environmental microbiology reports

Environmental Microbiology Reports (2022) **14**(1), 130–137 doi:10.1111/1758-2229.13030

Brief Report

Mutualistic relationship between *Nitrospira* and concomitant heterotrophs

Chiho Murakami,[1,2,3†] Koshi Machida,[4] Yoichi Nakao,[4] Tomonori Kindaichi,[1] Akiyoshi Ohashi[1] and Yoshiteru Aoi[2,3*]

[1]*Department of Civil and Environmental Engineering, Graduate School of Engineering Hiroshima University, Hiroshima, Japan.*

[2]*Unit of Biotechnology, Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan.*

[3]*Institute for Sustainable Science and Development, Hiroshima University, Hiroshima, Japan.*

[4]*Waseda Research Institute for Science and Engineering, Waseda University, Tokyo, Japan.*

Summary

Nitrifying chemoautotrophs support the growth of diverse concomitant heterotrophs in natural or engineered environments by supplying organic compounds. In this study, we aimed to investigate this microbial association, especially (i) to distinguish whether the relationship between nitrifying chemoautotrophs and heterotrophs is commensal or mutualistic, and (ii) to clarify how heterotrophs promote the growth of autotrophic nitrite-oxidizing bacteria (*Nitrospira*). Pure cultured *Nitrospira* (*Nitrospira* sp. ND1) was employed in this study. Heterotrophs growing with metabolic by-products of *Nitrospira* as a sole carbon source were isolated from several environmental samples and used to test the growth-promoting activity of *Nitrospira*. Furthermore, liquid chromatography–mass spectrometry analysis was conducted to evaluate how heterotrophs consumed chemical compounds produced by *Nitrospira* and newly produced during co-cultivation. Notably, *Nitrospira* growth was stimulated by co-cultivation with some heterotrophs and the addition of spent media of some strains, suggesting that not only heterotrophs but also *Nitrospira* received benefits from their mutual co-existence. Furthermore, the data suggested that some of the growth-promoting heterotrophs provided as-yet-unidentified growth-promoting factors to *Nitrospira*. Overall, *Nitrospira* and heterotrophs thus appear to exhibit a mutualistic relationship. Such mutualistic relationships between autotrophs and heterotrophs would contribute to the stability and diversity of microbial ecosystems.

Received 9 November, 2020; accepted 10 November, 2021. *For correspondence. E-mail yoshiteruaoi@hiroshima-u.ac.jp; Tel. +81-82-424-7892; Fax +81-82-424-7000.

†**Present address:** Chiho Murakami, Faculty of Pharmacy, Yasuda Woman's University, Hiroshima, Japan.

Introduction

Nitrifying chemoautotrophs (ammonia and nitrite oxidizers) play important roles in the biological nitrogen cycle and wastewater treatment processes. Nitrifying chemoautotrophs constitute primary producers in natural environments as they assimilate inorganic carbon and release organic carbon compounds to microbial ecosystems. In nitrifying microbial ecosystems, concomitant heterotrophs are always observed representing approximately 50% of microbial communities even under conditions lacking organic carbon supply (Rittmann *et al*., 1994; Okabe *et al*., 1999; Okabe *et al*., 2002; Kindaichi *et al*., 2004; Martiny *et al*., 2005; Fujitani *et al*., 2013). The growth of diverse heterotrophs has been shown to be supported by nitrifying chemoautotrophs through the utilization of soluble microbial products derived from metabolic by-products and decaying biomass (Noguera *et al*., 1994; Rittmann *et al*., 2002; Kindaichi *et al*., 2004; Okabe *et al*., 2005; Matsumoto *et al*., 2010). Nevertheless, although the in situ ecophysiological interactions between nitrifying chemoautotrophs and heterotrophs based on cross-feeding have been well studied using cultivation-independent approaches, a better understanding of the ecophysiological interactions between nitrifying chemoautotrophs and heterotrophs is expected to clarify the factors that control the efficiency and stability of nitrification.

However, because of the difficulty in cultivating nitrifying microorganisms, analysis of the microbial associations

based on pure cultures has been limited. For example, although it is well-established that the growth of concomitant heterotrophs completely depends on nitrifying chemoautotrophs in the ecosystems, it remains unclear how the nitrifying chemoautotrophs benefit in turn from such dependent heterotrophs.

Notably, some heterotrophs exhibit the potential to promote the growth of ammonia-oxidizing bacteria or archaea (Keluskar *et al*., 2013; Sedlacek *et al*., 2016; Bayer *et al*., 2019), whereas the association with nitrite-oxidizing bacteria has not been reported. The key nitrite oxidizers *Nitrospira* may represent candidate species able to benefit from microbial associations as they are widespread both in the natural ecosystem and engineered environments and play a significant role in the nitrogen cycle (Wagner *et al*., 1996; Burrell *et al*., 1998; Hovanec *et al*., 1998; Juretschko *et al*., 1998; Bartosch *et al*., 1999). Nevertheless, despite their importance, a comprehensive understanding of their physiological properties has been limited because they generally resist cultivation, especially into pure cultivation (Nowka *et al*., 2015; Daims *et al*., 2016).

