ORIGINAL ARTICLE

Phylogenomics of *Rhodobacteraceae* reveals evolutionary adaptation to marine and non-marine habitats

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Marine *Rhodobacteraceae* (*Alphaproteobacteria*) are key players of biogeochemical cycling, comprise up to 30% of bacterial communities in pelagic environments and are often mutualists of eukaryotes. As '*Roseobacter* clade', these 'roseobacters' are assumed to be monophyletic, but non-marine *Rhodobacteraceae* have not yet been included in phylogenomic analyses. Therefore, we analysed 106 genome sequences, particularly emphasizing gene sampling and its effect on phylogenetic stability, and investigated relationships between marine versus non-marine habitat, evolutionary origin and genomic adaptations. Our analyses, providing no unequivocal evidence for the monophyly of roseobacters, indicate several shifts between marine and non-marine habitats that occurred independently and were accompanied by characteristic changes in genomic content of orthologs, enzymes and metabolic pathways. Non-marine *Rhodobacteraceae* gained high-affinity transporters to cope with much lower sulphate concentrations and lost genes related to the reduced sodium chloride and organohalogen concentrations in their habitats. Marine *Rhodobacteraceae* gained genes required for fucoidan desulphonation and synthesis of the plant hormone indole 3-acetic acid and the compatible solutes ectoin and carnitin. However, neither plasmid composition, even though typical for the family, nor the degree of oligotrophy shows a systematic difference between marine and non-marine *Rhodobacteraceae*. We suggest the operational term '*Roseobacter* group' for the marine *Rhodobacteraceae* strains.
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Introduction

Rhodobacteraceae (Garrity *et al.*, 2005), one of the major subdivisions of *Alphaproteobacteria*, include >100 genera and >300 species with very diverse physiologies (Pujalte *et al.*, 2014). Giovannoni and Rappé (2000) assigned most *Rhodobacteraceae* to the *Roseobacter* group ('roseobacters'), which now includes >70 validly named genera, >170 validly named species (Pujalte *et al.*, 2014) and numerous additional isolates and 16S rRNA phylotypes (http://www.arb-silva.de). Most roseobacters originate from marine habitats but some from (hyper-)saline lakes or soil (Pujalte *et al.*, 2014). Other

Rhodobacteraceae include only few strains of marine origin (Pujalte *et al.*, 2014). Roseobacters show a very versatile physiology to dwell in greatly varying marine habitats (Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006; Brinkhoff *et al.*, 2008; Newton *et al.*, 2010; Sass *et al.*, 2010; Laass *et al.*, 2014; Luo and Moran, 2014; Collins *et al.*, 2015) and account for large proportions of bacterioplankton communities (Selje *et al.*, 2004; West *et al.*, 2008; Alonso-Gutiérrez *et al.*, 2009; Giebel *et al.*, 2011; Buchan *et al.*, 2014; Gifford *et al.*, 2014; Wemheuer *et al.*, 2015).

Genome plasticity might explain adaptability and diversity of roseobacters (Luo and Moran, 2014). Pelagic roseobacters show streamlined genomes (Voget *et al.*, 2015) and possibly other adaptations to an oligotrophic lifestyle. Extrachromosomal replicons (ECRs) comprise chromids and genuine plasmids (Harrison *et al.*, 2010; Petersen *et al.*, 2013). Four replication systems (RepA, RepB, RepABC, DnaA-like) with about 20 phylogenetically distinguishable

compatibility groups were identified in roseobacters (Petersen *et al.*, 2013), but a comprehensive analysis of *Rhodobacteraceae* genome architecture is lacking. Whereas extensive studies of physiological, genetic and genomic features of roseobacters were performed, only scarce and unsystematic information is available on how these traits are distributed among marine and non-marine *Rhodobacteraceae*.

The *Roseobacter* group is presumed to be monophyletic and thus frequently called '*Roseobacter* clade' (Buchan *et al.*, 2005; Newton *et al.*, 2010; Luo *et al.*, 2013; Pujalte *et al.*, 2014). Strains within this group share >89% identity of the 16S rRNA gene (Brinkhoff *et al.*, 2008; Luo and Moran, 2014) but a reliable delineation of this group from other *Rhodobacteraceae* cannot be carried out with this gene (Breider *et al.*, 2014), as branch support and other rationales for suggested 16S rRNA gene-derived *Rhodobacteraceae* lineages (Pujalte *et al.*, 2014) are lacking.

Even though more comprehensive phylogenetic analyses of the *Roseobacter* group have been conducted (Newton *et al.*, 2010; Tang *et al.*, 2010; Luo *et al.*, 2012, 2013; Luo and Moran, 2014; Voget *et al.*, 2015), an analysis of the phylogenetic affiliation of this group as a component of the entire *Rhodobacteraceae* has not yet been carried out. In the majority of phylogenomic analyses of roseobacters (Luo *et al.*, 2012, 2014; Luo and Moran, 2014; Voget *et al.*, 2015), non-roseobacter *Rhodobacteraceae* were missing, and a single set of selected genes was concatenated and analysed with a single inference method such as Maximum Likelihood (ML) assuming a single amino-acid substitution model for all genes. However, selections of genes, strains and other factors influence the resulting phylogenies (Jeffroy *et al.*, 2006; Philippe *et al.*, 2011; Salichos and Rokas, 2013; Breider *et al.*, 2014). Even though evolutionary aspects of gene gains and losses of the *Roseobacter* group were analysed previously (Luo *et al.*, 2013; Luo and Moran, 2014), these analyses did not consider the content of specific genomic features as adaptations to the environmental settings.

We carried out phylogenomic analyses of 106 sequenced *Rhodobacteraceae* genomes using distinct inference algorithms and distinct gene selections ranging from the analysis of the core genes to the 'full' supermatrix, to address the following questions: (i) How is the *Roseobacter* group related to other *Rhodobacteraceae*? (ii) Is this relationship robust against variations in gene selection and phylogenetic inference? (iii) Do stable subgroups exist within this family that are supported by various phylogenomic approaches? and (iv) Do genomic characters correlate with marine or non-marine habitats?

Materials and methods

Genome-scale phylogenetic analysis
All applied methods are detailed in Supplementary File S1. Among the 106 genome-sequenced strains investigated, 13 strains of the *Labrenzia*/*Stappia*

group taxonomically assigned to *Rhodobacteraceae* but rather placed within *Rhizobiales* in 16S rRNA gene analyses (Pujalte *et al.*, 2014) were used as outgroup. An extended data set including 132 genomes was phylogenetically analysed using Genome BLAST Distance Phylogeny (Auch *et al.*, 2006; Meier-Kolthoff *et al.*, 2014). Digital DNA:DNA hybridization was used to check all species affiliations (Auch *et al.*, 2010; Meier-Kolthoff *et al.*, 2013a). Pairwise 16S rRNA gene similarities (Meier-Kolthoff *et al.*, 2013b) were determined after extraction with RNAmmer version 1.2 (Lagesen and Hallin, 2007). The proteome sequences were phylogenetically investigated using the DSMZ phylogenomics pipeline (Anderson *et al.*, 2011; Breider *et al.*, 2014; Frank *et al.*, 2014; Stackebrandt *et al.*, 2014; Verbarg *et al.*, 2014). Alignments were concatenated to three main supermatrices: (i) 'core genes', alignments containing sequences from all proteomes; (ii) 'full', alignments containing sequences from at least four proteomes; and (iii) 'MARE', the full matrix filtered with that software (Meusemann *et al.*, 2010). The core genes were further reduced to their 50, 100, 150 and 200 most conserved genes (up to 250 without outgroup). Long-branch extraction (Siddall and Whiting, 1999) to assess long-branch attraction artefacts (Bergsten, 2005) was conducted by removing the outgroup strains, generating the supermatrices anew and rooting the resulting trees with LSD version 0.2 (To *et al.*, 2015).

