Phylogenetic constraints on elemental stoichiometry and resource allocation in heterotrophic marine bacteria

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Summary

The objective of this study was to evaluate the contribution of evolutionary history to variation in the biomass stoichiometry and underlying biochemical allocation patterns of heterotrophic marine bacteria. We hypothesized that phylogeny significantly constrains biochemical allocation strategy and elemental composition among taxa of heterotrophic marine bacteria. Using a 'common-garden' experimental design, we detected significant interspecific variation in stoichiometry, macromolecule allocation and growth rate among 13 strains of marine *Proteobacteria*. However, this variation was not well explained by 16S rRNA phylogenetic relationships or differences in growth rate. Heterotrophic bacteria likely experience C-limitation when consuming resources in Redfield proportions, which consequently decouples growth rate from allocation to rRNA and biomass P content. Accordingly, overall bacterial C : nutrient ratios (C : P = 77, C : N = 4.9) were lower than Redfield proportions, whereas the average N : P ratio of 17 was consistent with the Redfield ratio. Our results suggest that strain-level diversity is an important driver of variation in the C : N : P ratios of heterotrophic bacterial biomass and that the potential importance of non-nucleic acid pools of P warrants further investigation. Continued work clarifying the range and controls on the stoichiometry of heterotrophic marine bacteria will help improve understanding and predictions of global ocean C, N and P dynamics.

Introduction

Current models of ocean biogeochemistry assume that carbon (C), nitrogen (N) and phosphorus (P) cycle according to the 'Redfield ratio' (molar C : N : P = 106:16:1) (Moore *et al.*, 2004; Aumont and Bopp, 2006; Follows *et al.*, 2007). This canonical ratio was derived from Alfred Redfield's observations of the stoichiometric similarity between dissolved nutrients in seawater and the biomass of marine plankton (Redfield, 1934). Subsequent studies investigating the biomass stoichiometry of marine plankton have detected variability across space and time, as well as between different species (e.g. Karl *et al.*, 2001; Michaels *et al.*, 2001; Quigg *et al.*, 2003; 2011; Martiny *et al.*, 2013a). Most of these studies have examined autotrophic phytoplankton, yet the contribution of heterotrophic bacteria to marine microbial biomass can occasionally surpass that of autotrophic phytoplankton (Ducklow, 1999; Pomeroy *et al.*, 2007; Buitenhuis *et al.*, 2012). Compared with that of autotrophs, the stoichiometry of heterotrophic bacteria remains poorly understood.

In marine systems, remineralization of organic C, N and P is primarily driven by heterotrophic bacteria (Cotner and Biddanda, 2002; Kirchman, 2008) and is influenced by the stoichiometry of microbial biomass. For example, physiological regulation of biomass C : N : P composition relative to environmental supply determines whether marine bacteria are sources or sinks of mineral nutrients (Goldman *et al.*, 1987; Tezuka, 1990). Bacterial stoichiometry also influences the trophic transfer of nutrients when bacteria are consumed (Güde, 1985; Martinussen and Thingstad, 1987; Shannon *et al.*, 2007). Additionally, the elemental composition of bacterial biomass can impact the autotrophic component of the food web by affecting the nutrient that limits phytoplankton growth (Daufresne and Loreau, 2001; Danger *et al.*, 2007).

Observed variation in C : N : P stoichiometry of marine plankton communities is likely due to a combination of physiological plasticity and phylogenetic constraints. For marine phytoplankton, classic experiments demonstrated that nutrient supply ratio could drive biomass stoichiometry (Rhee, 1978). The flexibility in
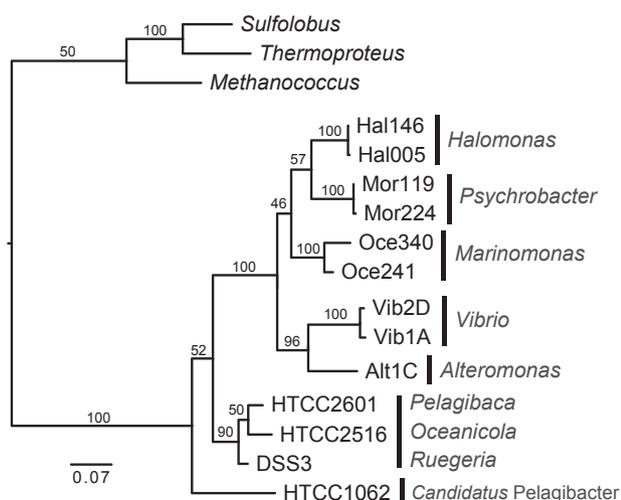
Fig. 1. Phylogenetic relationships between the bacterial strains used in this study inferred by maximum-likelihood estimation from 16S rRNA gene sequence comparisons. Bars show taxonomic associations at the family level with genus names on the right. Three archaeal sequences, *Thermoproteus tenax*, *Sulfolobus solfataricus* and *Methanococcus vannielii*, were used as an outgroup. Values at the nodes show the bootstrap support from 100 resampled data sets. Scale bar represents branch length.

stoichiometric ratios (plasticity) is dependent on growth rate, with more constrained ratios at maximum growth rates (Klausmeier *et al.*, 2008). Evolutionary history (phylogeny) imposes taxon-specific constraints on plasticity (Quigg *et al.*, 2003; 2011), resulting from phylogenetically conserved differences among taxa in cellular architecture or ecological strategy. Accordingly, phytoplankton taxonomic composition can be a significant determinant of community biomass stoichiometry (Arrigo, 1999; Weber and Deutsch, 2010) and may be a significant contributor to global patterns in marine C : N : P ratio (Martiny *et al.*, 2013a).

Differences in stoichiometry due to physiological plasticity or phylogenetic conservation are likely linked to differences in biochemical allocation of cellular resources. Variation in cellular elemental composition is controlled by the differential partitioning of C, N and P resources to various macromolecules according to ecological strategy (Klausmeier *et al.*, 2004; 2008; Arrigo, 2005). For example, the growth rate hypothesis (GRH) predicts that greater allocation to P-rich ribosomes for growth will drive a cell to become enriched in P, reducing overall biomass C : P and N : P ratios (Elser *et al.*, 1996; Sterner and Elser, 2002). However, evidence for the GRH has been derived largely from studies of zooplankton, phytoplankton or *Escherichia coli* (Elser *et al.*, 2000; 2003). It remains unclear to what extent the GRH applies to marine heterotrophic bacteria, and consequently, to what extent macromolecule allocation is linked to the biomass stoichiometry of marine bacteria.