In this study, we tested the growth promotion effect of heterotrophs on nitrifying chemoautotrophs, especially *Nitrospira*, to clarify the microbial association; in particular, to (i) distinguish whether the relationship between nitrifying chemoautotrophs and heterotrophs is commensal or mutualistic, and (ii) clarify how heterotrophs promote the growth of *Nitrospira*. For this purpose, pure cultured *Nitrospira* (*Nitrospira* sp. ND1) belonging to sublineage I was employed in this study (Fujitani *et al*., 2014).

The effects of co-cultivation with several selected concomitant heterotrophs grown with the spent (conditioned) medium of *Nitrospira* as a carbon source on the growth of *Nitrospira* were analysed by focusing on specific growth rate and lag time. In addition, the spent medium of each heterotroph was applied to pure *Nitrospira* culture to clarify how growth-promoting heterotrophs affect the growth of *Nitrospira*; i.e., to distinguish whether such strains secrete growth-promoting factors (GPF). Finally, the fate of chemical compounds during the cultivation of heterotrophs with the spent medium of *Nitrospira* culture were comparatively analysed among the strains using liquid chromatography–mass spectrometry (LC–MS) to evaluate how heterotrophs consumed compounds produced by *Nitrospira* and produced during the cultivation.

Results and discussion

Isolating and pre-screening candidate heterotrophs

A total of 84 strains of heterotrophs were isolated from three different samples using the culture media included spent medium from *Nitrospira* culture (NSM) (Fig. 1). This medium contains 15.9 $\pm$ 6.3 mg-C L^{-1} (S.D.) of dissolved organic carbon derived from metabolic by-products of

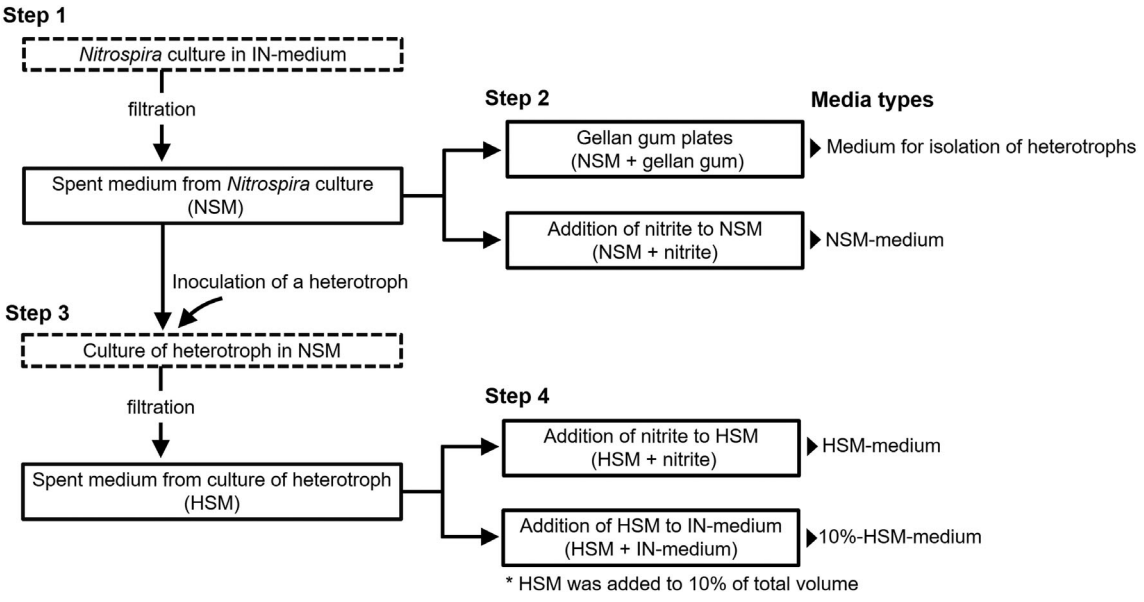

Fig. 1. Flowchart of procedure for the preparation of culture media used in this study. Step 1: the spent medium from *Nitrospira* culture (NSM) was prepared by filtration of *Nitrospira* culture after cultivation with IN-medium. Step 2: NSM was employed for two types of culture medium; (i) the medium for isolation of heterotrophs was prepared as gellan gum plates (NSM with gellan gum); (ii) NSM-medium was prepared by adding nitrite to NSM. Step 3: each tested heterotroph was cultivated in NSM. Then, the spent medium from each culture of heterotroph (HSM) was prepared by filtration. Step 4: HSM was employed for two types of culture medium; (i) HSM-medium was prepared by adding nitrite to HSM; (ii) 10% HSM-medium was prepared by adding HSM to IN-medium (HSM was added to IN medium to 10% of the total volume).

Nitrospira as a sole carbon source. Among the 84 strains, 15 strains that promoted the nitrite oxidation activity of *Nitrospira* were selected based on the pre-screening test. The type strain, *Pseudomonas putida* (NBRC14164) was also employed as strain No.0, as it has been reported to enhance the growth of several autotrophic bacteria such as ammonia and methane-oxidizing bacteria (Ho *et al*., 2014; Sedlacek *et al*., 2016). Finally, a total of 16 strains were employed for further analysis to investigate the effect of heterotrophs on the nitrite oxidation activity of *Nitrospira* (Table 1).