ML and maximum parsimony (MP) phylogenetic trees were inferred as described (Andersson *et al.*, 2011; Breider *et al.*, 2014; Frank *et al.*, 2014; Stackebrandt *et al.*, 2014; Verbarg *et al.*, 2014) but MP tree search was conducted with TNT version 1.1 (Goloboff *et al.*, 2008). Additionally, best substitution models for each gene and ML phylogenies were calculated with ExaML version 3.0.7 (Stamatakis and Aberer, 2013). Ordinary and partition bootstrapping (Siddall, 2010) was conducted with 100 replicates except for full/ML for reasons of running time. To assess conflict between the genomic data and roseobacter monophyly, site-wise ML and MP scores were calculated from unconstrained and accordingly constrained best trees, optionally summed up per gene, and compared with Wilcoxon and *T*-tests (R Core Team, 2015) and, for ML, with the approximately unbiased test (Shimodaira and Hasegawa, 2001). Potential conflict with the 16S rRNA gene was measured using constraints derived from the supermatrix trees. Major sublineages were inferred from the phylogenomic trees non-arbitrarily as the maximally inclusive, maximally and consistently supported subtrees.

Analysis of character evolution
Phylogenetic correlations between pairs of binary characters (Pagel, 1994) were detected with Bayes-Traits version 2.0 (Pagel *et al.*, 2004) in conjunction with the rooted ML phylogenies. Ratios of the

estimated rates of change were calculated to verify the tendency toward marine or non-marine habitats. Three distinct genome samplings were used to detect an influence of only partially sequenced genomes; only results stable with respect to topology and genome sampling were considered further. Evolution of selected genomic characters was visualized using Mesquite v2.75 (Maddison and Maddison, 2011). Habitat assignments (Supplementary File S2) found in the literature only allowed for distinguishing marine and non-marine habitats but this distinction was fully supported by isolation location, physiology and environmental sequencing wherever available (Supplementary File S3). Habitats with a salt concentration comparable to the sea were considered marine (Hiraishi and Ueda, 1994; Brinkhoff and Muyzer, 1997).

The EnzymeDetector (Quester and Schomburg, 2011) was used for initial enzyme annotations of the genomes, improved by strain-specific information from BRENDA and AMENDA (Schomburg et al., 2013), BrEPS (Bannert et al., 2010) and BLAST (Altschul et al., 1990) search against UniProt (UniProt Consortium, 2013). To validate the completeness of each proteome, its proportion of enzymes was calculated (see Supplementary File S2). Enzymes were mapped on MetaCyc pathways (Caspi et al., 2012) as previously described (Chang et al., 2015). A pathway with ⩾75% of its enzymes present was initially assumed to be present too; for pathways discussed in detail, this was manually refined considering the enzymes essential for pathway functionality. The genomic clusters of orthologous groups (COGs) were taken from Integrated Microbial Genomes (Mavromatis et al., 2009) Prodigal annotations (Hyatt et al., 2010). Plasmid replication systems and compatibility groups were determined as described (Petersen et al., 2009, 2011), as were flagellar gene clusters and flagellar types (Frank et al., 2015a). For details, as well as for tests for phylogenetic inertia (Diniz-Filho et al., 1998) and quantification of oligotrophy (Lauro et al., 2009), see Supplementary File S1.

Results and discussion

Genome-scale phylogenetic analysis
The core-genes analyses under LG (Le and Gascuel, 2008) as single ML model yielded seven maximally and consistently supported, maximally inclusive ingroup clades and four deeply branching strains (Figure 1, Supplementary File S3). Support was maximal for most branches in the distinct analyses (Supplementary File S3) but they differed regarding the backbone of the tree. The core-gene topology was almost identical to that of earlier phylogenomic analyses (Newton et al., 2010; Luo and Moran, 2014; Voget et al., 2015), provided the strains additionally included here were removed. Here the Roseobacter group appeared not monophyletic but

paraphyletic, as clade 2 harbouring the non-roseobacter genera was nested within this group.

All core-gene inference and bootstrapping approaches showed 95% support for strains HTCC2255 and HTCC2150 branching first. Support for Planktomarina temperata RCA23ᵀ and Litoreibacter arenae DSM 18583ᵀ also branching before clade 2 was similarly high but collapsed under ML when partition bootstrapping or individual substitution models were applied (Figure 1). Average support was also lower under these settings; for details, see Supplementary File S3, which also provides revised genus and species affiliations. The extracted 16S rRNA gene sequences showed >89% similarity between all Rhodobacteraceae, not only between roseobacters (Supplementary File S3). The overall best 16S rRNA gene trees were not significantly better than the best constrained trees, confirming that the gene does not significantly support the Roseobacter clade.

Phylogenetic inference without outgroup and LSD rooting yielded an ML topology (Supplementary File S3) identical to the one obtained by pruning the outgroup from the tree depicted in Figure 1, irrespective of whether or not the gene set was renewed after strain removal. The core-gene matrices reduced to 200 genes again showed the same topology, albeit a slightly reduced support, whereas with ⩽150 genes the backbone was not supported any more and distinct between ML and MP (Supplementary File S3). The topologies different from that in Figure 1 showed a monophyletic yet statistically unsupported Roseobacter clade and included a cluster comprising HTCC2255, HTCC2155, P. temperata RCA23 and L. arenae in conflict with their deep branching (as in Figure 1) in earlier phylogenomic studies (Newton et al., 2010; Luo et al., 2012; Luo and Moran, 2014; Voget et al., 2015).