Thus far, studies investigating the biomass stoichiometry of marine heterotrophic bacteria have been sparse and disparate (reviewed in Fagerbakke *et al.*, 1996; Fukuda *et al.*, 1998; Hochstädter, 2000). Evidence from several classic studies has shown that marine bacteria tend to be enriched in N and P relative to C, with C : N : P ratios below Redfield (Bratbak, 1985; Goldman *et al.*, 1987; Fagerbakke *et al.*, 1996; Goldman and Dennett, 2000; Vrede *et al.*, 2002), though substantial variation has been reported (Fukuda *et al.*, 1998; Løvdal *et al.*, 2008; Chan *et al.*, 2012). Furthermore, bacterial C : N ratios appear to be more constrained (Goldman *et al.*, 1987; Goldman and Dennett, 2000) and C : P ratios to be more flexible (Bratbak, 1985; Cotner *et al.*, 2010). These studies have demonstrated that like that of phytoplankton, the biomass stoichiometry of marine bacteria can vary in response to physical (e.g. temperature), chemical (e.g. resource ratio) and physiological (e.g. growth phase) parameters, as well as among species. However, no study has yet reported on whether variation in elemental ratios and macromolecule allocation in marine heterotrophic bacteria is phylogenetically conserved.

Therefore, the objective of our study was to explicitly evaluate the contribution of phylogeny (evolutionary history) to variation in biomass stoichiometry and underlying biochemical allocation patterns in marine bacteria. We tested the hypothesis that phylogeny significantly constrains biochemical allocation strategy and elemental composition among taxa by cultivating diverse strains of marine bacteria under standardized conditions. This 'common-garden' approach was designed to minimize the effect of physiological plasticity and isolate inherent taxonomic differences in allocation and stoichiometry.

Results

Using a common-garden design with standardized resource and growth conditions, we evaluated the relationships among biomass stoichiometry, macromolecule allocation, growth and phylogeny of 13 bacterial strains from two classes (*Alphaproteobacteria* and *Gammaproteobacteria*) within the *Proteobacteria* phylum, representing 7 prokaryotic families (Fig. 1). The strains included a representative of the SAR11 cluster, *Candidatus* Pelagibacter ubique strain HTCC1062 (Rappé *et al.*, 2002; Giovannoni *et al.*, 2005), as well as three members of the marine *Roseobacter* group: *Oceanicola granulosus* HTCC2516, *Pelagibaca bermudensis* HTCC2601 and *Ruegeria pomeroyi* DSS-3 (Cho and Giovannoni, 2004; 2006; Moran *et al.*, 2004). The remaining strains were isolated from coastal California seawater (see Supporting Information).

Biomass stoichiometry and relation to Redfield

Overall, marine bacteria tended to have low C : P and C : N ratios in relation to Redfield (molar C : N : P = 106:16:1) when supplied with carbon and nutrients in Redfield proportions. Strain-specific C : P ratio varied from 36 (HTCC1062) to 141 (DSS-3), with a geometric mean C : P ratio of 77 across all strains, significantly below the Redfield value of 106 ($P = 0.048$, Wilcoxon test, $n = 13$; Table 1). Nine strains had C : P ratios lower than Redfield (Fig. 2a), though only five differences were statistically significant (HTCC2516, Alt1C, Vib1A, Hal146, Hal005; $P < 0.05$), and one was marginally significant (HTCC1062; $P = 0.06$). Mor119 did not differ significantly from Redfield C : P ($P = 0.88$), while DSS-3, HTCC2601 and Vib2D had C : P ratios that were greater ($P < 0.05$, except $P = 0.06$ for Vib2D). The C : N ratio was more consistent across strains than C : P (Fig. 2c). The geometric mean ratio was significantly lower than Redfield (6.63) at 4.91 ($P = 0.001$, $n = 12$), and strain C : N ranged from 4.08 (Alt1C) to 7.35 (Vib2D). Nearly all strains had a C : N ratio below Redfield ($P < 0.05$, except $P = 0.06$ for Oce241, Oce340, Mor224 and Mor119), with the exceptions of DSS-3, which was not significantly different ($P = 0.81$), and Vib2D, which was greater ($P = 0.06$).

The geometric mean N : P molar ratio across all strains was 16.6, nearly equal to Redfield ($P = 0.42$, n = 12; Fig. 2b; Table 1), with N : P ranging from 9.6 (Alt1C) to 25 (Mor119). However, no individual strain N : P ratio was equivalent to Redfield. Five strains (HTCC2516, Alt1C, Vib1A, Hal146 and Mor224) had an N : P ratio below 16 ($P < 0.05$, except $P = 0.06$ for Mor224), while the remaining 7 strains had N : P ratios greater than Redfield ($P < 0.05$, except $P = 0.06$ for Vib2D, Oce241, Oce340 and Mor119).

C : P ($K = 0.05$, $P = 0.42$), N : P ($K = 0.03$, $P = 0.86$) and C : N ($K = 0.16$, $P = 0.34$) ratios were weakly associated with phylogeny, as determined by phylogenetic signal analysis using Blomberg's K-statistic (Blomberg *et al.*, 2003), which assumes a Brownian motion model of trait evolution. Non-parametric analysis of variance confirmed that C : P, N : P and C : N ratios varied significantly among the strains in our study ($P < 0.001$, Kruskal–Wallis ANOVAs); therefore, this weak association was not due to lack of variation.

Growth rate and allocation

Similarly to biomass stoichiometry, growth rate and biochemical allocation (whether normalized to cell abundance or C biomass) were weakly associated with phylogeny, though strains varied significantly in each trait ($P < 0.001$, Kruskal–Wallis ANOVAs). Growth rate varied among strains from 0.009 to 0.621 h^{-1} (HTCC1062 and Vib1A, respectively), with an overall mean of 0.227 h^{-1} (Fig. 3; Table 1). Growth rate differences among strains did not show significant phylogenetic signal ($K = 0.05$, $P = 0.69$), implying that evolutionary history was not a strong predictor of measured growth rate for this group of organisms. Likewise, differences in cellular C biomass (Table 1), a proxy for cell size, were not phylogenetically conserved ($K = 0.06$, $P = 0.65$), despite significant variation across strains ($P < 0.001$).

We also measured the concentration of DNA, RNA and proteins. Average cell quota for DNA among the

Table 1. Comparison of mean[a] growth rate; cellular macromolecule content; RNA : DNA ratio; percent total phosphorus (P) in RNA; cellular C, N and P quotas; and molar element ratios for strains of marine bacteria in this study.

