	0.88
<i>Rhodobacter</i>	47	0.55
<i>Rhodovulum</i>	29	0.43

analysed sample sizes were not large enough to draw conclusions on species or strain level, yet variation was shown.

Agar spot assays show inter- and intra-species microbial inhibition by PNSB

Once the genotypical potential was established, experimental verification of antimicrobials production by PNSB was required. A combination of PNSB obtained from culture collections and isolated PNSB were spotted on agar plates and overlaid with a second PNSB or non-PNSB strain (i.e. indicator strain). Areas around the spotted cultures clear of growth were an indication of the inhibitory activity (i.e. inhibition zone).

Inhibition zones were detected for two PNSB strains namely, *Rps. palustris* EBE1 and *Rb. blasticus* EBR2 (Table 2); the former against *Rps. palustris* EBR1 and *Escherichia coli* MG1655 K12 and the latter against the *Rps. palustris* EBE1. These inhibition zones could be due to several mechanisms including growth limitations due to competition of nutrients, changes in pH by the production or consumption of organic acids, inhibition by

undissociated organic acids and inhibition by antimicrobial compounds (Hibbing *et al.* 2010). Competition of nutrients can probably be excluded because a nutrient-rich medium was used for the agar plate experiments (section ‘Inocula and medium’). The pH of the agar can change during the experiment due to photoassimilation of acetate by PNSB. This might also result in inhibition. pH changes were probably not the driving mechanism for inhibition because the final pH values of the inhibition zones (pH 8.0–8.5) were similar to the rest of the plate. If pH would have been the driving factor, inhibition would have occurred independently of the spotted strain because all PNSB consume acetate. Inhibition by undissociated organic acids was also not possible because the carbon contained in the medium (acetate, propionate and butyrate) cannot be converted further through secondary fermentation reaction without H₂ or ethanol present (Agler *et al.* 2011). Inhibition was, therefore, likely due to antimicrobial activity. The type of antimicrobial secreted cannot be deduced, yet the inhibition is specific to certain strains. This is a key indicator for bacteriocin activity, which is often strain-specific (de Freire Bastos *et al.* 2015). This is also in line with the genome mining results (section ‘Mining of PNSB genomes’) and the study by Lee *et al.* (2009), who have shown bacteriocin production by a *Rb. capsulatus* strain.

During the agar spot assays, absence of competitive inhibition was observed for *Rps. palustris* EBE1 and *Rb. blasticus* EBR2 against some indicator strains. Variation in the size of the inhibition zone against *Rps. palustris* EBE1 ($p < 0.05$) was also detected (Table 2). The actual reason for the absence of inhibition or variation in the size of the inhibition zone cannot be deduced, yet research shows that multiple causes may be at the root of this. Resistance to bacteriocins, for example, is often found within the bacteriocin gene cluster (de Freire Bastos *et al.* 2015). Some bacteriocins, for example, have a very narrow spectrum of activity (Rea *et al.* 2010), possibly not targeting the

Table 2 Inhibition zone for the agar spot assays, for each strain combination showing averages \pm standard deviation. ($n = 9$). ‘–’ No inhibition observed. Spotted strain: microbe with potential antimicrobial activity; Indicator strain: microbe potentially sensitive towards antimicrobials produced by spotted strain

Indicator strain → Spotted strain ↓	<i>Rhodopseudomonas palustris</i> EBR1	<i>Rhodopseudomonas palustris</i> EBE1	<i>Rhodobacter blasticus</i> EBR2	<i>Rhodospirillum rubrum</i> S 1 H	<i>Escherichia coli</i> MG1655 K12
<i>Rhodopseudomonas palustris</i> EBE1	5.7 \pm 0.3 ^a	–	–	–	1.5 \pm 0.0 ^b
<i>Rhodobacter blasticus</i> EBR2	–	5.1 \pm 0.5 ^a	–	–	–
<i>Rhodopseudomonas palustris</i> EBR1	–	–	–	–	–
<i>Rhodospirillum rubrum</i> S 1 H	–	–	–	–	–

indicator strain. For Gram-positive bacteria, it was shown that the resistance to bacteriocins may either be innate or acquired and that the mechanisms are complex, even differing amongst strains of the same species (de Freire Bastos *et al.* 2015). The properties of the inhibiting compound might also be different. This may result in a different diffusion distance and might contribute to a different size in the inhibition zone (Gravesen *et al.* 2002). Another cause might be a difference in sensitivity of the indicator strain, with one target being more susceptible to the antimicrobial compound(s) than others. Finally, a different compound might be the cause of inhibition possibly not triggering bacteriocin production as heavily as other microbes (Maldonado-Barragán *et al.* 2013).

