Supplementary results

Identification of novel bacteriohopanepolyols

Six novel BHPs with the *m/z* 762.5, 656.5, 771.6, 748.5, 638.5, and 743.6 Da were tentatively identified using MS^2 spectra and molecular formulas derived from accurate masses (Table S2). All novel BHPs contained amino or nitro groups and yielded the characteristic fragment at *m/z* 191.2 fragment, indicating ring cleavage of hopanoids not methylated at C-2 or C-3. BHP-762.5 ($C_{43}H_{72}NO_{10}^+$, $[M+H]^+$) was tentatively identified as tetra-functionalized, containing three hydroxyl groups indicated by consecutive neutral loss of 42/60 (acetylated hydroxyl groups, $COCH_2$/CH_3COOH) and one nitro group (neutral loss of 47). The molecular formula indicates a lack of acetylation at one functional group, which is also observed in other BHPs such as adenosylhopane, and which could be related to steric effects. BHP-656.6 ($C_{40}H_{66}NO_6^+$, $[M+H]^+$) was tentatively identified as a tetra-functionalized BHP bearing an amino group within a lactone ring, indicated by neutral losses of 17 (NH_3) and 44 (CO_2; Crotti et al., 2005), respectively. The fragmentation pattern of BHP-771.6 ($C_{46}H_{79}N_2O_7^+$, $[M+H]^+$) suggests a tetra-functionalized BHP containing two hydroxyl groups (neutral loss of 42/60), an amino group (neutral loss of 59; Talbot et al., 2001), and an ether-bound aminopropanol group indicated by sequential neutral loss of 59 (acetamido; C_2H_5NO) plus 58 (C_3H_6O). BHP-748.5 ($C_{42}H_{70}NO_{10}^+$) produced a dominant in-source fragment in MS^1 mode at *m/z* 688.5 (neutral loss of 60). Fragmentation of 688.5 yielded two neutral losses of 60 as well as neutral losses of 75 ($CHNO_3$) and 93 (CH_3NO_4). Fragmentation spectra thus indicate that BHP-748.5 is tetra-functionalized and contains three hydroxyl groups and a fourth hydroxyl group that is bound to a nitrogen-containing functional group. Based on the neutral loss of 93, we speculate that the nitrogen-containing functional group could be nitroformic acid or nitroperoxymethane. BHP-638.5 could not be identified with confidence due to its low abundance

and consequent noisy spectra. However, accurate mass analysis suggests a molecular formula of $C_{40}H_{64}NO_5^+$ ($[M+H]^+$) and thus the presence of a nitrogen-containing functional group. BHP-743.6 produced a dominant MS^1 in-source fragment at *m/z* 684.5 (neutral loss of 59). Accurate mass analysis suggests a molecular formula of $C_{44}H_{75}N_2O_7^+$ ($[M+H]^+$), suggesting the presence of two nitrogen-containing functional groups. Fragmentation of *m/z* 684.5 yielded three neutral losses of 60 and a neutral loss of 31 (methylamine, CH_5N), suggesting that BHP-743.6 is penta-functionalized. Presence of one neutral loss of 59 but lack of a second neutral loss of 59 indicates that the second nitrogen-containing group is not acetylated. This suggests that the second nitrogen-containing group consists of an amino nitrogen bound to the hopanoid backbone and bound to a methyl group that prevents acetylation. BHP-743.6 thus represents a penta-functionalized hopanoid containing three hydroxyl groups, an amino group, and a methylamine group.

Supplementary Datafile captions

Supplementary Datafile S1 (Supplied as supplementary .xlsx file). Occurrence (+) and absence (-) of hopanoid biosynthesis genes in AOB and NOB genomes from cultures and environmental samples (metagenome-assembled genomes and single cell genomes) as well as from closely related non-nitrifying taxa. Accession numbers, ecological metadata (taxonomy, habitat, C-fixation pathway, metabolism), and references to original work describing genomic data are given.