Isolate	N	Growth rate (hr^{-1})	Macromolecule[b] (fg $cell^{-1}$)			RNA : DNA ratio	% P from RNA	Element quota (fg $cell^{-1}$)			Molar ratio		
			DNA	RNA	Protein			C	N	P	C : N	C : P	N : P
HTCC1062	6	0.009	NA	NA	NA	NA	NA	32.2	NA	2.9	NA	36	NA
DSS-3	5	0.210	2.56	27.4	62.9	10.7	99.3%	142	25.1	2.6	6.61	141	21
HTCC2516	6	0.125	1.88	9.33	30.5	4.9	19.8%	94.8	21.7	4.4	5.12	55	11
HTCC2601	6	0.293	3.21	30.0	78.5	9.1	74.0%	172	35.9	3.7	5.56	121	22
Alt1C	6	0.323	3.65	12.3	71.4	3.4	19.8%	88.6	25.3	5.8	4.08	39	9.6
Vib1A	6	0.621	3.08	6.35	66.6	2.1	11.0%	129	34.1	5.4	4.42	62	14
Vib2D	4	0.200	2.39	3.02	66.2	1.3	9.1%	168	26.8	3.1	7.35	139	19
Oce241	4	0.233	2.48	4.10	53.9	1.7	16.3%	86.0	21.7	2.4	4.63	94	20
Oce340	4	0.246	6.66	64.8	129	9.5	103%	203	51.4	5.9	4.60	89	19
Mor224	4	0.225	2.85	9.64	71.9	3.3	6.3%	292	74.3	14	4.57	54	12
Mor119	4	0.041	0.65	0.55	41	0.86	1.3%	163	48.9	4.1	4.08	103	25
Hal005	5	0.332	4.53	41.3	143.4	9.1	72.1%	190	49.8	5.6	4.47	92	21
Hal146	5	0.088	2.87	4.14	97.4	1.5	7.7%	127	33.7	5.0	4.40	65	15
Grand Mean		0.227	3.07	17.7	76	4.8	37%	145	37.4	5.0	4.91	77	17

a. Values shown represent arithmetic means, except that geometric means are reported for the elemental ratios.
b. Macromolecule mass based on standard curve concentrations of λ dsDNA, *E. coli* rRNA or BSA protein.
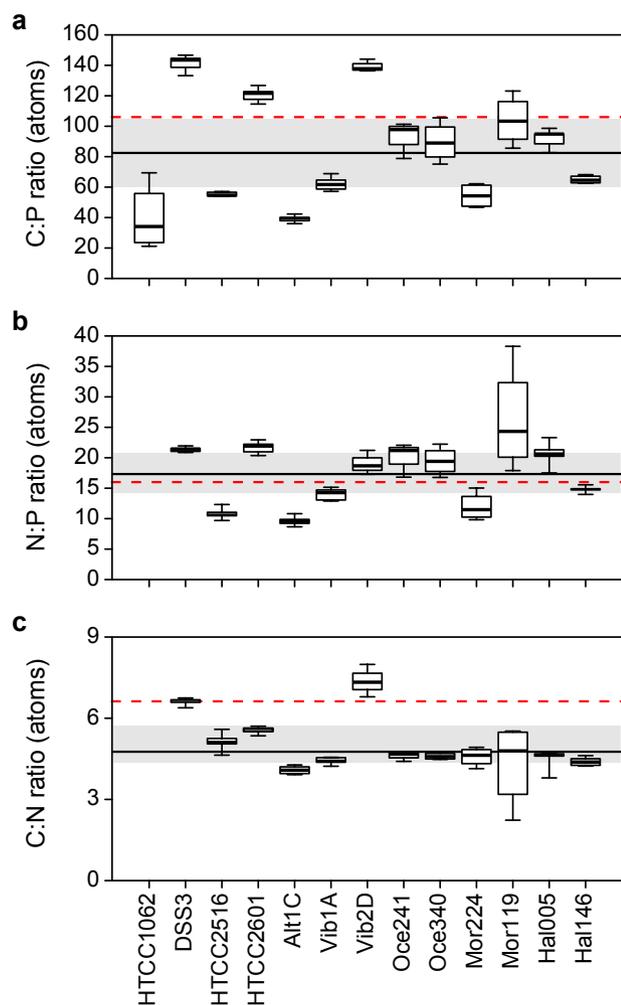NA, not applicable; value was below detection threshold.

Fig. 2. Elemental composition of marine bacteria in this study. Median values for each strain are shown along with the quartile, minimum and maximum values. Geometric means are given in Table 1. Dashed red lines depict Redfield ratios for (a) C : P = 106, (b) N : P = 16 or (c) C : N = 6.625. Solid black lines and shaded areas show estimated median ratios across all strains ± 95% CI. Overall, bacterial N : P was equal to Redfield ($P = 0.424$, Wilcoxon test), but C : P ($P = 0.048$) and C : N ($P = 0.001$) ratios were significantly different. No significant phylogenetic signal was detected for any of the ratios. Results of Kruskal–Wallis ANOVAs indicated significant variance in all ratios among the strains ($P < 0.001$).

$P = 0.54$), RNA ($K = 0.04$, $P = 0.68$) or protein ($K = 0.03$, $P = 0.79$) cell quotas.

When normalized to C biomass to account for potential variation in cell volumes among the strains (e.g. Edwards *et al.*, 2012) as well as possible inconsistencies between cell concentrations determined by flow cytometry and the actual number of cells retained on analysis filters, DNA content was substantially less variable among strains, ranging from 0.004 (Mor119) to 0.041 fg fg^{-1} C (Alt1C) with an overall mean of 0.022 fg fg^{-1} C (Fig. 4a; Table S1). By contrast, normalizing RNA and protein quotas to C biomass had little effect on variation across strains. RNA content ranged from 0.003 (Mor119) to 0.32 fg fg^{-1} C (Oce340), with an overall mean of 0.11 fg fg^{-1} C (Fig. 4b; Table S1). Average allocation to protein across strains was 0.52 fg fg^{-1} C and ranged from 0.25 (Mor119) to 0.81 fg fg^{-1} C (Alt1C; Fig. 4c; Table S1). When normalized to C biomass, protein content showed significant phylogenetic signal ($K = 0.60$, $P = 0.001$), as did DNA content ($K = 0.28$, $P = 0.01$), though neither was strongly associated with phylogeny (e.g. $K < 1$). RNA content did not show significant phylogenetic signal ($K = 0.05$, $P = 0.59$).