Exploring antimicrobial enhanced selectivity in liquid cultures

Liquid culture competition experiments

The spot assay and previous reports in the literature show that some PNSB strains possess antimicrobial activity on agar plates. This, however, does not imply similar behaviour in mixed photobioreactors with a suspended PNSB community. Experiments were, therefore, conducted to verify whether the inhibiting PNSB strains of the spot assays have a competitive advantage in liquid cultures (proxy for photobioreactor) and contribute to selective growth. The maximal specific growth rate of each strain was first determined under the same conditions as the competition experiment and was used to predict the theoretical abundance of each strain in case no inhibition occurs (Supporting Information S1).

According to the agar spot assays, the slower-growing *Rb. blasticus* EBR2 ought to inhibit the growth of *Rps. palustris* EBE1, yet no inhibition was observed (Figure 3). The relative abundance of *Rb. blasticus* EBR2 (19%) was lower than expected through the model (28%). During the agar spot assay (section 'Agar spot assay'), the spotted PNSB strains (i.e. antimicrobial producer) had no initial competition, allowing for the cost versus growth tradeoff to be less impactful (Riley and Gordon 1999). The antimicrobial compound concentration will be relatively high near the producing strain and, thus, more impactful. During the liquid culture experiments, however, both the indicator strain and the antimicrobial-producing strain were initially present in low biomass concentrations (0.025 g volatile suspended solids; VSS L⁻¹), making the energy cost of bacteriocin production a greater factor, as was shown by microbial ecology studies (Abrudan *et al.* 2012). Moreover, the antimicrobial-producing strain will benefit less from the antimicrobial-induced inhibition as the concentration is rapidly diluted due to mixing and diffusion. Lower antimicrobial compound concentration

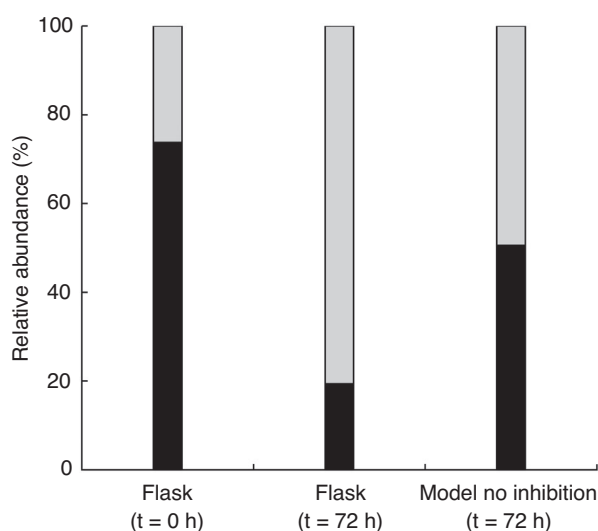


Figure 3 Liquid culture competitive growth experiment showing the relative abundance of *Rhodobacter blasticus* EBR2 (■) and the *Rhodopseudomonas palustris* EBE1 (□) at time (t) 0 h, 72 h and through the growth model. The PNSB abundancies in the growth model were calculated using the equation $N = N_0 * e^{\mu_{max} * (t_{final} - t_{lag})}$ without considering competitive inhibition. The abundance was corrected for 16S gene copies per genome.

will result in a lower effectiveness and thus less inhibition than expected based on the spot assays.

Assessing the genomic potential of the inhibiting PNSB strains

The initial bioinformatic screening used genomes from online databases (section 'Mining of PNSB genomes'). The genomes of the inhibiting strains *Rb. blasticus* EBR2 and the *Rps. palustris* EBE1 used in the agar spot assays were, therefore, also analysed using antiSMASH and BAGEL.