Supplementary Datafile S2 (Supplied as supplementary xlsx file). Sheet 1: Average relative abundance (%, +/- standard deviation, values below 0.01 rounded to 0.01) of bacteriohopanepolyols in triplicate cultures of nitrite-oxidizing bacteria grown under different conditions. b.d., below detection. Standard deviations omitted for experiments performed without replicates (*Nitrobacter vulgaris* and *Nitrococcus mobilis* with pseudovitamin B_{12}). Sheet 2: Average relative abundance (%, +/- standard deviation, values below 0.01 rounded to 0.01) of bacteriohopanepolyols (BHPs) and diploptene/methylated diploptene in tripliate cultures of nitrite-oxidizing bacteria grown under different conditions. b.d., not detected. Standard deviations omitted for experiments performed without replicates (*Nitrobacter vulgaris* and *Nitrococcus mobilis* with pseudovitamin B_{12}). Sheet 3: Average abundance of total hopanoids (BHPs + 2-Me Diploptene + 2-Me BHPs) in triplicate cultures normalized to mmol NO_2^- oxidized. Standard deviations omitted for experiments performed without three replicates (*Nitrobacter vulgaris* and *Nitrococcus mobilis* with pseudovitamin B_{12} as well as NO_2^--limited and O_2-limited experiments with *Nitrococcus mobilis* and *Nitrospina gracilis*).

Supplementary tables and figures

Table S1. Growth characteristics of nitrite-oxidizing bacteria in batch cultures (n.a., not available).

Strain	Growth condition	NO_2^--oxidation rate (mM d^{-1})
Nitrospira marina 295	Autotrophic, early stationary	0.22 ± 0.05
Nitrospira marina 295	Autotrophic, late stationary	n.a.
Nitrospira marina 295	Autotrophic, methionine, early stationary	0.11 ± 0.07
Nitrospira marina 295	Autotrophic, vitamin B12, early stationary	0.3 ± 0.14
Nitrospira marina 295	Mixotrophic, early stationary	0.58 ± 0.11
Nitrospira lenta BS10	Autotrophic, early stationary	n.a.
Nitrospira defluvii A17	Autotrophic, early stationary	n.a.
Nitrospira moscoviensis M-1	Autotrophic, early stationary	n.a.
Nitrospina gracilis Nb-3/211	Autotrophic, early stationary	0.74 ± 0.01
Nitrospina gracilis Nb-3/211	Autotrophic, methionine, early stationary	0.74 ± 0.01
Nitrospina gracilis Nb-3/211	Autotrophic, vitamin B12, early stationary	0.74 ± 0.02
Nitrococcus mobilis 231	Autotrophic, early stationary	1.16 ± 0.26
Nitrococcus mobilis 231	Autotrophic, late stationary	n.a.
Nitrococcus mobilis 231	Autotrophic, methionine, early stationary	1.2 ± 0.01
Nitrococcus mobilis 231	Autotrophic, vitamin B12, early stationary	1.25 ± 0.1
Nitrobacter vulgaris AB1	Autotrophic, mid-growth phase	1.89 ± 0.07
Nitrobacter vulgaris AB1	Autotrophic, early stationary	2.14 ± 0.1
Nitrobacter vulgaris AB1	Autotrophic, methionine, early stationary	2.34 ± 0.08
Nitrobacter vulgaris AB1	Autotrophic, vitamin B12, early stationary	2.62 ± 0.05
Nitrobacter vulgaris AB1	Autotrophic, vitamin B12/methionine/light, early stationary	2.66 ± 0.22
Nitrobacter vulgaris AB1	Mixotrophic, early stationary	n.a.
Nitrobacter vulgaris AB1	Heterotrophic aerobic, early stationary	n.a.
Nitrobacter vulgaris AB1	Heterotrophic anaerobic, early stationary	n.a.

Table S2. Growth characteristics of *Nitrobacter vulgaris* AB1 in chemostat experiments under NO_2^--limited and O_2-limited conditions (average of triplicates ± 1σ standard deviation; b.d., below detection ~ <0.1 ppm).