The proportion of total cellular P resources bound in RNA (RNA-P) differed markedly among the strains in our study ($P < 0.001$, Kruskal–Wallis ANOVA, Fig. 5). RNA-P represented a majority of cellular P content ($> 72\%$) for DSS-3, HTCC2601, Oce340 and Hal005, while RNA-P in the remaining strains represented 20% or less of the total P quota (Table 1). These differences did not depend on phylogenetic relationships among the strains ($K = 0.09$, $P = 0.40$).
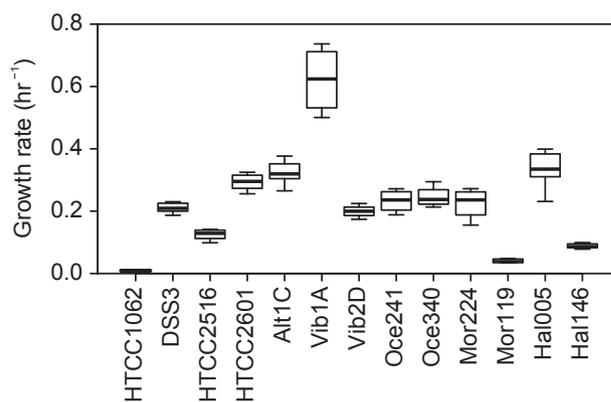
strains was 3.1 fg cell^{-1} (Fig. S1a; Table 1), ranging from 0.65 (Mor119) to 6.7 fg cell^{-1} (Oce340). RNA content was 4.8 times higher than DNA content on average. Cellular RNA quota varied 100-fold among the strains, from 0.55 (Mor119) to 64.8 fg cell^{-1} (Oce340), with a grand mean of 17.7 fg cell^{-1} (Fig. S1b; Table 1). Average protein allocation was 76 fg cell^{-1} and was less variable across strains, ranging from 31 (HTCC2516) to 143 fg cell^{-1} (Hal005; Fig. S1c; Table 1). No significant phylogenetic signal was detected for DNA ($K = 0.05$,

Fig. 3. Strain-specific growth rates of marine bacteria in this study. Median values for each strain are shown along with the quartile, minimum and maximum values. Means are given in Table 1. Blomberg's *K*-statistic for phylogenetic signal was not significant ($K = 0.045$, $P = 0.693$). Results of a Kruskal-Wallis ANOVA indicated significant variance in growth rate among the strains ($P < 0.001$).
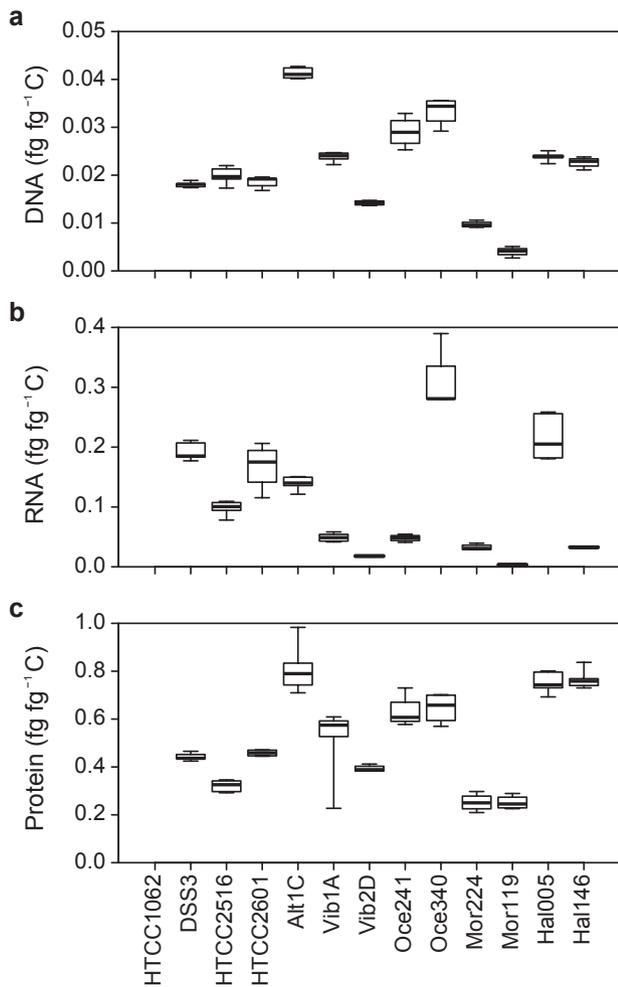
Fig. 4. Allocation to (a) DNA, (b) RNA and (c) protein (normalized to C biomass, w/w) of marine bacteria in this study. Median values for each strain are shown along with the quartile, minimum and maximum values. Means are given in Table S1. Blomberg's *K*-statistic for phylogenetic signal was significant for DNA ($K = 0.275$, $P = 0.007$) and protein content ($K = 0.602$, $P = 0.001$), but not RNA ($K = 0.050$, $P = 0.594$). Results of Kruskal-Wallis ANOVAs indicated significant variance in all traits among the strains ($P < 0.001$).

Linkages between growth, allocation and stoichiometry

We used Spearman's rank correlations to determine associations among elemental composition, macromolecule content and growth rate. DNA, RNA and protein allocation, whether normalized to cell abundance or C biomass, were not significantly associated with elemental ratios ($P > 0.05$). Likewise, growth rate was not significantly related to C : P ratios, N : P ratios or P content (normalized to C biomass, Fig. 6a–c, or cell abundance, Fig. S2a), as would be expected under the GRH. By contrast, growth rate was significantly and positively associated with RNA content ($P = 0.587$, $P = 0.049$ when normalized to C biomass, Fig. 6d; $P = 0.580$, $P = 0.052$

when normalized to cell abundance, Fig. S2b) across strains.

Discussion

As a means of understanding potential drivers of variation in the elemental composition of marine heterotrophic bacteria, the objective of this study was to explicitly evaluate the contribution of evolutionary history (phylogeny) to measured variation among bacterial strains. The goal of our common-garden approach was to minimize physiological plasticity in order to isolate taxonomic patterns in elemental stoichiometry, evaluate the linkages between stoichiometry and biochemical allocation strategy and assess the phylogenetic conservation of these traits. We hypothesized that variation in the biomass stoichiometry of individual strains would be related to macromolecule allocation and that both traits would follow phylogenetic relationships. Our results revealed significant strain-level variation in biomass stoichiometry and allocation strategy that was not strongly related to 16S ribosomal RNA phylogeny (i.e. long-term evolutionary history). Instead, resource allocation in marine heterotrophic bacteria likely varies at fine taxonomic resolution (i.e. on the scale of short-term evolutionary history). Consistent with previous observations of some heterotrophic bacteria (Neidhardt and Magasanik, 1960; Rosset *et al.*, 1966; Kemp *et al.*, 1993; Kerkhof and Ward, 1993; Poulsen *et al.*, 1993; Wagner, 1994; Bremer and Dennis, 1996), overall RNA content increased with measured growth rate (Fig. 6d, S2b). However, this relationship was not strong enough to significantly influence total biomass P content or C : P and N : P ratios as predicted by the GRH, at least under the conditions of our study (Fig. 6). Biomass stoichiometry and allocation to RNA may commonly be decoupled in
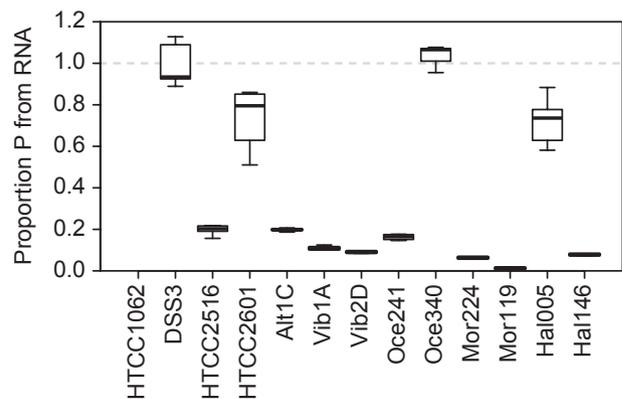