After removing the outgroup and re-estimating the root with LSD, all alternative trees showed an ingroup topology as in Figure 1. The six trees inferred from larger matrices (Supplementary File S3) that showed the Roseobacter group as monophyletic were also in conflict with earlier studies and, in the LSD test, showed a paraphyletic Roseobacter group as in Figure 1. Confirming Siddall (2010), conflicting branch support, if any, never originated from partition bootstrapping; analogously, conflict with the topology in Figure 1 assessed in paired-site tests was never significant when each gene was treated as a single character. Such alternative tests for topologies might help tackling incongruence in genome-scale phylogenetic analyses (Jeffroy et al., 2006). The topologies from distinct gene selections differed mainly regarding the placement of the outgroup, and distant outgroups are particularly frequently subject to long-branch attraction (Bergsten, 2005). Whereas the outgroup chosen here is more closely related to Rhodobacteraceae than Escherichia coli as used in Newton et al. (2010), we would recommend sampling even more and particularly non-marine Rhodobacteraceae genome sequences in future
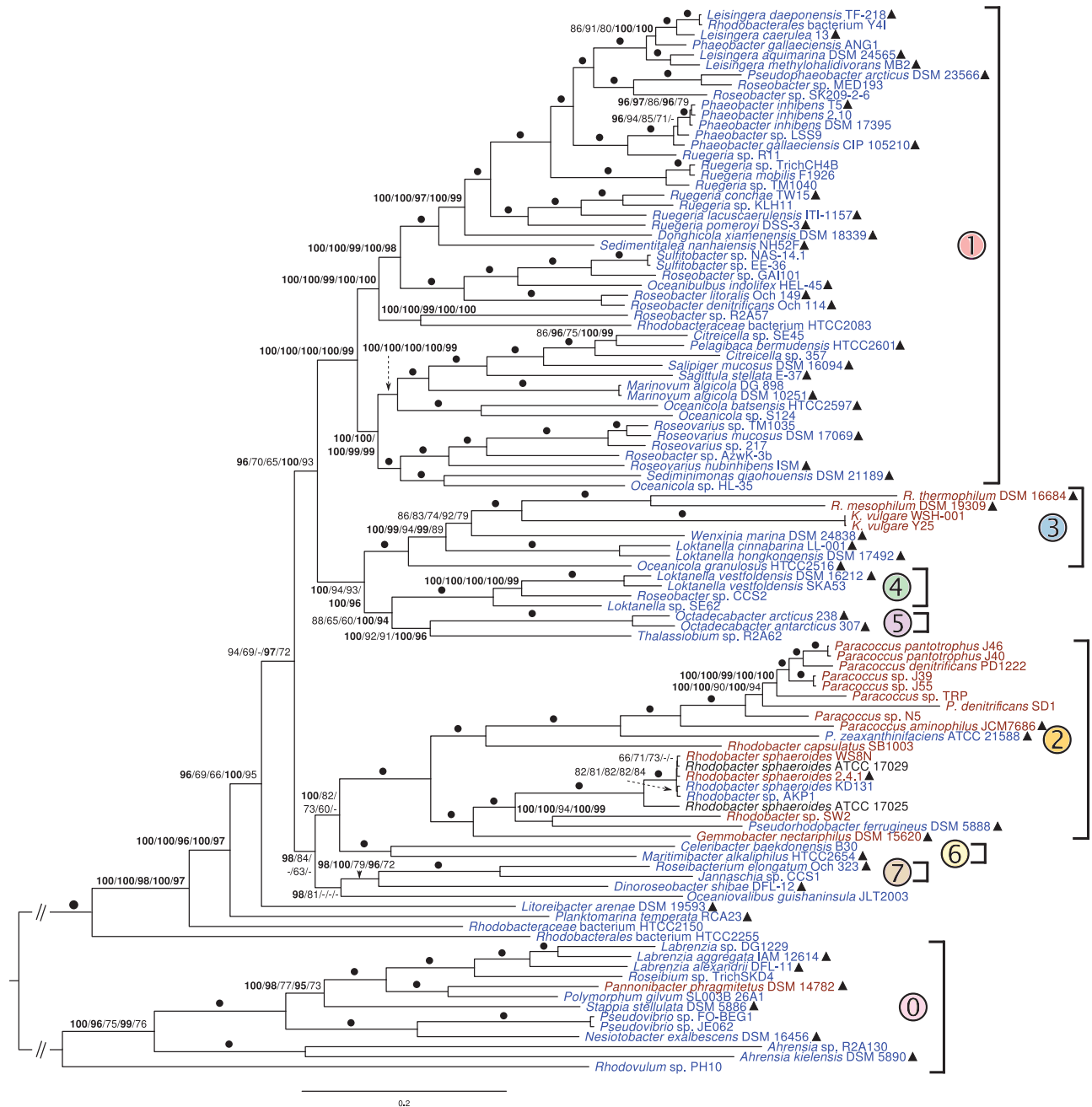
Figure 1 ML tree inferred from the core-gene matrix (208 genes, 80 578 characters) under a single overall model of amino-acid evolution and rooted with the included outgroup strains. The branches are scaled in terms of the expected number of substitutions per site; double slashes indicate branches shortened by 90%. Numbers above branches (from left to right) are bootstrapping support values if >60% from (i) ordinary bootstrap under ML with a single overall model of amino-acid evolution; (ii) ordinary bootstrap under ML with one model per gene; (iii) partition bootstrap under ML; (iv) ordinary bootstrap under MP; and (v) partition bootstrap under MP. Values >95% are shown in bold; dots indicate branches with maximum support under all settings. The inferred major clades are indicated with numbers and colours; clade 2 comprises all ingroup strains not assigned to the *Roseobacter* group. Triangles indicate type strains. The colours of the tip labels indicate the habitat: blue, marine; brown, non-marine; uncoloured, unknown.

studies. However, outgroup removal and LSD rooting indicated that the alternative topologies (Supplementary File S3) rather than the one in Figure 1 might suffer from long-branch attraction to the outgroup. The Genome BLAST Distance Phylogeny analysis of the larger data set also showed HTCC2255 branching first within the ingroup with high support, followed by HTCC2150 (Supplementary File S3).

Our extensive phylogenomic analyses hardly support a monophyletic *Roseobacter* group. Particularly the core-genes analysis, methodologically most similar to earlier studies, shows paraphyletic roseobacters. This finding is in conflict with literature claims but not actually with the underlying analyses (Buchan *et al.*, 2005; Newton *et al.*, 2010; Luo *et al.*, 2012; Luo and Moran, 2014; Pujalte *et al.*, 2014;

Voget *et al.*, 2015) because it is just due to increased strain sampling, particularly of *Paracoccus* and *Rhodobacter*. The tree shown in Figure 1 is not in conflict with previous analyses, whereas the topologies observed with some other gene sets are.

The *Roseobacter* group has been considered as the marine *Rhodobacteraceae* (Giovannoni and Rappé, 2000; Buchan *et al.*, 2005; Brinkhoff *et al.*, 2008; Luo *et al.*, 2012) but also includes non-marine genera such as *Rubellimicrobium* and *Ketogulonicigenium* (Buchan *et al.*, 2005; Brinkhoff *et al.*, 2008; Pujalte *et al.*, 2014). *Ketogulonicigenium*, however, also comprises marine ribotypes (Gifford *et al.*, 2014). *Paracoccus* and *Rhodobacter*, which form clade 2 in our analysis (Figure 1), are no roseobacters and mainly non-marine, whereas *P. zeaxanthinifaciens* ATCC 21588[T] (Berry *et al.*, 2003) and *R. sphaeroides* KD131 (Lim *et al.*, 2009) dwell in the sea. *P. denitrificans* has marine ribotypes (Gifford *et al.*, 2014), whereas strain PD1222 was derived from PD1001 (de Vries *et al.*, 1989), isolated from garden soil (Nokhal and Schlegel, 1983). Obviously, some *Paracoccus* and *Rhodobacter* strains became secondarily marine.

As a conclusion, definitions and uses of the terms 'Roseobacter' group' and 'Roseobacter' clade' need to be reconsidered. The *Roseobacter* clade is neither unambiguously supported by our in-depth analysis nor by previous studies, which either lacked branch support or sufficient strain sampling. We suggest using the term 'Roseobacter group', not 'clade', for the marine *Rhodobacteraceae*. This operational definition is consistent with the current general use of the name of the group and avoids overinterpreting the phylogenetic evidence. Our analysis further shows that a transition from marine to non-marine habitats occurred several times independently within *Rhodobacteraceae*. An equivalent step occurred only once in the evolution of the SAR11 clade, another prominent lineage of marine but strictly pelagic *Alphaproteobacteria* (Luo *et al.*, 2015). *Rhodobacteraceae* genomes often contain many transposable elements (Vollmers *et al.*, 2013), ECRs (Petersen *et al.*, 2013) and gene-transfer agents (Zhao *et al.*, 2009; Luo and Moran, 2014). The lack of these traits in the streamlined genomes of the SAR11 clade might explain just one transition between marine and freshwater habitats (Luo *et al.*, 2015). The more frequent habitat transitions within *Rhodobacteraceae* call for an analysis of the underlying genomic adaptations.