The *Rps. palustris* EBE1 analysed through antiSMASH returned four terpene clusters, two homoserine lactone clusters and a single betalactone cluster. *Rb. blasticus* EBR2 returned hits for two terpene clusters, a homoserine lactone cluster and a polyketide synthetase cluster. These hits are not directly linked to inhibitory peptides, yet the polyketide synthetase enzyme could possibly produce antimicrobials. Follow-up screening through BAGEL identified a microcin cluster in *Rb. blasticus* EBR2. The microcin cluster contained genes likely encoding for a leukotoxin-activating lysine acyltransferase (35% match, E value 6e-14). This transferase is required for the modification and activation of leukotoxin, which are pore-forming antimicrobial compounds (Narayanan *et al.* 2002). For the *Rps. palustris* EBE1, however, no relevant cluster hits were found. This is likely the case because little research

has been performed on antimicrobials produced by PNSB. Therefore, few identified core peptides are known, allowing for only limited hits with existing databases.

Peptidomics point towards the production of a 'non-ribosomal peptide' as antimicrobial

The bioinformatic screening showed that several PNSB lineages possess the genes for the production of antimicrobials. The spot assay test showed examples of intra- and inter-species microbial inhibition. This activity suggests that the genes related to antimicrobials may have been expressed. This section aims to provide evidence that antimicrobial compounds have been produced and that those compounds are likely of peptidic nature. However, further studies are required to provide a direct link between the occurrence of the identified candidates and antimicrobial activity.

Amino acid sequences of peptides reconstructed from filtered supernatant from the competition experiment between *Rps. palustris* EBE1 and *Rps. palustris* EBR2 did not provide any hits for bacteriocins through a direct blast on the bacteriocin database. This is likely the case because this database is biased towards well-studied compounds. The *de novo* peptide sequences were also aligned through the global non-redundant protein sequence database (NCBI) and the *Rps. palustris* sequence database. From the obtained matches to known proteins, one sequence provided a match (percentage identity 100%) with a peptide directly linked to antimicrobial activity (i.e. SVLQLLR). The sequence matched with three non-ribosomal peptide synthetases (Supporting Information S2), enzymes involved in the synthesis of antimicrobial peptides (Accession number: KAF9768165; KAF4336958; WP_071803044) (Felnagle *et al.* 2008).

Outlook

This research and previous reports have shown that certain PNSB strains possess the potential to inhibit microbial competitors through antimicrobial peptides. Competitive inhibition in photobioreactors, however, will probably be more difficult due to the dilution of the antimicrobials through mixing and diffusional limitations in bioaggregates. Traditional selection phenomena such as supplying fermented carbon sources, avoiding oxygen and visible light are probably more influential in driving microbial selectivity of PNSB, especially for suspended cultures (Capson-Tojo *et al.* 2020). In PNSB aggregates, which are flocs or granules generated in upflow reactors or sequencing batch reactors (Cerruti *et al.* 2020), it might be possible that dilution of the antimicrobials will play less of a role and, thus, antimicrobial enhanced selectivity might be more important. Stegman *et al.* (2021),

for instance, studied an upflow photobioreactor and observed a uniform microbial structure in the granules. The researchers, however, attributed the uniform interior microbial community structure to the metabolic versatility of PNSB (acidogenic fermentation in the granule interior) and the utilization of extracellular polysaccharides as a carbon source. Our research was mainly set up to understand the contribution of antimicrobials in suspended PNSB systems (e.g. raceway ponds and tubular photobioreactors). Dedicated research initiatives are necessary to elucidate whether antimicrobials contribute to selectivity in floccular and biofilm PNSB systems.

The antimicrobial peptides produced by PNSB are also relevant for aquaculture. Especially for shrimp breeding, there is a lot of research progress on the use of PNSB biomass as feed ingredient and/or as probiotic (Chumpol *et al.* 2018; Chumpol *et al.* 2019; Saejung *et al.* 2021; Alloul *et al.* 2021). Particularly, probiotic use of PNSB is interesting because the shrimp sector combats with outbreaks of pathogens related to *Vibrio*. These pathogens cause acute hepatopancreatic necrosis disease, which can result in up to 100% shrimp mortality (Tran *et al.* 2013). In previous research, we have shown through agar spot assays that strains of the species *Rb. capsulatus* and *Rps. palustris* can inhibit the growth of shrimp *Vibrio* pathogens (Alloul *et al.* 2021). There was, however, no enhanced survival against *Vibrio* pathogens for shrimp fed with PNSB in the *in vivo* challenge test, probably because there was no direct contact with the pathogen and PNSB species. Next to photoheterotrophy, PNSB are also able to grow anaerobic chemoheterotrophically in the dark through acidogenic fermentative conversions (Imhoff 2006). Aquaculture research should, therefore, study PNSB that can colonize the gastrointestinal tract of shrimp and, thus, provide potentially better protection against pathogens.