Fig. 5. Contribution of RNA-P to total biomass P of marine bacteria in this study. Median values for each strain are shown along with the quartile, minimum and maximum values. Means are given in Table 1. Blomberg's *K*-statistic for phylogenetic signal was not significant ($K = 0.088$, $P = 0.404$). Dotted line equals 1.
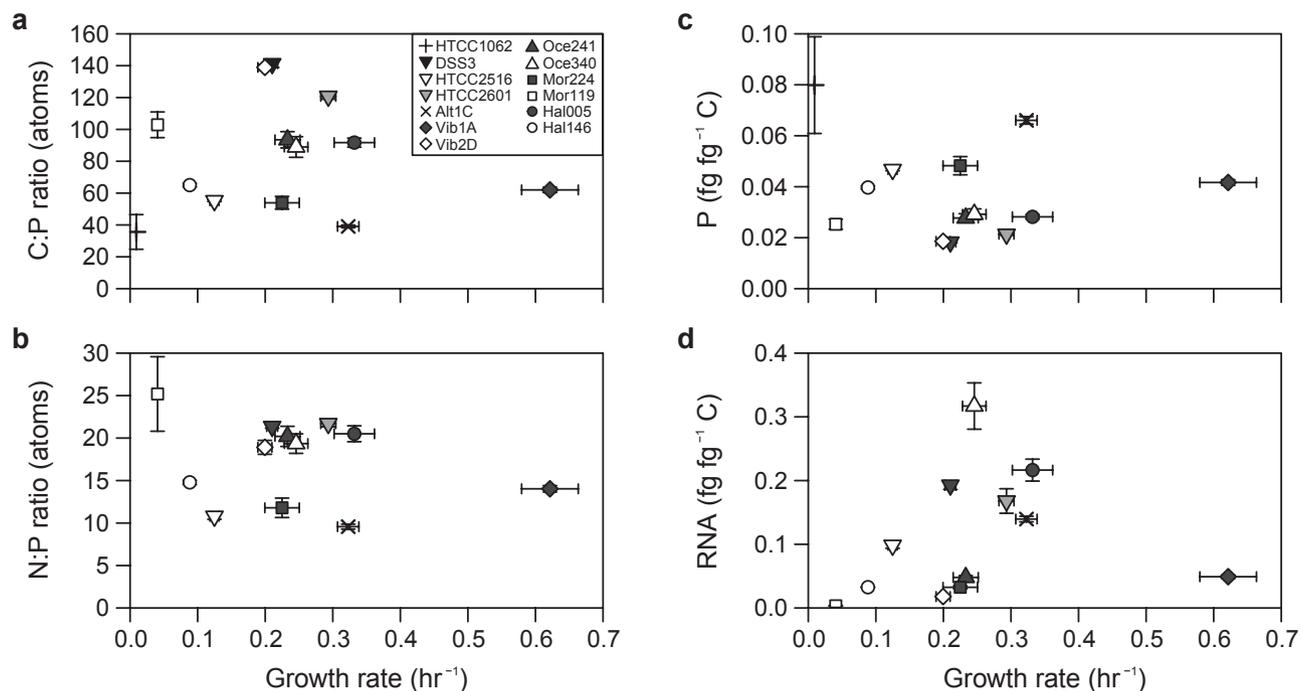
Fig. 6. Relationships between (a) C : P and (b) N : P elemental composition and growth rate, or (c) P and (d) RNA content (normalized to C biomass, w/w) and growth rate for strains of marine bacteria in this study. Points represent the arithmetic mean ± SEM for each strain, except that ratios are shown as geometric means. Shapes represent different families. Spearman's rank correlation indicated a significant association between RNA content and growth rate ($P = 0.05$, $\rho = 0.59$), but no significant associations were detected between C : P ($P = 0.94$) or N : P ($P = 0.68$) ratios and growth rate or between P ($P = 0.94$) content and growth rate.

heterotrophic marine bacteria if P is not the principal limiting resource and accumulates in biomass pools other than ribosomal RNA.

Overall, C : P and C : N biomass ratios of the strains in our study deviated from the Redfield ratios of C : P = 106 and C : N = 6.63, with a majority of the strains falling significantly below the Redfield value for both ratios (Fig. 2a,c). Without knowing the minimum cell quotas of C, N and P for the strains in our study, we speculate that this pattern reflects general carbon limitation, which appears to be common for marine heterotrophic bacteria (Kirchman, 1990; Cherrier *et al.*, 1996; Kirchman *et al.*, 2000; Carlson *et al.*, 2002). The average amount of biomass produced per mass of organic C consumed (bacterial growth efficiency, BGE) has been estimated at 22% for heterotrophic marine bacteria (Del Giorgio and Cole, 1998), though there is substantial variation due to biotic and abiotic influences (Carlson *et al.*, 2007). Assuming that all added organic C was consumed, BGE averaged 37% for the strains in our study. suggesting generally high C demand. Thus, C limitation and sub-Redfield C : P and C : N ratios may be prevalent among marine heterotrophs that consume resources with Redfield stoichiometry. By contrast, the average N : P ratio across strains was not statistically different from Redfield (Fig. 2b).

The GRH is one mechanism proposed for linking biomass stoichiometry and allocation strategy (Elser *et al.*, 1996; 2000). However, tight coupling among growth rate, RNA content and biomass P is primarily expected when P limits ribosome biogenesis and growth (Elser *et al.*, 2003; Makino *et al.*, 2003; Franklin *et al.*, 2011). Widespread C limitation would imply that the GRH is probably a poor predictor of biomass stoichiometry among marine heterotrophic bacteria. Accordingly, we did not observe the predicted inverse relationship between growth rate and C : P or N : P ratio (Fig. 6a,b) or the underlying positive relationship between biomass P and growth rate (Fig. 6c, Fig. S2a). The GRH assumes that rRNA is the primary pool of P biomass (Elser *et al.*, 2003; Vrede *et al.*, 2004), but total RNA was not a major determinant of biomass P for eight strains in our study (RNA-P = 11% of total P on average; Table 1). The other four strains (DSS-3, HTCC2601, Oce340 and Hal005) allocated a majority of their P resources to RNA (RNA-P = 87% of total P on average; Fig. 5; Table 1) and may therefore have been growing at or near maximum growth rates. However, it is increasingly apparent that the relationship between growth rate and rRNA in bacteria is complex (Blazewicz *et al.*, 2013).