Effect of heterotrophs on the growth activity of Nitrospira

We tested the effect of heterotrophs grown with NSM-medium (Fig. 1) as the sole carbon source on the nitrite oxidation activity of *Nitrospira* under two different conditions. These included (i) co-cultivation with tested heterotrophs in NSM-medium and (ii) pure cultivation in the spent medium from heterotroph culture (HSM-medium) derived from each tested heterotroph (Fig. 1).

Figure S1A and C shows representative time-course changes in nitrites during co-cultivation with heterotrophs (strains No.0, No.4 and No.71), and pure cultivation in HSM-media derived from each heterotroph respectively. The results of co-culture assays indicated that three heterotrophs (No.0, No.4 and No.71) apparently elevated the nitrite oxidation activity of *Nitrospira* (equivalent to the growth) (Table 1, Figs S1B and S2). On the other hand, 9 strains of heterotrophs (No.0, No.1, No.4, No.14, No.38, No.49, No.55, No.61 and No.71) elicited a positive effect on the growth of *Nitrospira* through their HSM-medium (Table 1, Figs S1D and S2).

Figure S3 shows the effect of co-cultivation with heterotrophs (Fig. S3A) or pure cultivation in HSM on the growth characteristics of *Nitrospira* (Fig. S3B). The specific growth rate of *Nitrospira* was distinctly higher in co-cultivation with three strains, No.0, No.61 and No.71 (p-values < 0.05, t-test) than that in pure culture. The lag time was distinctly shortened when *Nitrospira* was co-cultivated with strains No.0, No.4, No.49 and No.55 ($p < 0.05$) compared with that in pure culture. In contrast, the lag time was extended when *Nitrospira* was co-cultivated with strains No.11, No.74 and No.40. The specific growth rate of *Nitrospira* was lower in co-cultivation with strain No.38 than that in pure cultivation (Table 1, Fig. S3B). The specific growth rate of *Nitrospira* was distinctly higher when growing in the HSM from strain No.4 ($p < 0.05$). The relative lag time of *Nitrospira* was shortened when growing in HSMs from strains No.1, No.4, No.49, No.55, No.0, No.61 and No.71 ($p < 0.05$).

The results of co-culture assays indicated that three heterotrophs (No.0, No.4 and No.71) promoted the growth of *Nitrospira* (Table 1, Fig. S1A), whereas a larger number (9 strains) of heterotrophs (No.0, No.1, No.4, No.14, No.38, No.49, No.55, No.61 and No.71) elicited a positive effect on the growth of *Nitrospira* through their HSM-medium (Table 1, Fig. S1D). There can be several reasons for this difference as follows: (i) the competition for dissolved oxygen (Ohashi *et al*., 1995; Okabe

Table 1. Test strains used for assays and effect of those strains on the growth of *Nitrospira*.

Strain No.	Source	Closest cultured relative	Similarity (%)[a]	Accession No.	Co-culture			HSM			10% HSM		
					r.a.	μ	λ	*r.a.*	μ	λ	*r.a.*	μ	λ
0	CC	*Pseudomonas putida*	100	AP013070	+	+	+	+	±	+	+	+	+
4	EC1	*Mycobacterium grossiae*	99	CP043474	+	+	+	+	+	+	±	±	±
71	EC2	*Shinella fusca*	99	KM210268	+	+	±	+	±	+	+	+	+
49	EC2	*Pseudomonas putida*	100	CP026115	+**	±	±	+	±	+	+	+	+
55	EC2	*Ensifer adhaerens*	100	KT229738	+**	±	±	+	±	+	+	+	+
1	EC1	*Sphingopyxis alaskensis*	99	AY509241	±	±	±	+	±	+	±	±	±
14	AS	*Mycobacterium dioxanotrophicus*	99	CP020809	±	±	±	+	±	±	±	±	±
61	EC2	*Alicycliphilus denitrificans*	100	AB908107	±	±	±	+	±	+	±	±	±
38	EC2	*Gordonia sihwensis*	100	MN880097	−	−	±	+	±	±	n.d.	n.d.	n.d.
11	EC1	*Bradyrhizobium elkanii*	100	LC515847	−	±	−	±	±	±	n.d.	n.d.	n.d.
20	AS	*Gordonia austrails*	100	MK680170	±	±	±	±	±	±	n.d.	n.d.	n.d.
39	EC2	*Shinella fusca*	99	KM210268	±	±	±	±	±	±	n.d.	n.d.	n.d.
40	EC2	*Gordonia sihwensis*	100	MN880097	−	±	−	±	±	±	n.d.	n.d.	n.d.
63	EC2	*Sphingobacterium mizutaii*	100	MK253331	±	±	±	±	±	±	n.d.	n.d.	n.d.
64	EC2	*Paludibaculum fermantans*	94	NR134120	±	±	±	±	±	±	n.d.	n.d.	n.d.
74	EC2	*Reyranella massiliensis*	99	HM048834	−	±	−	±	±	±	n.d.	n.d.	n.d.