	NO_2^--limited	O_2-limited
O_2 dissolved, inflow (ppm)	8.6 ± 0.2	8.6 ± 0.2
O_2 dissolved, reactor (ppm)	6 ± 0.2	b.d.
NO_2^- dissolved, inflow (mM)	10	10
NO_2^- dissolved, reactor (mM)	0.009 ± 0.002	5.82 ± 0.14
Medium volume, reactor (L)	2.0	2.0
Medium inflow rate (ml min^{-1})	0.443	0.443
Growth rate (h^{-1})	0.013	0.013
Doubling time (h)	52.2	52.2

Table S3. Growth characteristics of *Nitrospina gracilis* Nb-211 and *Nitrococcus mobilis* Nb-231 in chemostat experiments under NO_2^--limited and O_2-limited conditions (average of quadruplicates ± 1σ standard deviation; b.d., below detection).

	N. gracilis		*N. mobilis*	
	NO_2^--limited	O_2-limited	NO_2^--limited	O_2-limited
NO_2^- dissolved, inflow (mM)	2.0	2.0	2.0	2.0
NO_2^- dissolved, outflow (mM)	b.d.	1.1	b.d.	1.1
Medium volume, reactor (L)	2.0	2.0	2.0	2.0
Medium in-/outflow rate (ml min^{-1})	0.37	0.37	0.37	0.37
Growth rate (h^{-1})	0.011	0.011	0.011	0.011
Doubling time (h)	62.5	62.5	62.5	62.5
Cell concentration (cells ml^{-1})	$1.04 \pm 0.21 \times 10^8$	$0.86 \pm 0.14 \times 10^8$	$1.83 \pm 0.46 \times 10^8$	$2.76 \pm 0.62 \times 10^8$
Specific NO_2^- ox. rate (fmol $cell^{-1}$ d^{-1})	20.0 ± 4.8	10.1 ± 1.7	11.5 ± 2.9	3.2 ± 0.7
Growth yield (cells mol NO_2^- $ox.^{-1}$)	$5.19 \pm 1.05 \times 10^{13}$	$1.01 \pm 1.59 \times 10^{14}$	$9.13 \pm 2.28 \times 10^{13}$	$3.20 \pm 0.71 \times 10^{14}$

Table S4. Observed mass, predicted mass, predicted sum formula, measurement error inferred from predicted and observed masses, and double-bond equivalents (Dbl. eq.) of base peak (underlined) and major fragment ions in MS^2 spectra of novel BHPs from *Nitrobacter vulgaris* AB1 and *Nitrococcus mobilis* 231.