Genomic traits of marine versus non-marine *Rhodobacteraceae*

Altogether 1835 enzymes with distinct EC numbers were predicted, 106 in all strains and 419 in >90%.
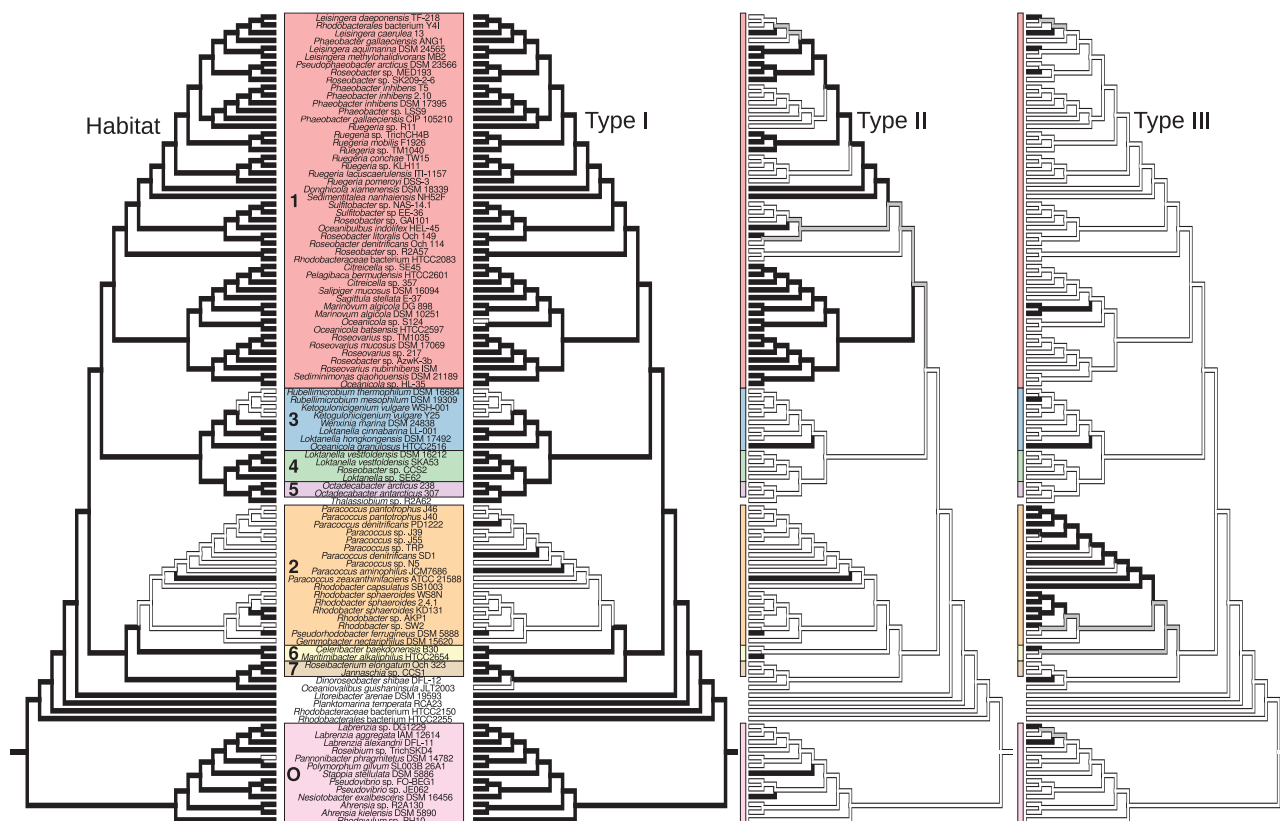

Figure 2 Ancestral character-state reconstruction under ordered MP for the presence (black) or absence (white) of H, marine or equivalent habitat; I, (S)-2-haloacid dehalogenase (EC 3.8.1.2); II, ectoine synthase (EC 4.2.1.108); and III, 6-phosphofructokinase (EC 2.7.1.11). The tree topology and the number and colour codes for the major clades are as in Figure 1. Grey shading indicates uncertainties in character-state assignment. The major types of phylogenetic distributions represented by the three genomic characters are: I, losses predominantly in non-marine strains; II, gains mainly in marine strains; and III, gains predominantly in non-marine strains.

Their proportion in each proteome ranged between 11.58% (*Paracoccus* sp. J55) and 24.05% (*Oceaniovalibus guishaninsula* JLT2003; for further details, see Supplementary File S2). The enzymes indicated 322 pathways overall, 18 present in all strains. The Integrated Microbial Genomes annotations yielded 4873 COGs overall, 345 present in all strains. Of the 106 strains, 85 were assigned to a marine or saline habitat, 19 to a non-marine one and for 2 a habitat assignment was not possible (Figure 1). Apparently, the marine state was lost and regained several times in evolution, even though it is phylogenetically conserved (Figure 2, Supplementary File S3). This result supports the claims of a predominantly marine roseobacter group, as the habitat can apparently be well predicted from the phylogenetic position of a strain and thus indirectly supports the reliability of our habitat assignments.

In contrast to χ^2 tests, considering phylogenies in comparative biology has a high chance to avoid false positives and false negatives (Pagel, 1994; Pagel *et al.*, 2004). The BayesTraits software estimates rates of changes in a phylogeny and, for a pair of discrete characters, statistically compares a model with independent rates of change to one with the changes in one character dependent on the states of the other. BayesTraits analyses allow for detecting genomic adaptations that accompany phylogenetically independent switches in habitat preferences. As the inferred tree topologies were not in agreement throughout (Figure 1, Supplementary File S3), only those BayesTraits results were considered further

Table 1 Selected genomic characters that were significantly ($\alpha = 0.01$) habitat-correlated in the BayesTraits tests under all conditions (for sulphoacetaldehyde acetyltransferase (EC 2.3.3.15) under most conditions), along with their overall type of evolution (as in Figure 2), sum of evolutionary rates estimated by BayesTraits indicating co-occurrence divided by overall sum of rates and percentage occurrences (on which the test is not based) in marine and non-marine strains

Feature	ID	Rate quotient	Marine	Non-marine
I. Lost in non-marine habitats				
Chloride channel protein	COG0038	0.834	96	32
Ca^{2+}/Na$^+$ antiporter	COG0530	0.783	99	42
NhaP-type Na$^+$/H$^+$ and K$^+$/H$^+$ antiporters	COG0025	0.829	76	21
(S)-2-haloacid dehalogenase	EC 3.8.1.2	0.751	94	21
Mercury (Hg) II reductase	EC 1.16.1.1	0.887	98	26
Carbon monoxide dehydrogenase	EC 1.2.99.2	0.654	93	42
Precorrin-8X methylmutase	EC 5.4.1.2	0.445	96	63
Precorrin-4 C11-methyltransferase	EC 2.1.1.133	0.687	95	68
Precorrin-6B methylase 2	COG2242	0.794	85	37
Predicted cobalamin binding protein	COG5012	0.596	90	42
Cobalamin biosynthesis protein CbiD	COG1903	0.774	38	5
Choline-glycine betaine transporter	COG1292	0.733	98	69
II. Gained in marine habitats				
Ectoine synthase	EC 4.2.1.108	0.979	41	0
Ectoine biosynthesis pathway	P101-PWY	0.954	38	0
Betaine-homocysteine *S*-methyltransferase	EC 2.1.1.5	0.763	25	0
Glycine betaine degradation	PWY-3661	0.883	94	74
Gamma butyrobetaine dioxygenase	EC 1.14.11.1	0.969	48	0
Trimethylamine-corrinoid protein Co-methyltransferase	EC 2.1.1.250	0.493	62	11
Trimethylamine-N-oxide reductase	EC 1.6.6.9	0.891	12	5
Probable taurine catabolism dioxygenase	COG2175	0.859	52	16
Nitrile hydratase	EC 4.2.1.84	0.749	62	0
Arylsulphatase	EC 3.1.6.1	0.752	61	11
Precorrin-3B synthase	EC 1.14.13.83	0.596	47	37
Unclear whether lost or gained				
ABC-type tungstate transport system, periplasmic component	COG4662	0.567	70	5
ABC-type tungstate transport system, permease component	COG2998	0.676	69	5
Sulphoacetaldehyde acetyltransferase	EC 2.3.3.15	0.434	69	53
Trimethylamine-N-oxide reductase (cytochrome c)	EC 1.7.2.3	0.763	34	26
III. Gained in non-marine habitats				
ABC-type sulphate transport system, permease component	COG4208	0.128	12	95
ABC-type sulphate transport system, periplasmic component	COG1613	0.129	10	95
ABC-type sulphate/molybdate transport systems, ATPase component	COG1118	0.13	10	95
Formaldehyde oxidation pathway II (glutathione-dependent)	PWY-1801	0.412	8	58
S-(hydroxymethyl)glutathione synthase	EC 4.4.1.22	0.372	8	63
Taurine dehydrogenase	EC 1.4.99.2	0.154	4	37
6-Phosphofructokinase	EC 2.7.1.11	0.398	16	58

P-values differ depending on the underlying tree and strain sampling and are provided in the according supplements. The characters are presence and absence of enzymes (as indicated by their EC number), COGs (as indicated by their COG number) and pathways (as indicated by their Metacyc IDs). Ancestral character-state reconstructions of the exemplary occurrences of (S)-2-haloacid dehalogenase (EC 3.8.1.2) for type I, ectoine synthase (EC 4.2.1.108) for type II and 6-phosphofructokinase (EC 2.7.1.11) for type III are depicted in Figure 2.

that were significant under all tested topologies and thus independent, for example, of a monophyletic or a paraphyletic *Roseobacter* group.