[a]16S rRNA similarity of each strain to the closest known species.
AS, activated sludge; CC, type strain; EC1, enrichment culture I; EC2, enrichment culture II; n.d., no data; *r.a*., relative activity.
+: positive; ±: no effect; −: negative; +**: positive (large S.D.).

et al., 1996; Nogueira *et al*., 2002), (ii) the effect of physical contact between the cells on the induction of secondary metabolite (Onaka *et al*., 2011; 2015) and (iii) predatory bacteria for *Nitrospira* (Dolinšek *et al*., 2013).

Effect of low-level supplementation of HSM (10% HSM-medium) on the growth activity of Nitrospira

The positive effect of heterotrophs on the growth of *Nitrospira* through their HSM-medium suggested that some of the heterotrophs secreted chemical compounds enhancing the growth of *Nitrospira*. However, the analysis left another possibility that this positive effect might occur by degradation of toxic compounds by heterotrophs. To confirm whether the heterotrophs produce chemical compounds enhancing the growth of *Nitrospira*, the effect of low-level supplementation with HSM on *Nitrospira* growth activity was examined. *Nitrospira* was cultivated with 10% HSM-medium composed of inorganic nitrite (IN)-medium with the addition of HSM from each heterotroph to 10% of the total volume (Fig. 1).

The results showed that the nitrite oxidation activity of *Nitrospira* in the 10% HSM-media from strains No.0, No.49, No.55 and No.71 was apparently promoted whereas that from strains No.1, No.4, No.14 and No.61 did not positively affect the nitrite oxidation activity (Table 1, Fig. 2). This indicated that four HSMs from among eight tested strains apparently contained chemical compounds facilitating the growth of *Nitrospira*. Thus, these 4 strains could be classified as GPF suppliers. Conversely, the lack of growth-promoting effects from the other four strains (No.1, No.4, No.14 and No.61) was likely due to (i) the addition of 10% HSM being insufficient to promote *Nitrospira* growth (containing less amount of growth-promoting chemical compounds), or (ii) the ability of these strains to promote the growth of *Nitrospira* being a consequence of the reduction (degradation) of growth-inhibiting compounds through scavenging of the metabolic by-products derived from *Nitrospira*, as previously hypothesized (Paerl and Pinckney, 1996; Paerl *et al*., 2000; Morris *et al*., 2008; Sher *et al*., 2011; Ho *et al*., 2014).

Although nitrate, the main metabolic product of *Nitrospira* inhibits the growth itself when the concentration was over 15 mM (Fig. S4), consistent with the previous observation (Nowka *et al*., 2015), the growth promotion observed in the present study was not likely due to the reduction of nitrate by heterotrophs. This is because there was no significant difference in the concentration of nitrate between NSMs and HSMs (data not shown).

Chemical species in NSM and HSMs as analysed by LC–MS

As heterotrophs grew with organic substrates released from *Nitrospira*, it can be expected that heterotrophs have the potential to consume various types of organic compounds in NSM by assimilation or dissimilation. They would also have the potential to release different types of organic compounds into HSMs as metabolic by-products that would not be contained in NSM.

To address these possibilities, we compared the chemical compounds from 100 to 2000 kDa contained in NSM and HSMs by LC–MS and signpost analysis to evaluate how each heterotroph consumed and produced chemical compounds during the cultivation in NSM (Fig. 3A).

The analysis results revealed that each HSM exhibited a different chemical profile. Signal intensities of several compounds detected in NSM disappeared or were significantly reduced in each HSM, suggesting that they were used as substrates for the growth of heterotrophs. In particular, some chemical compounds derived from NSM ($m/z = 403.13$, 546.20, 585.15, 602.18 and 690.23; blue arrows in Fig. 3A) disappeared or exhibited significantly decreased signal intensity in all HSMs, suggesting that these chemical compounds were commonly consumed by all heterotrophs, and were probably utilized as key substrates for the growth of the tested heterotrophs.

Some HSMs contained substantially more compounds than NSM or characteristic compounds that were not detected in NSM, suggesting that heterotrophs produced diverse organic compounds accompanied by the consumption of organic compounds in NSM and consequently their spent media exhibited unique chemical profiles. Among these compounds, several common chemical compounds highly produced by GPF suppliers (No.0, No.49, No.55 and No.71) in particular were identified (Fig. 3A). However, we were unable to identify chemical compounds exclusive to HSMs from GPF suppliers. Notably, the chemical compound ($m/z = 519.08$; red arrow in Fig. 3A) was present at particularly high levels in HSMs from three strains (No.49, No.55 and No.71), suggesting this compound as a candidate for the GPF for *Nitrospira*. Although strain No.0 should also be a GPF supplier, it likely produces different chemical compounds as GPFs than those of strains No.49, No.55 and No.71.