Materials and methods

Bioinformatic screening for bacteriocins and ribosomally synthesized peptides

A set of 166 reference PNSB genomes, obtained from Database Resources of the National Center for Biotechnology Information (NCBI), have been mined to uncover their potential to produce secondary metabolites (Genbank Assembly Accessions see Supporting Information S3). PNSB strains belonging to 28 recognized PNSB genera were included in the database except for *Roseospira*, *Phaeovibrio*, *Rhodobaculum*, *Rubribacterium*, *Rhodothalassium*, *Rhodocyclus* and *Rubrivivax*. The online secondary metabolite prediction tool antiSMASH was used to mine the genomes (Blin *et al.* 2019). The 'strict' setting was selected in the web client to obtain well-

defined clusters containing all required functional parts for a specific secondary metabolite. The FASTA files of the genomes were uploaded directly to antiSMASH, which uses GLIMMERHMM (Majoros *et al.* 2004) and Prodigal (Hyatt *et al.* 2010) to identify the putative bacterial gene clusters based on hidden Markov motifs (Medema *et al.* 2011). The feature KnownClusterBlast was also enabled, which automatically screened the identified putative gene clusters against the MIBig database to provide hits against known biosynthetic gene clusters (Medema *et al.* 2015). The number of copies of each gene cluster in a genome was then calculated.

To test whether PNSB have the genetic ability to produce bacteriocins or ribosomally synthesized and post-translationally modified peptides (RiPPs), the 166 PNSB genomes were mined using the bacteriocin prediction tool BAGEL (van Heel *et al.* 2018).

Inocula and medium

Four PNSB strains were used during the experiments: (i) *Rhodospirillum rubrum* S 1 H, (ii) *Rps. palustris* EBE1 obtained from a local pond based on the isolation protocol of Alloul *et al.* (2019) and two strains isolated by Ceruti *et al.* (2020) namely (iii) *Rps. palustris* EBR1 and (iv) *Rb. blasticus* EBR2. The reference strain *Escherichia coli* MG1655 K12 was used as a proxy for non-PNSB microorganisms. Growth conditions for the pre-cultivation are described in the next section 'Agar spot assay'.

A synthetic cultivation medium containing a mixture of volatile fatty acids at a pH of 7 was used for all experiments. The medium contained 0.9 g l⁻¹ acetate, 0.7 g l⁻¹ propionate, 0.5 g l⁻¹ butyrate, 0.8 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgCl₂·6H₂O, 0.1 g l⁻¹ CaCl₂·2H₂O, 0.7 g l⁻¹ Na₂SO₄, 1.2 g l⁻¹ NH₄Cl, 1.0 g l⁻¹ NaCl, and 0.3 g l⁻¹ NaHCO₃. Volumes of 0.001 l of trace elements and 0.001 l vitamin solutions, based on the composition given in Imhoff (2006), were also added per litre of medium. For the spot assays, 15 g l⁻¹ agar was added to the medium. *E. coli* was cultured aerobically in Lysogeny broth.

Agar spot assay

For the antimicrobial spot assays, PNSB cultures were first phototrophically pre-cultivated in 0.100-l infusion flasks on a shaker at 30°C (agitation speed 150 rpm). Light was provided with two halogen lamps on each side (light intensity 60 W m⁻²). The light was filtered for wavelengths above 700 nm through infrared transmission filters (Bay Plastics Ltd., United Kingdom). The headspace of each flask was flushed with argon to create an anaerobic environment. Agar plates were then spotted with 2 µl

liquid PNSB culture and incubated in an atmosbag (SigmaAldrich, the Netherlands). The atmosbag was flushed with nitrogen gas to create an anaerobic environment. A halogen lamp was placed outside the bag. White light was filtered to supply infrared wavelengths above 700 nm through an infrared transmission filter, resulting in an irradiance intensity of 40 W m⁻² at the surface of the bag (pyranometer, Kipp & Zonen, the Netherlands). After 5 days, the plates were overlaid with 0.005 l of soft agar (at 5 g l⁻¹) containing another diluted PNSB strain or *E. coli* at 0.05 g l⁻¹ VSS. 1 g VSS l⁻¹ corresponds with approximately 1.2–3.6 viable cells × 10¹¹ l⁻¹ (Degenaar *et al.* 2000). Optical density in relation to microbial biomass can be found in Supporting Information S4.