Up to 90 pathways were significantly habitat correlated (59 identified by all analyses; Supplementary File S4), 391 (255) enzymes (Supplementary File S4) and 563 (386) COGs (Supplementary File S4). As judged from the estimated rates of changes as well as MP reconstructions of ancestral character states on the major distinct topologies, significant characters exhibited three major types of phylogenetic distributions (Table 1, Figure 2, Supplementary File S3): (i) inheritance from the most recent common ancestor and loss in non-marine strains; (ii) absence in the ancestor, gain in marine strains; and (iii) absence in the ancestor, gain in non-marine strains. In few cases, ambiguity in character-state reconstruction prevented the distinction between types (i) and (ii).

Genomic traits predominantly lost in non-marine strains

The transition into non-marine habitats is reflected by adaptations to their different ecology and biogeochemistry such as losses (and lack of gains) of genetic traits directly related to the strongly reduced NaCl concentration from ~1.015 M in the sea to <10 mM in soil and freshwater habitats (Table 1, Figure 2). Marine bacteria require Na^+ for growth, and some use a Na^+ circuit for various functions (Kogure, 1998). The significant trend to evolutionary losses (and no gains) of Na^+ antiporters and a Cl^- channel protein in non-marine *Rhodobacteraceae* and thus their probably reduced capability to transport Na^+ and Cl^- across the cell membrane is consistent with the strongly reduced NaCl concentration in non-marine habitats and the fact that most *Paracoccus* and *Rhodobacter* strains do not require Na^+ for growth (Pujalte *et al.*, 2014).

The gene encoding (S)-2-haloacid dehalogenase showed a significant trend to be missing in non-marine genomes (Table 1). The great majority of organohalogens is produced by macroalgae, sponges, corals, tunicates, polychaetes and other marine organisms, even though terrestrial and freshwater cyanobacteria, fungi and bacteria can also produce them (Fielman *et al.*, 2001; Gribble, 2003, 2012). Particularly the halogenated metabolites produced by macroalgae are toxic for various animals and bacteria, such as *Vibrio* sp. and *Acinetobacter* sp. (Paul *et al.*, 2006; Cabrita *et al.*, 2010; Gribble, 2012). As many marine *Rhodobacteraceae* not only live in close association with microalgae and macroalgae, sponges and corals (Buchan *et al.*, 2005; Brinkhoff *et al.*, 2008; Raina *et al.*, 2009; Lachnit *et al.*, 2011; Webster *et al.*, 2011) but may also be exposed to particulate detrital and dissolved organohalogens, they presumably use dehalogenases for detoxifying and utilizing these compounds as substrates (Novak *et al.*, 2013). Conversely, the lack of (S)-2-haloacid

dehalogenase could reflect an adaptation to a reduced exposure to such toxic compounds.

The significantly correlated mercury-II reductase gene is missing in many non-marine but present in almost all marine strains (Table 1). Bacterial detoxification of mercury (Hg) by reducing oxidized Hg(I) to volatile Hg(0) is widely distributed in habitats with Hg(I) or Hg(II) (Barkay *et al.*, 2010). Under a reduced redox potential, mercury is present as Hg(0), and bacteria dwelling under these conditions often lack Hg reductase. The occurrence of non-marine *Rhodobacteraceae* such as *Paracoccus* in soil, compost or sewage under reduced or zero-oxygen conditions and of *Rhodobacter* in anaerobic freshwater conditions (Pujalte *et al.*, 2014) thus may explain their tendency to lose the ability to detoxify Hg(I/II).

The lack of the carbon monoxide dehydrogenase (CODH) gene was also significantly correlated with non-marine habitats (Table 1). It is often part of the bacterial enzyme complex to oxidize CO to CO_2 (King and Weber, 2007). The large subunit of the CODH complex (*coxL*) gene occurs in two forms. Nearly all marine *Rhodobacteraceae* harbour form II but only those which perform CO oxidation harbour form I (Cunliffe, 2011). Oxidation of this secondary green-house gas is an important process in pelagic marine ecosystems (Tolli *et al.*, 2006; Moran and Miller, 2007; Dong *et al.*, 2014), but the exact function of *coxL* form II is still unclear. Comparison with published sequences (King, 2003) showed that form I and form II were distinct clusters of orthologues in our data set; the BayesTraits test was always significant for form I, the one for form II only for half of the assessed combinations of trees and strain samplings (Supplementary File S4). Therefore, CO oxidation, an adaptation of a complementary mode of energy acquisition under nutrient-depleted marine conditions, appears unsuitable in non-marine lineages.

Absences of five genes encoding enzymes operating on cobalamin and precorrin and thus biosynthesis and binding of vitamin B_{12} were correlated with non-marine habitats (Table 1). The vitamin B_{12} biosynthetic pathway has previously been demonstrated in roseobacters (Newton *et al.*, 2010; Luo and Moran, 2014). In contrast to other major groups of marine bacteria such as the SAR11 clade and *Bacteroidetes* (Sañudo-Wilhelmy *et al.*, 2014), marine *Rhodobacteraceae* are major vitamin suppliers for B_{12}-auxotrophic prokaryotes and eukaryotic primary producers, such as chlorophytes, diatoms, dinoflagellates, coccolithophores and brown algae (Croft *et al.*, 2005; Wagner-Döbler *et al.*, 2010; Helliwell *et al.*, 2011; Bertand and Allen, 2012; Sañudo-Wilhelmy *et al.*, 2014). The loss of the ability to produce vitamin B_{12} in non-marine *Rhodobacteraceae* could be due to more dominant bacteria, adapted to lakes and soil for an evolutionary longer period and major other producers of this vitamin. In fact, it has been shown that the ability to

produce vitamin B12 can be lost rapidly in a freshwater alga when exposed to a continuous supply of B12 (Helliwell *et al.*, 2015). This scenario appears to be applicable to the non-marine *Rhodobacteraceae*. Soil *Rhizobiales* are known to produce vitamin B_{12} (Kazamia *et al.*, 2012; Sañudo-Wilhelmy *et al.*, 2014), whereas *Rhodobacter* often dwells in eutrophic lakes or biofilms where cyanobacteria as vitamin-B_{12} producers are abundant. Macrophytes not requiring vitamin B_{12} (Helliwell *et al.*, 2011) often dominate as primary producers in soil and freshwater ecosystems, which may also result in its reduced demand.

Gains and losses of both permease and periplasmic component of the ATP-binding cassette (ABC)-type tungstate transport system were significantly correlated to the habitat, with ambiguous MP reconstructions indicating either losses in non-marine *Rhodobacteraceae* or gains by marine ones (Table 1). Oxyanions of molybdenum (MoO_4^{2-}) and tungsten (WO_4^{2-}) are the main sources of these essential trace metals in bacterial cells (Johnson *et al.*, 1996; Hille, 2002). The ubiquitous Mo-containing enzymes have important roles in the global cycles of nitrogen, carbon and sulphur (Kisker *et al.*, 1997). High-affinity molybdate/tungstate ABC-type transporters (Schwarz *et al.*, 2007) allow bacteria to scavenge these oxyanions in the presence of sulphate, whose concentration in seawater is ca. 10^5 times higher than that of molybdate and ca. 10^8 times higher than that of tungstate (Bruland, 1983). In terrestrial ecosystems, the concentration of tungstate is much higher (Senesi *et al.*, 1988), and owing to its similarity to molybdate, both may be transported by the same carrier (Bevers *et al.*, 2006; Taveirne *et al.*, 2009; Gisin *et al.*, 2010). Therefore, loss of this highly specific transport system appears to be an adaptation to non-marine habitats.