Compound	Ion	Observed mass (Da)	Predicted mass (Da)	Predicted formula	Error (ppm)	Error (mDa)	Dbl. eq.
BHP-762.5	$[M+H]^+$	<u>762.5101</u>	762.5151	$C_{43}H_{72}NO_{10}^+$	6.5	5.0	8.5
	$[M-CH_3COOH]^+$	702.4886	702.4939	$C_{41}H_{68}NO_8^+$	7.6	5.3	8.5
	$[M-CH_3COOH-CH_2CO]^+$	660.4766	660.4834	$C_{39}H_{66}NO_7^+$	10.3	6.8	7.5
	$[M-2xCH_3COOH-CH_2CO]^+$	600.4557	600.4622	$C_{37}H_{62}NO_5^+$	10.9	6.6	7.5
	$[M-2xCH_3COOH-CH_2CO-NO_2H]^+$	553.4653	553.4615	$C_{37}H_{61}O_3^+$	-6.8	-3.8	7.5
	$[M-3xCH_3COOH-CH_2CO-NO_2H]^+$	493.4438	493.4404	$C_{35}H_{57}O^+$	-6.9	-3.4	7.5
	$[M-3xCH_3COOH-CH_2CO-NO_2H-H_2O]^+$	475.4339	475.4298	$C_{35}H_{55}^+$	-8.6	-4.1	8.5
BHP-656.5	$[M+H]^+$	<u>656.4950</u>	656.4885	$C_{40}H_{66}NO_6^+$	-10	-6.5	8.5
	$[M-CO_2]^+$	612.5040	612.4986	$C_{39}H_{66}NO_4^+$	-8.8	-5.4	7.5
	$[M-CO_2-CH_3COOH]^+$	552.4824	552.4775	$C_{37}H_{62}NO_2^+$	-8.9	-4.9	7.5
	$[M-CO_2-2xCH_3COOH]^+$	492.4600	492.4564	$C_{35}H_{58}N^+$	-7.4	-3.6	7.5
	$[M-CO_2-2xCH_3COOH-NH_3]^+$	475.4363	475.4298	$C_{35}H_{55}^+$	-13.6	-6.5	8.5
BHP-771.6	$[M+H]^+$	<u>771.5944</u>	771.5882	$C_{46}H_{79}N_2O_7^+$	-8.1	-6.2	8.5
	$[M-CH_3CONH_2]^+$	712.5506	712.5511	$C_{44}H_{74}NO_6^+$	0.7	0.5	8.5
	$[M-CH_3CONH_2-CH_3CHCH_2O]^+$	654.5143	654.5092	$C_{41}H_{68}NO_5^+$	-7.8	-5.1	8.5
	$[M-CH_3CONH_2-CH_3CHCH_2O-CH_3COOH]^+$	594.4902	594.4881	$C_{39}H_{64}NO_3^+$	-3.6	-2.1	8.5
	$[M-CH_3CONH_2-CH_3CHCH_2O-2xCH_3COOH]^+$	534.4707	534.4669	$C_{37}H_{60}NO^+$	-7	-3.8	8.5
	$[M-CH_3CONH_2-CH_3CHCH_2O-2xCH_3COOH-CH_3CONH_2]^+$	475.4352	475.4298	$C_{35}H_{55}^+$	-11.3	-5.4	8.5
BHP-748.5	$[M+H]^+$	748.4982	748.4994	$C_{42}H_{70}NO_{10}^+$	1.6	1.2	8.5
	$[M-CH_3COOH]^+$	<u>688.4800</u>	688.4783	$C_{40}H_{66}NO_8^+$	-2.5	-1.7	8.5
	$[M-2xCH_3COOH]^+$	628.4577	628.4572	$C_{38}H_{62}NO_6^+$	-0.9	-0.5	8.5
	$[M-3xCH_3COOH]^+$	568.4321	568.4360	$C_{36}H_{58}NO_4^+$	6.9	3.9	8.5
	$[M-3xCH_3COOH-CHNO_3]^+$	493.446	493.4404	$C_{35}H_{57}O^+$	-11.4	-5.6	7.5
	$[M-3xCH_3COOH-CH_3NO_4]^+$	475.4311	475.4298	$C_{35}H_{55}^+$	-2.7	-1.3	8.5
BHP-638.5	$[M+H]^+$	<u>638.4823</u>	638.4779	$C_{40}H_{64}NO_5^+$	-6.9	-4.4	9.5
BHP-743.6	$[M+H]^+$	743.5651	743.5569	$C_{44}H_{75}N_2O_7^+$	-11.1	-8.2	8.5
	$[M-CH_3CONH_2]^+$	<u>684.5213</u>	684.5198	$C_{42}H_{70}NO_6^+$	-2.2	-1.5	8.5
	$[M-CH_3CONH_2-CH_3COOH]^+$	624.5041	624.4986	$C_{40}H_{66}NO_4^+$	-8.7	-5.5	8.5
	$[M-CH_3CONH_2-2xCH_3COOH]^+$	564.4848	564.4775	$C_{38}H_{62}NO_2^+$	-12.9	-7.3	8.5
	$[M-CH_3CONH_2-3xCH_3COOH]^+$	504.4550	504.4564	$C_{36}H_{58}N^+$	2.7	1.4	8.5
	$[M-CH_3CONH_2-3xCH_3COOH-CH_3N]^+$	473.4252	473.4142	$C_{35}H_{53}^+$	-23.3	-11	9.5