Previous studies have indicated that the proportion of biomass P represented by RNA can vary substantially

in other species of bacteria. In P-limited cultures of *Corynebacterium bovis*, Chen (1974) measured ~30% of total cellular P bound as RNA. Makino *et al.* (2003) demonstrated that the allocation of P resources to RNA can vary in cultures of *E. coli* from 40–50% at lower growth rates up to 70–80% at higher growth rates. The magnitude of variation we measured in RNA-P among strains of marine bacteria is more similar to the variation reported for communities of lake bacteria (Makino and Cotner, 2004). In these communities, both growth (dilution) rate and substrate ratio influenced the contribution of RNA-P to total P, which varied from 25–43% under C-limitation to 76–93% under P-limitation. We recognize that there may be uncertainty in our RNA-P data because we did not account for potential differences in nucleic acid extraction efficiency among strains. Furthermore, the mass balance of intracellular resource pools is subject to technical limitations and influenced by uncertainty in the methods used to quantify various resource pools (e.g. Aschar-Sobbi *et al.*, 2008). In spite of these potential limitations, our results suggest that pools of P other than nucleic acids, such as phospholipids or polyphosphate (Sterner and Elser, 2002; Makino *et al.*, 2003; Makino and Cotner, 2004; Cotner *et al.*, 2006), may be important in determining the C : P and N : P ratios of heterotrophic bacterial biomass. Similarly, non-nucleic acid P made up 3–70% of total biomass P in lake bacteria (Makino and Cotner, 2004), and polyphosphate production appears to be a widely distributed trait among heterotrophic bacteria (Harold, 1966; Kornberg *et al.*, 1999). Unlike *Cyanobacteria*, heterotrophic marine bacteria probably cannot substitute phospholipids to reduce physiological P demand (Van Mooy *et al.*, 2009).

We used our common-garden experiment to explicitly evaluate the contribution of evolutionary history to variation in biomass stoichiometry, macromolecule allocation and growth rate among marine bacteria. Traits were considered phylogenetically conserved when the values among related organisms were significantly more similar than expected by Brownian motion trait evolution (phylogenetic signal *K*-statistic > 1; Blomberg *et al.*, 2003). Overall, 16S rRNA phylogeny was not an important determinant of strain-level differences in elemental composition (Fig. 2) or allocation to DNA, RNA or protein content (Fig. 4). We observed this result regardless of whether we analysed elemental and macromolecule content per cell or normalized to C biomass. Furthermore, we did not detect significant phylogenetic signal in growth rate among the strains in our study (Fig. 3), even though specific growth rate represents an integrated parameter of general life history strategy (Arendt, 1997). This lack of phylogenetic signal was not due to a lack of variation. Non-parametric analysis of variance confirmed that

biomass stoichiometry, macromolecule allocation and growth rate all varied significantly among the strains in our study ($P < 0.001$), indicating that within the *Proteobacteria*, there is substantial diversity at the strain level.

Our interpretation of phylogenetic signal is tempered by some limitations inherent in our approach; hence, we do not imply that there can never be a relationship between phylogeny and growth rate (and possibly elemental ratios). The lack of phylogenetic signal in growth rate may be influenced by our selection of strains or the particular metric used to represent growth rate. For example, lineages like SAR11 may have an inherently different growth strategy compared with *Vibrio*, though it appears there may be substantial variation in growth rates within the SAR11 clade (Campbell *et al.*, 2011). Expanding the phylogenetic breadth of the organisms in our analysis could increase the likelihood of detecting significant phylogenetic conservation in bacterial traits; however, distinct patterns supporting conservation of habitat preference and genome size, for example, have been demonstrated with less phylogenetic diversity than in our study (Ettema and Andersson, 2009). Likewise, our results may have differed if we were able to express growth rate relative to an empirically or theoretically determined maximum rate for each strain. Chrzanowski and Grover (2008) showed that relative growth rate, when measured as a percentage of maximum growth rate, explained a significant amount of the variance in the cellular C, N and P quotas of *Pseudomonas fluorescens*.

Another consideration of our experimental approach is that C was provided as a mixture of defined sources in the growth media. The use of simple C substrates is highly variable among closely related strains of bacteria (Martiny *et al.*, 2013b). Thus, it is probable that the organisms in our study could have differentially specialized on the various C sources provided, which has the potential to influence growth efficiency unequally across the strains. While this may have affected our results by generating additional variation in growth rate and stoichiometry, we consider potential differences in C resource use to be inherent characteristics of each strain, analogous to strain-specific differences in cell quotas, and therefore valid contributions to our observed results. Furthermore, strain-specific differences in C use are likely common in the ocean, where the combination of C compounds is variable and transient, influencing growth in numerous ways. Importantly, the strains in our study were supplied with the same resource combination under identical environmental conditions to meet our objective of evaluating the contribution of evolutionary history to variation in the stoichiometry and biochemical allocation patterns of marine bacteria.

Despite a weak phylogenetic signal, we observed significant strain-level variation in the biomass stoichiometry and allocation strategies of marine heterotrophic bacteria. This variation suggests that the genes controlling stoichiometric traits are evolving more rapidly than 16S rRNA. In bacteria, many traits related to resource use (Martiny *et al.*, 2013b) or 'adaptability processes' (Ettema and Andersson, 2009) are associated with specific taxa or ecotypes at ≥ 97% 16S rRNA gene sequence identity and are therefore difficult to resolve from traditional rRNA relationships. This pattern of fine-scale 'microdiversity' (e.g. Martiny *et al.*, 2006) may result from local adaptation or niche specialization and likely reflects differential adaptation to environmental conditions among closely related organisms. Protein allocation appeared to be an exception and was generally consistent within families (Fig. 4c), suggesting that constrained protein content in relation to C biomass may be a general pattern among marine bacteria (Simon and Azam, 1989; Kirchman, 2012). Overall, interspecific variation should be considered when evaluating or predicting bacterial contributions to ecosystem processes. Such variation may be especially important in low-diversity ecosystems that are dominated by few bacterial species with substantially different elemental ratios.

In general, bacterial biomass P content is more variable than C or N content (Bratbak, 1985; Kirchman, 2000; Vrede *et al.*, 2002; Cotner *et al.*, 2010). This distinction may in part be due to the similar N content in proteins and nucleic acids, such that organisms differing in protein : nucleic acid ratios do not differ substantially in N content (Elser *et al.*, 1996; Sterner and Elser, 2002). Cellular protein contents measured in our study are comparable to previously published values for other marine bacteria (Simon and Azam, 1989; Jeffrey *et al.*, 1996; Zubkov *et al.*, 1999), but our values are lower than the cellular protein content measured for rapidly growing *E. coli* (Bremer and Dennis, 1996; Kirchman, 2012). On average, C and N in nucleic acids represented a small proportion of total N (7%) and even less of total C (4%) in our study. The contribution of C and N bound in proteins to total cellular C and N quotas, by contrast, represented more significant biomass pools of both elements, constituting 28% of total C and 37% of total N on average across strains. One of the limitations of our method is that it does not account for the extraction efficiency of total protein; thus we may have underestimated the proportion of cellular C and N bound in protein. However, cells contain other pools of cellular C and N, including lipids, polysaccharides and peptidoglycan (e.g. Vollmer *et al.*, 2008). These pools can contribute substantially to bacterial biomass (Sterner and Elser, 2002) and may influence species-level differences in elemental composition.