Genomic traits predominantly gained in marine strains
The ectoine biosynthesis pathway and ectoine synthase distributions were significantly habitat-correlated; in contrast to the characters discussed above, MP reconstructions indicated the absence of ectoine synthesis in the common ancestor and gains predominantly in marine genomes (Table 1). Ectoine is a compatible solute that helps surviving extreme osmotic stress by acting as an osmolyte and is synthesized by a wide range of bacteria (Ventosa *et al.*, 1998; Reshetnikov *et al.*, 2004; Trotsenko *et al.*, 2007). Independent gains by several marine lineages (Figure 2) indicate that, whereas osmolytes are mandatory in the ocean, several alternatives can be used, as confirmed by the results for the following characters.

Carnitine, glycine betaine and proline are other osmolytes widespread in prokaryotes (Welsh, 2000; Hoffmann and Bremer, 2011). The enzyme mediating the last step in the biosynthesis of carnitine, gamma butyrobetaine dioxygenase, shows the same type of evolutionary changes as the ectoine-related characters (Table 1). Carnitine can also be taken up by a betaine/carnitine/choline transporter (Lidbury *et al.*, 2014), which was present in the ancestor and predominantly lost by non-marine strains (Table 1). Thus marine *Rhodobacteraceae* originally relied on carnitine uptake and tended to additionally gain the ability to synthesize it.

Carnitine can be decomposed by various ways to glycine betaine (Kleber, 1997; Welsh, 2000; Wargo and Hogan, 2009). Catabolism of glycine betaine and the enzyme mediating its first step, betaine-homocysteine *S*-methyltransferase, show gains by marine strains (Table 1). In contrast, almost all genomes encoded ABC transporters for proline/glycine betaine such as COG4176, which do not show a significant relationship to the habitat (Supplementary File S4). As biosynthetic traits for the osmolytes ectoine and carnitine were lost and gained several times independently by marine strains, they might be exposed to distinct magnitudes of osmotic stress, as typical for coastal areas.

Genes encoding enzymes involved in the reduction of trimethylamine-*N*-oxide (TMAO) and demethylation of trimethylamine (TMA) were significantly correlated with the habitat, with gains mainly occurring in the ocean (Table 1). TMAO is well known as terminal electron acceptor in anaerobic microbial respiration (Arata *et al.*, 1992; Gon *et al.*, 2001) and as osmolyte in a variety of marine biota (Seibel and Walsh, 2002; Gibb and Hatton, 2004; Treberg *et al.*, 2006). A TMAO-specific ABC transporter and genes encoding TMAO decomposition via TMA, dimethylamine and monomethylamine are widespread in marine metagenomic libraries and bacteria, including roseobacters and the SAR11 clade (Lidbury *et al.*, 2014, 2015). Most marine *Rhodobacteraceae* can use TMA and TMAO as sole N source and probably oxidize the methyl groups to CO_2 as a complementary pathway to conserve energy (Lidbury *et al.*, 2015), whereas *Roseovarius* can even use TMA and TMAO as sole C source (Chen, 2012). Such traits are unknown from aerobic freshwater environments (Treberg *et al.*, 2006).

The probable dioxygenase as a key enzyme in taurine catabolism was gained significantly often during transitions to marine habitats (Table 1). Taurine is an important organosulphur compound and compatible solute in marine and freshwater invertebrates and fish (Treberg *et al.*, 2006; Lidbury *et al.*, 2015). Freshwater and soil bacteria use taurine predominantly as S but not as C source, whereas marine bacteria, including *Rhodobacteraceae*, can use taurine both as S and as C and energy source (King and Quinn, 1997; Kertesz, 2000). In fact, taurine ABC transporters are major transport systems in marine bacterial communities comprising large proportions of *Alphaproteobacteria*, including *Rhodobacteraceae* (Gifford *et al.*, 2012; Williams *et al.*, 2012). Taurine as S source requires an oxygenation with taurine dioxygenase as key enzyme and

cleavage to aminoacetaldehyde and sulphite (Kertesz, 2000). This pathway might be encoded as COG2175 (probable taurine catabolism dioxygenase). However, taurine dioxygenase (EC 1.14.11.17) was not significantly habitat-correlated (Supplementary File S4). Taurine as C and energy source can be metabolized via two pathways (Kertesz, 2000; Denger et al., 2009). One involves taurine-pyruvate aminotransferase (EC 2.6.1.77) as key enzyme to generate alanine and sulphoacetaldehyde. This pathway was encoded in most genomes, and its gains and losses showed no significant dependency on the habitat (Supplementary File S4). In an alternative pathway, taurine is directly deaminated to sulphoacetaldehyde by a dehydrogenase, which was significantly habitat-correlated but more frequent in non-marine strains (Table 1). In both pathways, sulphoacetaldehyde is cleaved to acetylphosphate and sulphite by a sulphoacetaldehyde acetyltransferase, which was significantly habitat-correlated in the majority of the analyses and more frequently occurred in marine Rhodobacteraceae (Table 1). Thus taurine seems more important as C and energy source than as a general S source, with significant differences in the metabolic pathways between marine and non-marine strains.

An arylsulphatase was gained significantly more frequently in the sea (Table 1). Arylsulphatases cleave sulphate from phenolic compounds and, in a fungus, from breakdown products of the polysaccharide fucoidan, that is, sulphated fucose as monomers or oligomers (Shvetsova et al., 2015). Fucoidan is a major component of marine macroalgae, including Fucus, Laminaria and Macrocystis (Deniaud-Bouët et al., 2014), but not known from freshwater or soil. Marine Rhodobacteraceae are major colonizers of Fucus and other macroalgae and can grow on fucoidan (Lachnit et al., 2011; Bengtsson et al., 2011). If their arylsulphatase could also target fucoidan, marine Rhodobacteraceae would apparently benefit directly on macroalgae or detrital particles and colloids by utilizing breakdown products of fucoidan after cleaving the sulphate group.

Gains of nitrile hydratase were also correlated with marine habitats (Table 1). This enzyme is involved not only in acrylonitrile degradation (Kato et al., 2000) but also in the indole-3-acetonitrile pathway for the biosynthesis of indole-3-acetic acid (IAA), a plant hormone. Three pathways can lead to IAA, which is synthesized by Rhizobiaceae (Ghosh et al., 2011) and by a marine Sulfitobacter strain, enhancing growth of the abundant diatom Pseudonitzschia multiseries (Amin et al., 2015). In marine metatranscriptomic data sets from the Pacific, Roseobacter group-specific transcripts of all three IAA biosynthetis pathways were detected, mainly from the indole-3-acetonitrile pathway (Amin et al., 2015). This is consistent with IAA biosynthesis being significantly gained by marine Rhodobacteraceae, and its potential role in their symbiosis with phytoplankton and possibly also macroalgae, corresponding to the role of Rhizobiaceae for higher plants.

## Genomic traits predominantly gained in non-marine strains

Similar to the characters discussed above, the following ones were significantly phylogenetically correlated with the habitat but tended to be gained by non-marine Rhodobacteraceae (Table 1), as revealed by the estimated rates of changes and the MP reconstructions (Figure 2). These genomic traits must be interpreted particularly carefully because most Rhodobacteraceae are marine and thus the non-marine ones are less representative for the multitude of non-marine bacteria than the ones dwelling in the ocean for the plethora of marine strains.