Inhibition zones were measured from the edge of the PNSB colony until the end of the zone once growth was visible on the overlay agar (after *c.* 5 days). Samples of agar were dissolved in distilled water in a 1 : 1 mass ratio for pH measurement. Standard methods were used to analyse the concentrations of total suspended solids and VSS in liquid cultures (Greenberg *et al.* 1992).

Microbial competition experiment in liquid cultures

The microbial competition experiments aimed to explore the potential contribution of antimicrobials on the selective growth of PNSB. Maximal specific growth rates were first determined for all PNSB strains by incubating the cultures in 0.100-l flasks at 0.05 g VSS l⁻¹ under anaerobic conditions (setup see section 'Inocula and medium'). The absorbance at 660 nm was measured to construct growth curves (DR3900, Hach, Germany). For the actual competition experiment, co-cultures of two different PNSB strains inoculated each at 0.025 g VSS l⁻¹ were performed in 0.100-L flasks and incubated anaerobically in the light (setup see section 'Inocula and medium'). The experiment was halted after 72 h. This time was selected to ensure that all strains reach the stationary phase. Initial and final samples were taken and frozen at -20°C for further analysis.

Microbial community analysis and whole genome sequencing

To study the effect of antimicrobials on microbial selectivity, PNSB abundancies of the liquid culture competition experiment had to be compared to a simple growth model, which does not include competitive inhibition. The pools of genomic DNA of the initial and final co-cultures were extracted using the DNeasy UltraClean microbial extraction kit according to the manufacturer's instructions (Qiagen, Venlo, the Netherlands). The wetlab and dry-lab workflows for the 16S rRNA gene sequencing

were performed according to the protocol described by Alloul *et al.* (2021). In brief, the V3–V4 hypervariable region of the bacterial 16S rRNA gene pool of the DNA extracts was amplified by polymerase chain reaction (PCR) using the pair of 341f/806r primers before sequencing of PCR products using a HiSeq 2500 sequencer (Illumina) at Novogene (United Kingdom). The data have been deposited with links to BioProject accession number PRJNA807846 in the NCBI BioProject database. These relative abundances of the PNSB strains have been corrected for the number of 16S rRNA gene copies per strain using the average from comparable genomes on ribosomal RNA operon database (Stoddard *et al.* 2015). Final relative abundances of both strains were predicted from the initial relative abundances, assuming no competitive inhibition ($N = N_0 * e^{\mu_{\max} * (t_{\text{final}} - t_{\text{lag}})}$; N : final biomass concentration; N_0 : initial biomass concentration; μ_{\max} : maximal specific growth rate; t_{lag} : lag-phase) and using the earlier obtained maximal specific growth rates in pure cultures (see previous section ‘Microbial competition experiment’).

The extraction protocol was also used to obtain DNA of *Rps. palustris* EBE1 and *Rb. blasticus* EBR2 for whole genome sequencing. High-throughput genome sequencing was carried out on the Illumina platform HiSeq 2500 at Novogene (United Kingdom). The resulting paired read libraries were uploaded and processed on PATRIC, a platform for the analysis of microbial sequencing data (Brettin *et al.* 2015). The quality of the reads was checked using the FastQC pipeline and adaptors were trimmed using the trim option. Afterwards, genome assembly was executed using the genome assembly service, specifying the Illumina platform and the auto assembly strategy option. The resulting FASTA file for the pond isolate was then subjected to taxonomy identification.

Peptidomics

Peptidomics was conducted to search for peptide fragments of bacteriocin compounds or synthetases. The final broth of the competition with the co-culture *Rps. palustris* EBE1 and *Rps. palustris* EBR1 was used. Biomass was removed through centrifugation at 10 g for 15 min and filtered using a 10 kDa cutoff filter. Free peptides present in the supernatant were enriched using OASIS HLB solid-phase extraction cartridges (Waters, UK) and dried using a speed vacuum concentrator. The extract was solubilized in LC–MS grade H₂O containing 3% acetonitrile plus 0.01% trifluoroacetic acid. An aliquot of approximately 30% of the extract was analysed using a one-dimensional shotgun proteomics approach (Kleikamp *et al.* 2021). Briefly, the sample was analysed using a nano-liquid-chromatography system (EASY nano-LC 1200) equipped