Some organic compounds, such as amino acids, pyruvate and glucose can enhance the growth of autotrophic nitrifying organisms (Clark and Schmidt, 1966; Clark and Schmidt, 1967a,b; Pan and Umbreit, 1972; Kim *et al*., 2016; Bayer *et al*., 2021), it is likely that the growth-promoting heterotrophs provided such organic compounds for utilization as metabolic substrates. In addition, it is expected that the heterotrophs provided vitamins, siderophores or signal-like molecules that affect phenotypic

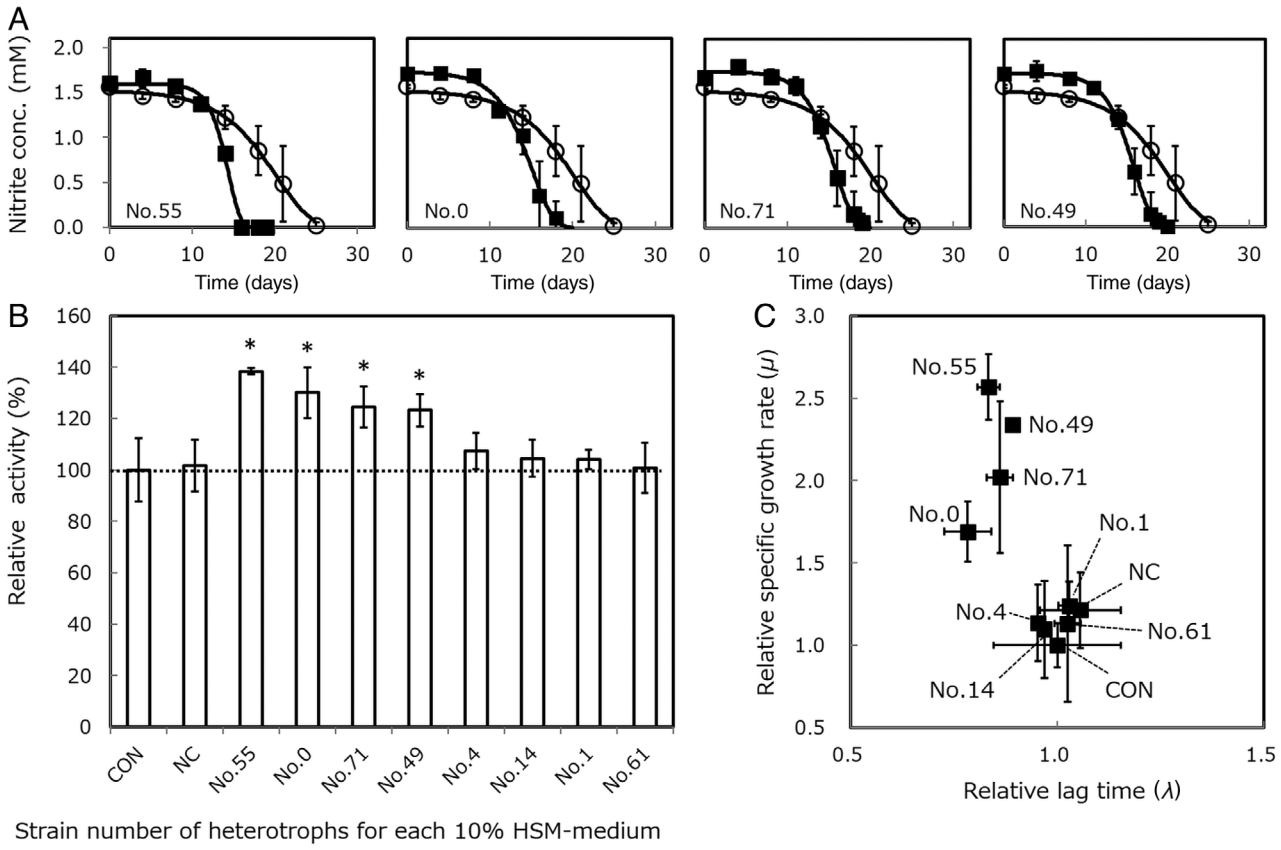

Fig. 2. The effect of cultivation of *Nitrospira* with 10% HSM-medium from each heterotroph on nitrite oxidation activity.
A. The time course change in nitrite during cultivation of *Nitrospira* with 10% HSM-medium (closed square with dashed line) and with IN-medium (closed circle). Data from cultivation with HSM-medium derived from strain No.0, No.49, No.55 and No.71 are shown.
B. Comparison of the nitrite oxidation activity of *Nitrospira* among cultivation with 10% HSM-media from each heterotroph, IN-medium (as a control, CON) and 10% NSM-medium (as a negative control, NC). The data are expressed as relative values with that with IN-medium defined as 100%. Nitrite oxidation activity was calculated based on the time required for reduction of nitrite concentration below the standard value, in the same manner as shown in Figs S2 and S3.
C. The comparison of specific growth rate (μ) and lag time (λ) of *Nitrospira* among cultivation with IN-medium, 10% NSM-medium and 10% HSM-media derived from each heterotroph. The data are expressed as relative values with that in IN-medium defined as 1.0. Error bars indicated the standard deviation of the biological triplicate. Asterisk indicates a value significantly different from the control ($p < 0.05$).

expression including growth activity (Batchelor *et al*., 1997; Burton *et al*., 2005; Keluskar *et al*., 2013).

Principal component analysis of chemical profiles revealed that the strong GPF suppliers, strains No.49, No.55 and No.71, belonged to the same cluster, whereas strain No.0 was positioned far from this cluster. Moreover, strains No.1, No.14, No.38 and No.61 belonged to the same cluster and were not grouped as strong GPF suppliers, with strain No.4 being located apart from any clusters (Fig. 3B). This further suggested that these strains enhanced the growth of *Nitrospira* differently compared with the GPF suppliers.