Distinct genes encoding ABC sulphate transporters showed a significant and positive relationship with non-marine habitats. This markedly differs from a sulphate permease of the SulP superfamily (COG0659) encoded in almost all genomes, which showed no habitat correlation (Supplementary File S4) and is also widespread among other bacteria (Aguilar-Barajas et al., 2011). Sulphate concentrations in marine waters are around 28 mM, whereas in common freshwater systems they are in the sub-millimolar range (Wetzel, 2001). A marine Rhodobacter strain can take up sulphate at concentrations ranging from 50 μM to 2 mM, obviously applying two transport systems with different affinities (Imhoff et al., 1983; Warthmann and Cypionka, 1996). This is in line with the finding that non-marine rather than marine Rhodobacteraceae harbour high-affinity sulphate uptake systems in addition to sulphate permease to cope with the low sulphate concentrations in freshwaters. For many marine Rhodobacteraceae, sulphate is probably not the major S source because uptake of dimethylsulphoniopropionate, occurring at concentrations in the nanomolar range, meets most of their sulphur demand (Simo et al., 2002; Malmstrom et al., 2004; Moran et al., 2012). Marine dimethylsulphoniopropionate is the major global source of organic sulphur, including the climatically relevant dimethylsulphide, whereas this and other organic S compounds are also produced in freshwater and terrestrial systems but to much lower extent (Schäfer et al., 2010). Accordingly, we did not find a significant dependency of the occurrence of genes encoding dimethylsulphoniopropionate decomposition and the habitat (Supplementary File S4).

Gains of S-(hydroxymethyl)glutathione synthase were also significantly related to non-marine habitats (Table 1). This enzyme catalyses the first of the three reactions of the equally significant glutathione-dependent formaldehyde oxidation II pathway for detoxifying formaldehyde (Gonzalez et al., 2006). For methylotrophic bacteria such as Paracoccus or Rhodobacter, formaldehyde is also a central intermediate for oxidizing methanol or methylamine (Ras et al., 1995; Harms et al., 1996; Barber et al., 1996; Chistoserdova, 2011). Although the enzymes catalysing the second and third reaction were

encoded in almost all genomes and showed no dependency on the habitat (Supplementary File S4), *S*-(hydroxymethyl)glutathione synthase has mainly

been gained in *Paracoccus* and *Rhodobacter*. The first reaction of the formaldehyde oxidation pathway II is spontaneous *in vivo* but can be
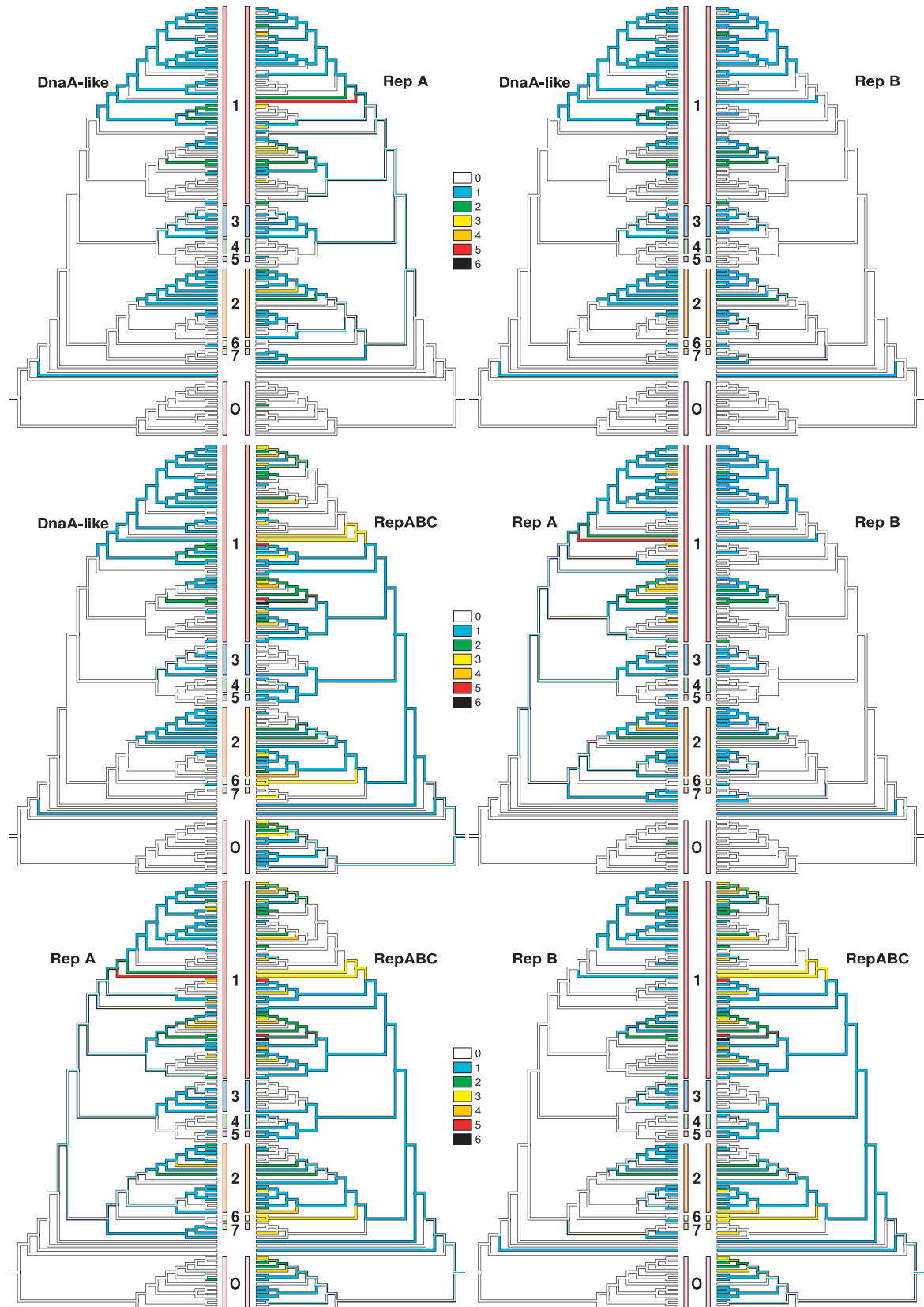
accelerated by the enzyme (Goenrich et al., 2002). It might be present only in strains that produce and consume large amounts of intracellular formaldehyde, whereas the spontaneous reaction could be sufficient for detoxifying exogenous formaldehyde (Goenrich et al., 2002). Roseobacters might use methanol as a supplementary energy rather than C source (Chen, 2012), hence the significant correlation with non-marine habitats might be due to the specific physiologies of Paracoccus and Rhodobacter.

Non-marine habitats were significantly related to gains of the gene encoding 6-phosphofructokinase (Table 1), the key enzyme of glucose metabolism via the Embden–Meyerhof–Parnas pathway (EMPP) (Flamholz et al., 2013). Marine Rhodobacteraceae, Gammaproteobacteria and Flavobacteria lack the EMPP and catabolize glucose exclusively via the Entner–Doudoroff pathway (EDP; Klingner et al., 2015), thus yielding slightly less ATP but, in contrast to the EMP, also NADPH (Flamholz et al., 2013). Klingner et al. (2015) showed that, even when the EMPP was additionally encoded in the genome, glucose was completely catabolized via the EDP. Rhodobacter sphaeroides behaves similarly (Fuhrer et al., 2005). Use of the EDP was interpreted as protection against oxidative stress, which is of major importance in near-surface marine habitats (Klingner et al., 2015). The EDP is evolutionary older (Romano and Conway, 1996), in accordance with its wide distribution in Proteobacteria (Flamholz et al., 2013) and its presence in ancestral Rhodobacteraceae as shown here. Apparently 6-phosphofructokinase was acquired several times independently by Rhodobacteraceae, most prominently in lineages with non-marine strains (Figure 2). Thus our analysis fully supports previous physiological studies and highlights the evolutionary importance of the EDP for the breakdown of glucose in the sea.