with an Acclaim PepMap RSLC RP C18 separation column (50 $\mu\text{m} \times 150 \text{ mm}$, 2 μm) and a QE plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany). The flow rate was maintained at 350 nl min^{-1} over a linear gradient from 5% to 30% solvent B over 40 min, and finally to 60% B over 15 min, followed by back equilibration to starting conditions. Solvent A was LC/MS grade H₂O containing 0.1% formic acid and solvent B consisted of 80% acetonitrile in LC/MS grade H₂O and 0.1% formic acid. The Orbitrap was operated in data-dependent acquisition mode acquiring peptide signals from 385–1250 m/z at 70 K resolution with a maximal injection time of 100 ms and an automatic gain control (AGC) target of 3e6. The top 10 signals were isolated at a window of 2.0 m/z and fragmented using a normalized collision energy of 28. Fragments were acquired at 17 K resolution with a maximal injection time of 75 ms and an AGC target of 2e5. Mass spectrometric raw data were analysed against the proteome sequence database from *Rps. palustris* (UniprotKB, Taxonomy ID: 1076) using PEAKS Studio X (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) allowing for 20 ppm parent ion and 0.02 m/z fragment ion mass error, 2 missed cleavages, methionine oxidation and N/Q deamidation as variable modifications. Peptide spectrum matches were finally filtered against a 1% false discovery rate. Alternatively, signals were de novo sequenced using PEAKS Studio X (Bioinformatics Solutions Inc.), allowing oxidation and deamidation as variable modifications. Only the top sequence hit was considered for further analysis using sequence alignment by BLASTP (protein–protein BLAST). Sequences were aligned against the global non-redundant protein sequence database or the *Rps. palustris* sequence database (Taxonomy ID: 1076), using default parameters for short sequences (NCBI, 1988). The hits against the global non-redundant protein sequence database as well as those against the *Rps. palustris* sequence database were screened by name for antimicrobial compounds. Unidentified and hypothetical proteins have been explored on NCBI to search for identified structures.

Statistical analyses

The post-hoc pairwise comparisons using the Tukey’s range test and the analysis of variance test were conducted for multiple comparisons. The Shapiro–Wilk normality test and Levene’s test were used to test normality and homogeneity of variances, respectively. The non-parametric Kruskal–Wallis rank sum test and posthoc pairwise comparisons using the Mann–Whitney U test (p -values were adjusted using the Benjamini–Hochberg correction) were performed if normality was rejected. In case of unequal sample size or if homoscedasticity was rejected, the Welch’s

t-test was conducted. A significance level of $p < 0.05$ was chosen. All analyses were performed in R (ver. 4.1.3) using RStudio (RStudio®, Boston, MA) for Windows.

Acknowledgements

The authors kindly acknowledge (i) the Start-Up Grant of the TU Delft Department of Biotechnology (D.G.W.), (ii) the project 'Saraswati 2.0' (821427) funded by the European Union's Horizon 2020 Research and Innovation program on EU-India water co-operation, for financial support of A.A. between the University of Antwerp and the TU Delft, (iii) the project 'PurpleRace' (40207) funded by IOF (Industrieel onderzoeksfonds) from the University of Antwerp for financial support of A.A., and (iv) the Research Foundation Flanders (Fonds Wetenschappelijk Onderzoek - Vlaanderen) for supporting A.A. with a postdoctoral fellowship (12W0522N).

Authors contributions

A.A. and D.G.W. designed the experiments, analysed the data and wrote the paper. W.V.K. collected the samples, analysed the data, performed the experiments and supported the writing process. M.C. supported the sample collection and helped write the paper. S.W. helped with the bioinformatic analysis. M.P. was involved in the proteomic and data analysis. All contributing authors have read and approved the final version of the manuscript.

Conflict of Interest

No conflict of interest declared.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting Information S1 Maximal specific growth rate of purple non-sulphur bacteria strains.

Supporting Information S2 Sequence alignment of the 'SVLQLLR' peptide fragment.

Supporting Information S3 Genbank Assembly Accession of purple non-sulphur bacteria genomes.

Supporting Information S4 Microbial biomass in relation to optical density.

Supporting Information S5 Liquid culture competition experiments.