We found that heterotrophic marine bacteria grown in the same resource environment show significant strain-level differences in biomass stoichiometry, allocation strategy and growth rate. However, this diversity was not correlated with long-term evolutionary history, as represented by 16S rRNA phylogenetic relationships. Our results suggest that the elemental stoichiometry of marine plankton may depend on the taxonomic identity of heterotrophic bacteria in the community. Our results also suggest that consumption of resources with near-Redfield stoichiometry likely causes C limitation of marine heterotrophs and could result in C : nutrient ratios below Redfield. Low C : P and C : N biomass ratios indicate that heterotrophic bacteria may function as sinks of mineral nutrients (relative to C) in marine systems. This stoichiometric pattern may have consequences for material exported to the deep ocean, transfer of resources to higher trophic levels (Güde, 1985; Martinussen and Thingstad, 1987; Shannon *et al.*, 2007) and limitation of algal growth (Daufresne and Loreau, 2001; Danger *et al.*, 2007). These potential consequences warrant more explicit consideration of heterotrophic bacteria in ocean biogeochemical models. Additional empirical studies are also needed to characterize spatial and temporal variation in the abundance and stoichiometry of marine heterotrophic bacteria. Together, these efforts should improve predictions of broad patterns in global ocean biogeochemistry.

Experimental procedures

Phylogenetic tree construction

16S rRNA gene sequences for strains HTCC1062, DSS-3, HTCC2516 and HTCC2601 were obtained from the Silva database (accession numbers CP000084, CP000031, AAOT01000021 and AATQ01000003 respectively; http://beta.arb-silva.de/; Pruesse *et al.*, 2007; Quast *et al.*, 2013) and aligned with the consensus sequences for all Newport Beach isolates using the SINA aligner (http://beta.arb-silva.de/aligner/; Pruesse *et al.*, 2012). Three archaeal 16S rRNA gene sequences were included in the alignment as an outgroup (*Thermoproteus tenax*, GenBank accession no. M35966; *Sulfolobus solfataricus*, D26490; and *Methanococcus vannielii*, M36507). The phylogenetic tree topology was inferred from the multiple-sequence alignment by maximum-likelihood estimation with a search for the best tree, global rearrangements allowed, a transition/transversion ratio of 2.0, a constant rate of variation among sites, and nucleotide frequencies that were estimated from the data. Bootstrap proportions were determined from 100 resamplings using the same maximum-likelihood parameters. Phylogenetic tree construction was carried out with the PHYLIP software package (v3.69; Felsenstein, 2005). The FIGTREE program (v1.4.0; http://tree.bio.ed.ac.uk/software/figtree/) was used for visualization and to root the phylogeny at the midpoint to meet requirements for further phylogenetic analyses.

Culture conditions

All strains were revived on ½ yeast–tryptone–sea salt agar plates (González and Moran, 1997; Hardwick *et al.*, 2003), and a single colony was picked to ensure purity prior to experiments. HTCC1062 was revived and grown only in liquid low-nutrient heterotrophic medium (Connon and Giovannoni, 2002) and monitored via flow cytometry (details further on) for growth and contamination. Fresh cultures were used to initiate growth in a standard seawater medium (SSM; Table S2) containing 87.5 μM sodium acetate, 29.17 μM D-glucose, 58.35 μM glycerol, 80 μM NH₄Cl, 5 μM K₂HPO₄, 1 nM L-methionine, 15 μM Na₂-EDTA, 95 μM Na₂CO₃, vitamins (10^{-4} dilution of stock; Rappé *et al.*, 2002) and SN trace metals (10^{-3} dilution of stock; Table S6). Seawater used to prepare SSM for all growth experiments was collected from the San Pedro Ocean Time-Series Station (SPOTS; 33°33′00″N, 118°24′00″W) in January 2012, filtered (0.2 μm) and autoclave-sterilized, then diluted with high-purity water (18.2 MΩ·cm) to 75% (by weight). SSM and culture conditions were designed to facilitate growth of a wide range of marine microorganisms (oligotrophs and copiotrophs), rather than to be optimal for any one organism, as well as to provide carbon (C), nitrogen (N) and phosphorus (P) resources in the molar Redfield ratio of ~106:16:1 (Redfield, 1958). Cultures were maintained in SSM in the dark at 20°C through several transfers to ensure proper growth prior to experiments. Growth profiles were established for each strain to determine the approximate time and culture concentration at the transition from log to stationary phase and were later used to guide sample collection. Cell abundance was estimated by flow cytometry (Marie *et al.*, 2001) on an Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), which was calibrated with beads once per week. Culture aliquots were fixed with glutaraldehyde (0.1% final concentration) and stained with SYBR Green-I nucleic acid dye (Molecular Probes, Eugene, OR, USA), then run in duplicate for one minute at a flow rate of 35 μL min⁻¹. Cytograms of green fluorescence versus side scatter were used to count and distinguish cells from media background. Cell abundances determined by flow cytometry were comparable to cell counts determined from dilution plating (unpublished data).

Common-garden experiments and growth rate analysis

Replicate experimental cultures (n = 6) of each bacterial strain were grown in SSM with gentle mixing using the same 20°C incubator. Experimental cultures were initiated with 1.3×10^4 (HTCC1062) to 1.3×10^5 (Vib2D) cells ml⁻¹ from 24–48 h liquid cultures. Growth was monitored in near-real time with flow cytometry, and samples were collected during late log to early stationary phase when each culture reached approximately 75% maximum abundance. Culture concentrations were monitored after sample collection to confirm that collection occurred at the appropriate growth phase. All culture samples were collected onto precombusted (450°C, 5 h) 0.3 μm-pore-size glass fibre filters (Sterlitech, Kent, WA, USA) under gentle filtration. For nucleic acid and protein determinations, duplicate samples were collected from each culture replicate, flash-frozen in liquid nitrogen and stored at −80°C. Additional duplicate samples were collected from

each culture replicate for evaluation of C/N (simultaneously) and P content and stored at −20°C in acid-washed and precombusted glass scintillation vials. Media blanks of sterile SSM were also collected as described.