Genomic traits unrelated to habitats
The comparison of habitat-related characters with genomic traits such as ECRs that show other distributional types provides additional valuable insights. The current study represents the most comprehensive comparison of ECRs in Rhodobacteraceae and revealed up to 12 replicons (chromosome, chromids, plasmids; Harrison et al., 2010) in a single bacterium (Supplementary File S2), in agreement with previous results (Pradella et al., 2004, 2010; Frank et al., 2015a), as well as 53 DnaA-like, 79 RepA, 52 RepB and 140 RepABC replication modules. Their phylogeny-based classification indicated distinct compatibility groups in the outgroup (Supplementary File S2), whereas Bayes-Traits analysis showed no correlation between ECRs

and habitat (Supplementary File S5). The replication systems were not distinct between marine and non-marine Rhodobacteraceae (Petersen et al., 2009), in line with our phylogenetic findings (Figure 1).

Occurrences of ECR types DnaA-like, RepA and RepB but not RepABC significantly depended on each other (Figure 3, Supplementary File S5). Only RepB and DnaA-like showed a significant phylogenetic inertia under all conditions (Supplementary File S3). An explanation might be that RepABC-type ECRs do not frequently occur on chromids (Harrison et al., 2010) but on genuine plasmids (Dogs et al., 2013; Frank et al., 2014), which often contain type-IV secretion systems enabling horizontal transfer via conjugation (Petersen et al., 2013). Significant positive correlations between ECRs and type-IV secretion systems were indeed found (Supplementary File S2). The significant positive correlation between the dTDP-L-rhamnose biosynthesis pathway and RepA (Supplementary File S6) is in agreement with previous studies (Frank et al., 2015b; Michael et al., 2016), much like the one between the archetypal type-1 flagellum and TDP-L-rhamnose biosynthesis (Frank et al., 2015a, b). L-rhamnose is essential for proteobacterial surface polysaccharides (Giraud and Naismith, 2000) and the typical swim-or-stick lifestyle of surface-associated roseobacters (Belas et al., 2009; Michael et al., 2016).

The oligotrophy index neither exhibited a habitat-specific distribution nor phylogenetic inertia (Supplementary File S2), in line with the distinct positions of oligotrophic, genome-streamlined strains such as Planktomarina temperata RCA23[T], HTCC2255, HTCC2150, Oceanicola batsensis HTCC2597[T], HTCC2083 and Sulfitobacter sp. NAS-14.1 (Figure 1). Evolutionary plasticity regarding the oligotrophic lifestyle might have helped Rhodobacteraceae to dwell in diverse habitats. Genome size and the number of replication systems were not correlated with oligotrophy, but its variance decreased for large genomes (Supplementary File S3). This result does not support the finding of Lauro et al. (2009), possibly because not only oligotrophy but also eutrophic and constant environments can cause genome streamlining (van de Guchte et al., 2006; Giovannoni et al., 2014).

Conclusion

Our comprehensive analysis provides new insights into phylogenomics and the evolutionary adaptation of Rhodobacteraceae to marine and non-marine habitats. It builds on previous phylogenomic analyses of the Roseobacter group, extends them to the entire Rhodobacteraceae and elucidates that

Figure 3 Ancestral character-state reconstruction under ordered MP for the number of ECR replicases of the distinct types DnaA, RepA, RepB and RepABC and according pairwise phylogenetic cross-comparisons of their abundances in each genome. For the labels of the tips, see Figure 2, where the same tree topology is depicted in exactly the same layout. The number and colour codes between the trees refer to the clades as indicated in Figure 1 (O = outgroup). The colours depicted on the topologies indicate the number of replicases of each type as follows: white, 0; blue, 1; green, 2; yellow, 3; orange, 4; red, 5; and black, 6. Presences and absences alone are correlated between DnaA, RepA and RepB but not between RepABC and the others.
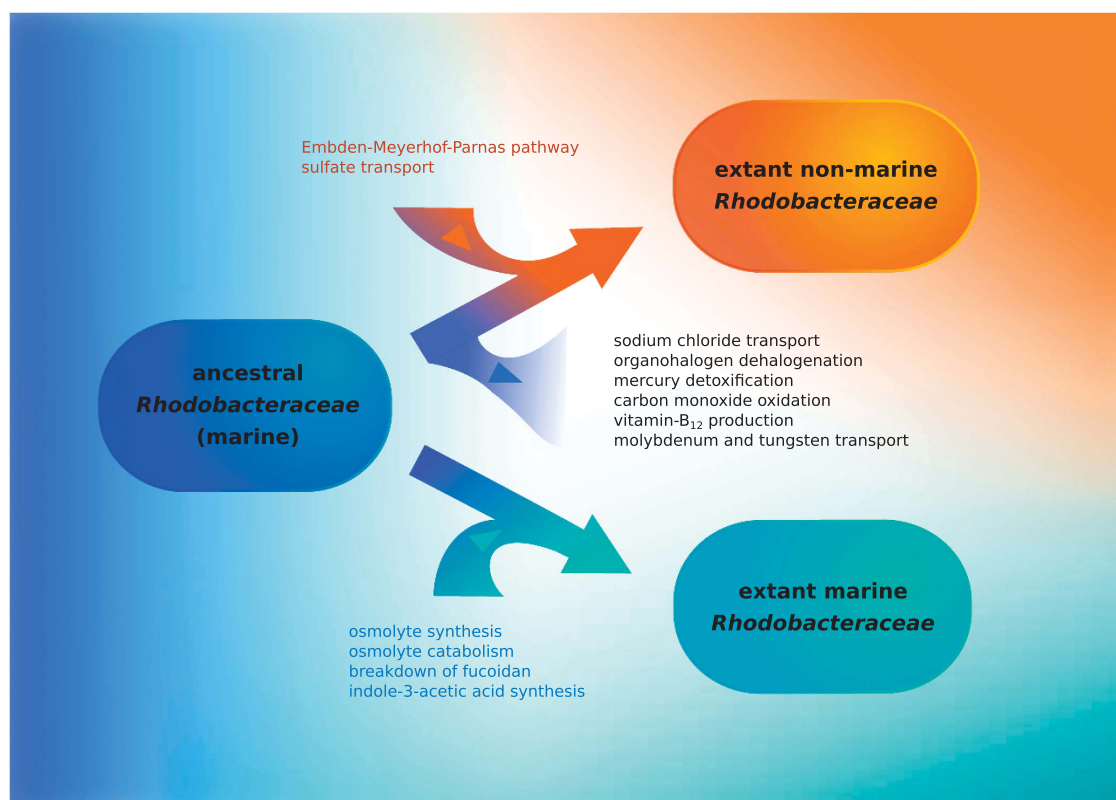
Figure 4 A summary of the interpretation of the genetic traits of the most recent marine ancestor of *Rhodobacteraceae* significantly gained or lost during adaptation to non-marine and gained during better adaptation to marine habitats.

evolutionary adaptations to marine and non-marine habitats of the most recent common ancestor were accompanied by distinct gains and losses of genes (summarized in Figure 4), obviously improving fitness of these lineages in these habitats. Of course, not all genomic features of relevance could be addressed here. For instance, quite a few roseobacters are well known to carry out aerobic anoxygenic photosynthesis (Wagner-Döbler and Biebl, 2006; Voget *et al.*, 2015). However, non-marine *Rhodobacteraceae* do not carry out this form of energy acquisition (Koblizek, 2015), and *Rhodobacter* is even an archetypal anaerobic anoxygenic photosynthetic bacterium (Koblizek, 2015). Therefore, this trait was beyond the scope of our study. Nevertheless, our comprehensive analysis forms a profound and stimulating basis for further and refined research on specific aspects of these adaptations.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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