Table S5. Absence (-) of marker genes for the aerobic (*cobF*, *cobG*) and anaerobic (*cbiD*, *cbiG*) cobalamin biosynthesis pathways as well as genes for cobalamin-dependent (*metH*) and cobalamin-independent (*metE*) methionine synthase in seven species of nitrite-oxidizing bacteria, indicating lack of cobalamin biosynthesis in four of these organisms. Presence of marker genes was determined through blast (Altschul et al., 1990) analysis using protein query sequences from *Salmonella typhimurium* LT2, *Escherichia coli* K12, *Pseudomonas denitrificans*, and *Halobacterium salinarum*.

	cobF	*cobG*	*cbiD*	*cbiG*	*metE*	*metH*
Nitrospira marina 295	-	-	-	-	-	+
Nitrospina gracilis 3/211	-	-	-	-	-	+
Nitrobacter vulgaris AB1	-	-	-	-	+	+
Nitrococcus mobilis 231	-	-	-	-	+	+
Nitrospira defluvii A17	-	-	+	+	-	+
Nitrospira lenta BS10	-	-	+	+	-	+
Nitrospira moscoviensis M-1	-	-	+	+	-	+

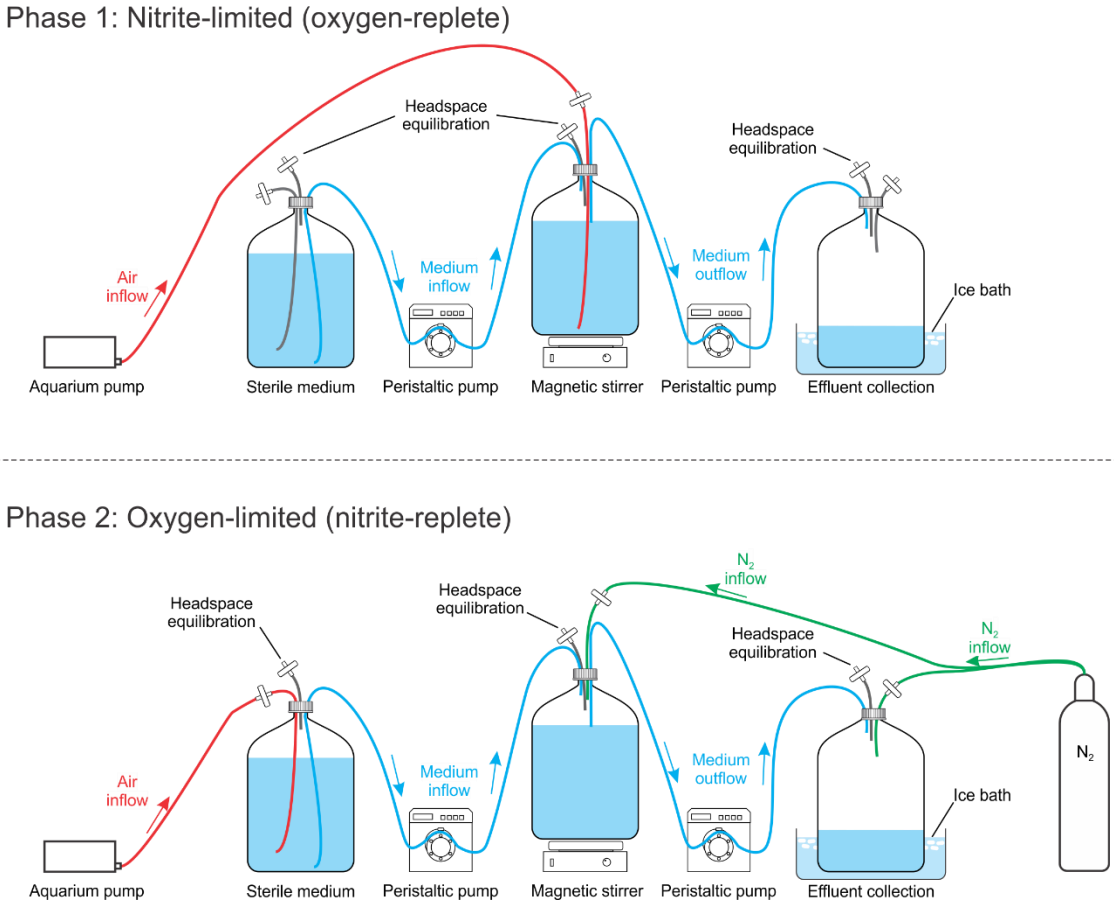

Fig. S1. The chemostat setup used to grow *N. gracilis, N. mobilis and N. vulgaris* in continuous culture under nitrite-limited and oxygen-limited conditions.