All growth analyses were carried out in R using the 'car' (Fox and Weisberg, 2011) and 'stats' packages (R Core Team, 2012). Growth curves were established individually for each culture replicate by fitting a logistic growth model to the data: $y = \theta_1/(1 + \exp[-(\theta_2 + \theta_3 \cdot x)])$, where the response, *y*, is the culture concentration, and the predictor, *x*, is hours. Initial parameters were estimated visually for the asymptote (θ_1), and the self-starting SSlogis function (Pinheiro and Bates, 2000) was used to estimate the value of *x* at the point of inflection (θ_2) and the scale parameter (θ_3). Maximum abundance was determined from the model and used to calculate growth rate as the exponential rate of change: μ (h⁻¹) $= \ln(N_{75} - N_{25})/dt$, where N_{75} and N_{25} are the culture concentrations at 75% and 25% maximum abundance respectively, and *dt* is the time interval (in hours) between observations. This method allowed us to target the most linear portion of each growth curve, and we excluded any individual replicates that showed unusual growth profiles. Additionally, we repeated all analyses with the subset of strains that were clearly collected during exponential growth (referred to as the 'exponential-only' data set) to ensure that slight differences in growth phase were not significantly altering our results. This subset included HTCC1062, HTCC2516, HTCC2601, Alt1C, Vib2D, Oce241, Oce340, Mor224 and Mor119. As all statistical results were similar with the full and exponential-only data sets, we presented all strains in the main text, but included results from the exponential-only subset in the Supporting Information (Tables S3, S4) for comparison of the two datasets.

Analytical methods: elemental composition

Biomass C and N content was determined using a CHN analyser (Thermo Finnigan EA 1112, Bremen, Germany) after samples were treated with HCl (0.2 M) to remove inorganic material and dried overnight at 65°C. Sample C/N mass was calculated from chromatogram area using atropine standards and corrected for media blanks. P content was determined using an ash-hydrolysis method with MgSO₄ (0.017 M) treatment as previously described (Solorzano and Sharp, 1980; Lomas *et al.*, 2010). Sample P was calculated from an asymptotic regression of absorbance versus known concentrations of potassium phosphate standards (0–0.5 μmol) and corrected for media blanks. Atomic ratios were calculated for the C, N and P content of samples, and the geometric means are reported for each strain. The amount of C, N and P bound in specific macromolecules was calculated assuming nucleic acids are 33% C, 15% N and 9% P, whereas protein is 53% C and 17% N on average (Sterner and Elser, 2002).

Analytical methods: macromolecule content

The quantification of DNA, RNA and protein content was modeled after a previously published method (Berdalet *et al.*, 2005) and designed to avoid potential loss and degradation resulting from the repeated isolation and cleaning

steps of traditional extraction procedures. High-sensitivity macromolecule-specific Quant-iT fluorophores (Molecular Probes) were used following a crude lysis to detect total DNA, RNA and protein released from cells. Dye selectivity and sensitivity as well as tolerance of contaminating substances are available from the manufacturer. Standards, buffers and reagents were stored and used according to the manufacturer's suggestions.

Briefly, nucleic acids and proteins were released from filters by mechanical lysis (MP FastPrep-24 bead beater, MP Biomedicals, Solon, OH, USA) in a solution of Tris buffer (5 mM) and RNA preservative (saturated ammonium sulfate solution). Sample supernatant was used to prepare assays in 96-well microplates with fluorescent dye, buffer and prediluted standards provided with each kit (*E. coli* rRNA, λ dsDNA or BSA protein). Potential interference from cell debris was tested using samples of *Roseovarius* sp. TM1035 collected as described above and spiked with known amounts of each target macromolecule. Nearly 100% of added RNA and DNA was detected (Fig. S3a,b). Only the protein fluorophore showed significant interference from cell debris (detected only $70 \pm 5\%$ of added BSA protein; Fig. S3c), so a spiked control of strain Mor224 was included in each assay. Macromolecule concentrations were calculated based on standard curve regressions of fluorescence versus known standard concentrations. See Supporting Information for additional details.

Phylogenetic and statistical analyses

Trait conservation was measured by phylogenetic signal using Blomberg's *K*-statistic (Blomberg *et al.*, 2003), which assumes a Brownian motion model of trait evolution where the expected covariance between species' trait values is proportional to the shared evolutionary history. *K* has an expected value of 1 when the traits have evolved by Brownian motion (e.g. descent with modification). *K* values < 1 indicate low phylogenetic dependence, whereas *K* values > 1 indicate that traits are more similar in related species than expected for Brownian-motion evolution. We considered *K* values > 1 as evidence of phylogenetic trait conservation. Phylogenetic signal analyses were completed with the R package 'picante' (Kembel *et al.*, 2010) using 999 randomizations for significance tests. We considered all statistical analyses to be significant when $P < 0.05$.

We used a two-sided Wilcoxon signed-rank test to test whether elemental ratios across all strains ($n = 12$ or 13, depending on specific ratio) differed from Redfield proportions (C : P = 106, N : P = 16 or C : N = 6.63). We then used Wilcoxon tests to determine which individual strains ($n = 4$–6 replicates) deviated significantly from Redfield stoichiometry. We tested for significant strain-level variance in growth rate, elemental ratios or macromolecule allocation using Kruskal–Wallis ANOVAs with strain as a random factor. Linkages among elemental composition, macromolecule content and growth rate were quantified using Spearman's rank correlations. Tests were implemented with the default 'stats' package in R (R Core Team, 2012). Phylogenetic and statistical analyses of DNA, RNA and protein allocation were done on data normalized to cell abundance as well as C biomass.

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Supporting information

Additional supporting information is available from the online version of this article at the publisher's web-site:

Fig. S1. Allocation to (a) DNA, (b) RNA and (c) protein (normalized to cell abundance) of marine bacteria in this study. Median values for each strain are shown along with the quartile, minimum and maximum values. Means are given in Table 1. Blomberg's *K*-statistic for phylogenetic signal was not significant.

Fig. S2. (a) P and (b) RNA content (normalized to cell count) plotted in relation to growth rate for strains of marine bacteria in this study. Points represent the mean ± SEM for each strain. Shapes depict different families. Spearman's rank correlation indicated a significant association between RNA content and growth rate ($P = 0.05$, $\rho = 0.58$), but not P content and growth rate ($P = 0.15$).

Fig. S3. Addition and recovery of pure (a) DNA, (b) RNA or (c) protein to cell lysate from *Roseovarius* sp. TM1035. Points represent the means ± SEM from triplicate determinations. Detection values have been adjusted for the baseline signal from unamended cell lysate.

Table S1. Comparison of mean growth rate and macromolecule content normalized to carbon (C) biomass (w/w) for strains of marine bacteria in this study.

Table S2. Components of standard seawater medium (SSM).

Table S3. Comparison of Wilcoxon signed-rank tests for departures from Redfield ratios[1] between all strains and the subset of strains in exponential growth.

Table S4. Comparison of phylogenetic signal between all strains and the subset of strains in exponential growth.

Table S5. Calculated DNA extraction efficiency based on genome size.

Table S6. SN trace metal stock solution used for preparation of isolation media and SSM.

Appendix S1. Experimental procedures.