Significance of the mutualistic relationship between Nitrospira *and heterotrophs*

The evidence provided herein that some heterotrophs promote the growth of *Nitrospira* suggests that the relationship between autotrophs and some heterotrophs is mutualistic rather than commensal. Not only *Nitrospira* but also other nitrifying microorganisms may, therefore, exhibit a similar mutualistic relationship with various heterotrophs. In addition, the possibility remains that other tested heterotrophs would also have the potential to promote the growth of *Nitrospira* but not exhibited under the tested condition because some heterotrophs did not grow efficiently. In the present study, we utilized NSM-medium derived from the supernatant of *Nitrospira* culture for the cultivation of heterotrophs without the addition of extra carbon sources. This is to clarify whether heterotrophs could exert their potential to promote the growth of nitrifiers under the conditions in natural ecosystems where heterotrophs grow using metabolic by-products of *Nitrospira* as a sole carbon source (Fig. 1). Therefore, the results obtained in this study were considered to

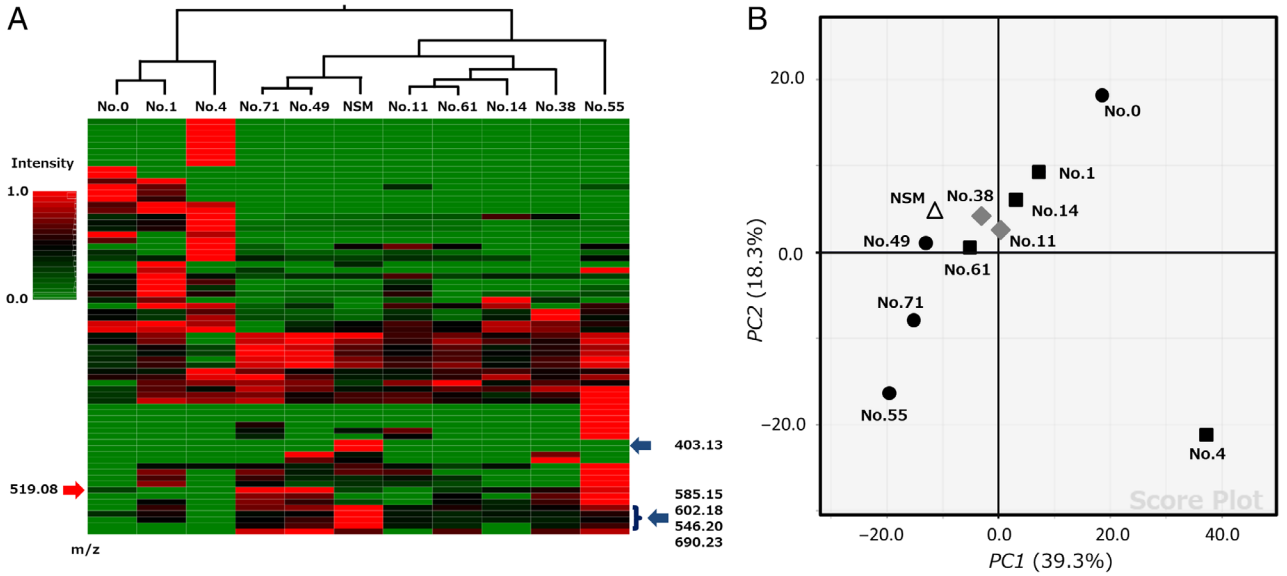

Fig. 3. Chemical profiles of the spent medium from *Nitrospira* (NSM) and each heterotroph (HSMs) analysed by LC–MS.
A. Hierarchical cluster analyses and heatmap of chemical profiles of NSM and HSMs from each heterotroph. The *x*-axis represents the type of spent medium (showing strain number or NSM), whereas the *y*-axis represents the chemical compounds in media. Green represents no difference in relative abundance and red represents maximum relative abundance. The red arrow indicates a chemical compound particularly strongly detected in HSM derived from No.49, No.55 and No.71.
B. Principal component analysis plot of chemical profiles. GPF-suppliers are represented with black closed circles (No.0, No.49, No.55 and No.71); NSM is represented with an open triangle; growth-promoting heterotrophs excluding GPF-suppliers (No.1, No.4, No.14 and No.61) are represented with closed black square; other heterotrophs (No.11 and No.38) are represented with grey square diamond.

closely reflect the actual interactions between nitrifying organisms and heterotrophs in natural ecosystems.

In the present study, we revealed that some heterotrophs supplied chemical compounds promoting *Nitrospira* growth (GPF-supplier). The results of LC–MS analysis suggested the existence of chemical compounds produced by GPF suppliers. However, these substances have not been completely characterized in the present study. Moreover, the mechanism of growth promotion might be varied among the microbial types in addition to supplying GPF but remained unclarified. Therefore, additional studies are required to obtain a comprehensive understanding of interactions based on these chemical compounds.