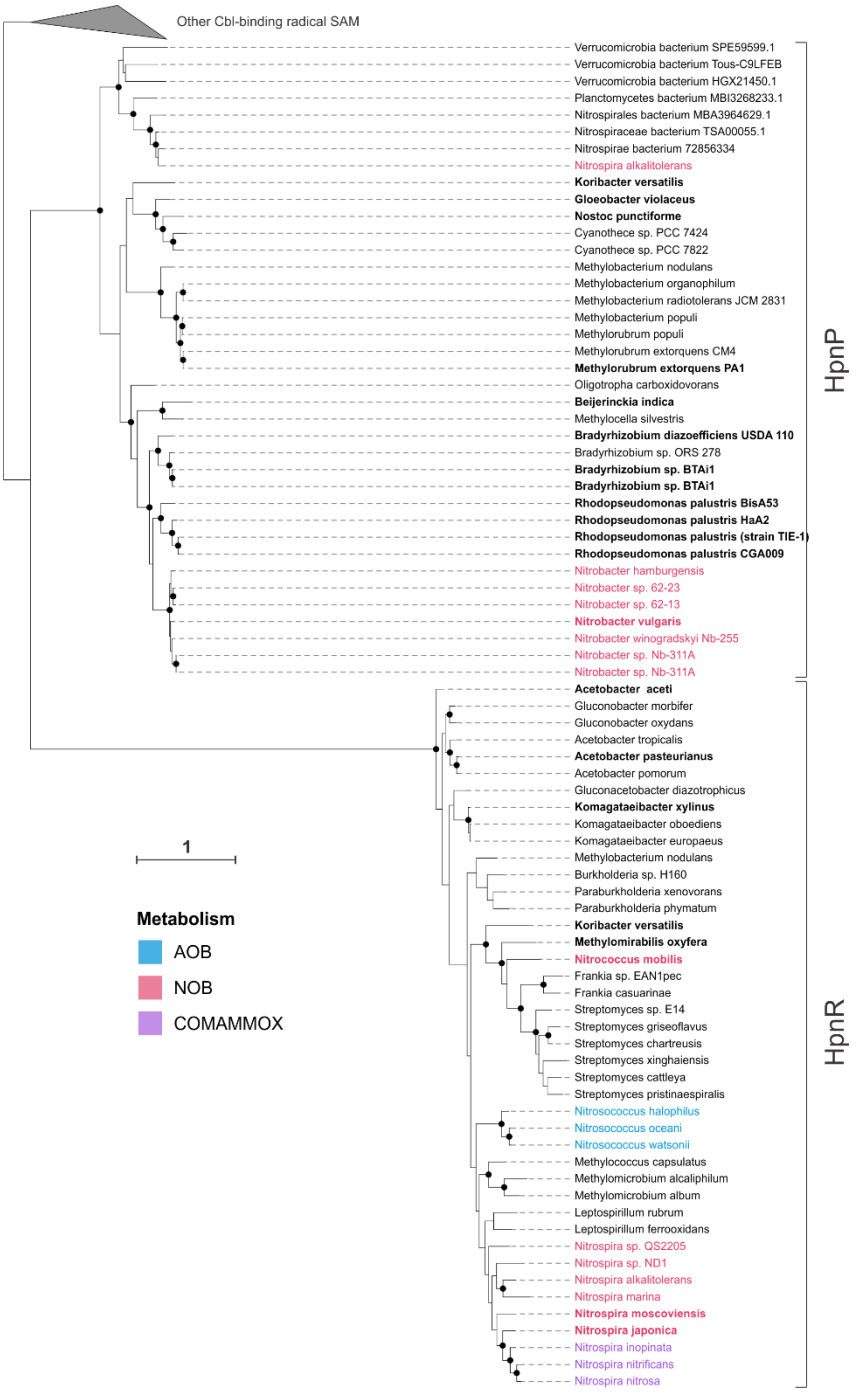

Fig. S2. Phylogenetic tree of hopanoid C-2 (*hpnP*) and C-3 (*hpnR*) methylase homologues in bacteria. Colors indicate nitrite-oxidizing bacteria (NOB; red), ammonia-oxidizing bacteria (AOB; cyan), and complete ammonia-oxidizing bacteria (comammox; purple). Strains where presence of C-2 or C-3 methyl hopanoids was tested and detected are highlighted in bold (based on this study and Rohmer et al., 1984; Welander et al., 2010; Welander and Summons, 2012; Kool et al., 2014; Sinninghe Damsté et al., 2017) and strains that were not