As it is impracticable to test every microbial type among the diverse heterotrophs in various environments, it is not clear how such diverse concomitant heterotrophs affect the growth of *Nitrospira*. However, the results obtained in the present study suggest that heterotrophs positively affecting the growth of *Nitrospira* are not rare; rather, a considerable proportion of heterotrophs in environments are likely to positively affect the growth of nitrifying chemoautotrophs.

Notably, one of the bottlenecks toward obtaining pure cultures of *Nitrospira* is the tendency of *Nitrospira* to form inseparable aggregates with heterotrophs when they are enriched from the environment (Spieck *et al*., 2006; Lebedeva *et al*., 2008; Fujitani *et al*., 2013; Fujitani *et al*., 2014; Nowka *et al*., 2015). However, this also suggests that *Nitrospira* and heterotrophs are strongly associated to promote their growth. Thus, the complex and diverse interactions revealed in this study may partly underlie the resistance of *Nitrospira* to isolation. Furthermore, these interactions may contribute to the stabilization of nitrifying microbial ecosystems in nature and engineered systems.

Acknowledgements

This research was supported by a JSPS KAKENHI Grant Numbers JP26450093, JP18K19181 and the NAGASE Science Technology Foundation. C.M. was a Research Fellow (RPD) of the Japan Society for the Promotion of Science.

References

Bartosch, S., Wolgast, I., Spieck, E., and Bock, E. (1999) Identification of nitrite-oxidizing bacteria with monoclonal antibodies recognizing the nitrite oxidoreductase. *Appl Environ Microbiol* **65**: 4126–4133.

Batchelor, S., Cooper, M., Chhabra, S., Glover, L., Stewart, G., Williams, P., and Prosser, J.I. (1997) Cell density-regulated recovery of starved biofilm populations

of ammonia-oxidizing bacteria. *Appl Environ Microbiol* **63**: 2281–2286.

Bayer, B., Pelikan, C., Bittner, J., Reinthaler, T., Könneke, M., Herndl, J., and Offre, P. (2019) Proteomic response of three marine ammonia-oxidizing archaea to hydrogen peroxide and their metabolic interactions with a heterotrophic alphaproteobacterium. *mSystems* **4**: e00181-19.

Bayer, B., Saito, A., McIlvin, R., Lücker, S., Moran, M., Lankiewicz, S., *et al*. (2021) Metabolic versatility of the nitrite-oxidizing bacterium *Nitrospira marina* and its proteomic response to oxygen-limited conditions. *ISME J* **15**: 1025–1039.

Burrell, P., Keller, J., and Blackall, L. (1998) Microbiology of a nitrite-oxidizing bioreactor. *Appl Environ Microbiol* **64**: 1878–1883.

Burton, E., Read, H., Pellitteri, M., and Hickey, W. (2005) Identification of acyl-homoserine lactone signal molecules produced by *Nitrosomonas europaea* strain Schmidt. *Appl Environ Microbiol* **71**: 4906–4909.

Clark, C., and Schmidt, E. (1966) Effect of mixed culture on *Nitrosomonas europaea* simulated by uptake and utilization of pyruvate. *J Bacteriol* **91**: 367–373.

Clark, C., and Schmidt, E. (1967a) Growth response of *Nitrosomonas europaea* to amino acids. *J Bacteriol* **93**: 1302–1308.

Clark, C., and Schmidt, E. (1967b) Uptake and utilization of amino acids by resting cells of *Nitrosomonas europaea*. *J Bacteriol* **93**: 1309–1315.

Daims, H., Lücker, S., and Wagner, M. (2016) A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends Microbiol* **24**: 699–712.

Dolinšek, J., Lagkouvardos, I., Wanek, W., Wagner, M., and Daims, H. (2013) Interactions of nitrifying bacteria and heterotrophs: identification of a Micavibrio-like putative predator of *Nitrospira* spp. *Appl Environ Microbiol* **79**: 2027–2037.

Fujitani, H., Aoi, Y., and Tsuneda, S. (2013) Selective enrichment of two different types of *Nitrospira*-like nitrite-oxidizing bacteria from a wastewater treatment plant. *Microbes Environ* **28**: 236–243.

Fujitani, H., Ushiki, N., Tsuneda, S., and Aoi, Y. (2014) Isolation of sublineage I *Nitrospira* by a novel cultivation strategy. *Environ Microbial* **16**: 3030–3040.

Ho, A., Roy, K., Thas, O., Neve, J., Hoefman, S., Vandamme, P., *et al*. (2014) The more, the merrier: heterotroph richness stimulates methanotrophic activity. *ISME J* **8**: 1945–1948.

Hovanec, T., Taylor, L., Blakis, A., and Delong, E. (1998) *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. *Appl Environ Microbiol* **64**: 258–264.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K., Pommerening, R.A., Koops, H., and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* **64**: 3042–3051.

Keluskar, R., Nerurkar, A., and Desai, A. (2013) Mutualism between autotrophic ammonia oxidizing bacteria (AOB) and heterotrophs present in an ammonia-oxidizing colony. *Arch Microbiol* **195**: 737–747.

Kim, G., Park, J., Sinninghe, S., Schouten, S., Rijpstra, I., Jung, Y., *et al*. (2016) Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea. *Proc Natl Acad Sci U S A* **113**: 7888–7893.