tested are set in normal font. Circles indicate branches with >85% support based on 500 bootstrap analyses. The scale bar represents 1 substitution per amino acid.

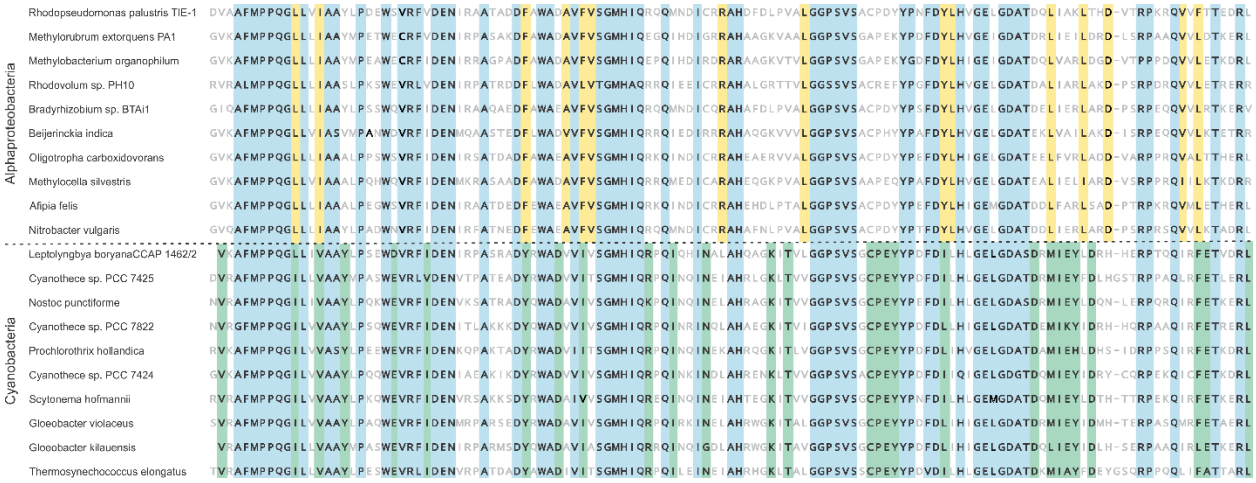

Fig. S3. Alignment of the cobalamin-binding domain of HpnP amino acid sequences from selected alphaproteobacteria and cyanobacteria. Highlighted are residues conserved in ≥90% of either cyanobacteria (green) or alphaproteobacteria (yellow). Also highlighted are residues universally conserved in both clades (blue).

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