Kindaichi, T., Ito, T., and Okabe, S. (2004) Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Appl Environ Microbiol* **70**: 1641–1650.

Lebedeva, E., Alawi, M., Maixner, F., Jozsa, P., Daims, H., and Spieck, E. (2008) Physiological and phylogenetic characterization of a novel lithoautotrophic nitrite-oxidizing bacterium, '*Candidatus* Nitrospira bockiana'. *Int J Syst Evol Microbiol* **58**: 242–250.

Martiny, C., Albrechtsen, J., Arvin, E., and Molin, S. (2005) Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with *Nitrospira* sp. *Appl Environ Microbiol* **71**: 8611–8617.

Matsumoto, S., Katoku, M., Saeki, G., Terada, A., Aoi, Y., Tsuneda, S., *et al*. (2010) Microbial community structure in autotrophic nitrifying granules characterized by experimental and simulation analyses. *Environ Microbiol* **12**: 192–206.

Morris, J., Kirkegaard, R., Szul, J., Johnson, I., and Zinser, R. (2008) Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by helper heterotrophic bacteria. *Appl Environ Microbiol* **74**: 4530–4534.

Noguera, R., Araki, N., and Rittmann, E. (1994) Soluble microbial products (SMP) in anaerobic chemostats. *Biotechnol Bioeng* **44**: 1040–1047.

Nogueira, R., Melo, F., Purkhold, U., Wuertz, S., and Wagner, M. (2002) Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. *Water Res* **36**: 469–481.

Nowka, B., Off, S., Daims, H., and Spieck, E. (2015) Improved isolation strategies allowed the phenotypic differentiation of two *Nitrospira* strains from widespread phylogenetic lineages. *FEMS Microbiol Ecol* **91**: fiu031.

Ohashi, A., Silva, V., Mobarry, B., Manem, J., Stahl, D., and Rittmann, B. (1995) Influence of the substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs. *Water Sci Technol* **32**: 75–84.

Okabe, S., Hiratia, K., Ozawa, Y., and Watanabe, Y. (1996) Spatial microbial distributions of nitrifiers and heterotrophs in mixed-population biofilms. *Biotechnol Bioeng* **50**: 24–35.

Okabe, S., Satoh, H., and Watanabe, Y. (1999) In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Appl Environ Microbiol* **65**: 3182–3191.

Okabe, S., Satoh, H., Naitoh, H., and Watanabe, Y. (2002) Structure and function of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Water Sci Technol* **46**: 233–241.

Okabe, S., Kindaichi, T., and Ito, T. (2005) Fate of C-14-labeled microbial products derived from nitrifying

bacteria in autotrophic nitrifying biofilms. *Appl Environ Microbiol* **71**: 3987–3994.
Onaka, H., Mori, Y., Igarashi, Y., and Furumai, T. (2011) Mycolic acid-containing bacteria induce natural-product biosynthesis in *Streptomyces* species. *Appl Environ Microbiol* **77**: 400–406.
Onaka, H., Ozaki, T., Mori, Y., Izawa, M., Hayashi, S., and Asamizu, S. (2015) Mycolic acid-containing bacteria activate heterologous secondary metabolite expression in *Streptomyces lividans*. *J Antibiot (Tokyo)* **68**: 594–597.
Paerl, H., and Pinckney, J. (1996) A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. *Microb Ecol* **31**: 225–247.
Paerl, W., Pinckney, L., and Steppe, F. (2000) Cyanobacterial-bacterial mat consortia: examining the functional unit of microbial survival and growth in extreme environments. *Environ Microbiol* **2**: 11–26.
Pan, P., and Umbreit, W. (1972) Growth of obligate autotrophic bacteria on glucose in a continuous flow through apparatus. *J Bacteriol* **109**: 1149–1155.
Rittmann, B., Regan, J., and Stahl, D. (1994) Nitrification as a source of soluble organic substrate in biological treatment. *Water Sci Technol* **30**: 1–8.
Rittmann, E., Stilwell, D., and Ohashi, A. (2002) The transient-state, multiple-species biofilm model for biofiltration processes. *Water Res* **36**: 2342–2356.
Sedlacek, J., Nielsen, S., Greis, D., Haffey, D., Revsbech, P., Ticak, T., *et al*. (2016) Effects of bacterial community members on the proteome of the ammonia-oxidizing bacterium *Nitrosomonas* sp. strain Is79. *Appl Environ Microbiol* **82**: 4776–4788.
Sher, D., Thompson, J.W., Kashtan, N., Croal, L., and Chisholm, S.W. (2011) Response of *Prochlorococcus* ecotypes to co-culture with diverse marine bacteria. *ISME J* **5**: 1125–1132.
Spieck, E., Hartwig, C., McCormack, I., Maixner, F., Wagner, M., Lipski, A., and Dimes, H. (2006) Selective enrichment and molecular characterization of a previously uncultured *Nitrospira*-like bacterium from activated sludge. *Environ Microbiol* **8**: 405–415.
Wagner, M., Rath, G., Koops, H., Flood, J., and Amann, R. (1996) In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Sci Technol* **34**: 237–244.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Experimental procedures.