ABSTRACT Microbial communities, through their metabolism, drive carbon cycling in marine environments. These complex communities are composed of many different microorganisms including heterotrophic bacteria, each with its own nutritional needs and metabolic capabilities. Yet, models of ecosystem processes typically treat heterotrophic bacteria as a “black box,” which does not resolve metabolic heterogeneity nor address ecologically important processes such as the successive modification of different types of organic matter. Here we directly address the heterogeneity of metabolism by characterizing the carbon source utilization preferences of 63 heterotrophic bacteria representative of several major marine clades. By systematically growing these bacteria on 10 media containing specific subsets of carbon sources found in marine biomass, we obtained a phenotypic fingerprint that we used to explore the relationship between metabolic preferences and phylogenetic or genomic features. At the class level, these bacteria display broadly conserved patterns of preference for different carbon sources. Despite these broad taxonomic trends, growth profiles correlate poorly with phylogenetic distance or genome-wide gene content. However, metabolic preferences are strongly predicted by a handful of key enzymes that preferentially belong to a few enriched metabolic pathways, such as those involved in glyoxylate metabolism and biofilm formation. We find that enriched pathways point to enzymes directly involved in the metabolism of the corresponding carbon source and suggest potential associations between metabolic preferences and other ecologically relevant traits. The availability of systematic phenotypes across multiple synthetic media constitutes a valuable resource for future quantitative modeling efforts and systematic studies of interspecies interactions.

IMPORTANCE Half of the Earth’s annual primary production is carried out by phytoplankton in the surface ocean. However, this metabolic activity is heavily impacted by heterotrophic bacteria, which dominate the transformation of organic matter released from phytoplankton. Here, we characterize the diversity of metabolic preferences across many representative heterotrophs by systematically growing them on different fractions of dissolved organic carbon. Our analysis suggests that different clades of bacteria have substantially distinct preferences for specific carbon sources, in a way that cannot be simply mapped onto phylogeny. These preferences are associated with the presence of specific genes and pathways, reflecting an association between metabolic capabilities and ecological lifestyles. In addition to helping understand the importance of heterotrophs under different conditions, the phenotypic fingerprint we obtained can help build higher resolution quantitative models of global microbial activity and biogeochemical cycles in the oceans.
Three quarters of Earth’s surface is covered in water, making the ocean the biggest continuous environment and home to extraordinary biodiversity (1). In stark contrast to terrestrial biomes, approximately 70 percent of the biomass in marine ecosystems is microbial (versus 96% plant on land) (2); accordingly, their submicroscale processes have global-scale consequences on ecosystem services critical to human society (3). Half of the Earth’s annual primary production is accomplished in the surface ocean by phytoplankton that harvest light to fix carbon dioxide (3–5). Heterotrophic bacteria are important players in these processes, as they impact carbon cycling in the marine environment along at least two main axes: first, phytoplankton function is modulated by interactions with heterotrophs in ways that drastically affect primary productivity (6–9). For example, heterotrophic bacteria provide nutrients essential for the long-term survival of some phytoplankton (10–13). Second, heterotrophs dominate the transformation of organic matter released from phytoplankton, and their metabolic activity ultimately determines the fate of organic carbon in the marine environment (6, 14).

The marine dissolved organic carbon (DOC) pool is the primary source of organic carbon for marine heterotrophs, containing an estimated 0.2 Pg of labile organic compounds, which can be readily metabolized by these bacteria (15). Analyses of bulk seawater reveal the enormous complexity and heterogeneity of marine DOC, enumerating a minimum of tens of thousands of distinct organic compounds (16–18). As the primary suppliers of marine DOC, phytoplankton transfer approximately 50% of their photosynthate to heterotrophs (4, 19) via a variety of active and passive mechanisms, including leakage (20), exudation (21), photosynthetic overflare (22), and cell death caused by viral lysis and protist grazing (23, 24). A number of studies indicate that the taxonomic composition of microbial communities is influenced by the provenance of organic material, suggesting that individual heterotrophs vary in their ability to utilize broadly defined classes of macromolecules (25, 26) and that these metabolic preferences may contribute to community structure (27–31). For example, heterotrophs associated with phytoplankton have shown preferences for amino acids, small sulfur-containing compounds, and one-carbon compounds (32–36). However, little is known about the specific preferences of individual heterotrophs for individual classes of compounds, making it difficult to understand the role that specific clades play in utilizing DOC in different environments. This limited knowledge also makes it challenging to lay the groundwork for mechanistic models that could help explain how such interactions shape the phylogenetic and functional composition of the community. Identifying the metabolic links between DOC and heterotrophs is key to understanding the ecological drivers underpinning carbon cycling in ocean ecosystems. This may also help improve global-scale models of marine microbial processes, where heterotrophs are often assumed to perform overall similar metabolic tasks (37, 38), an assumption likely reasonable for certain goals (e.g., modeling global primary productivity) but not others (e.g., modeling community composition or genetic capacity).

In principle, information on the substrate preferences of different heterotrophs could be inferred from their genomes; however, sequencing data alone have demonstrated a limited ability to predict microbial phenotypes and community functions in practice (39–43). For example, in a recent survey of human gut bacteria, metabolic models recapitulated growth for only 10 of the 40 strains tested, suggesting that genomic information combined with knowledge from the literature is insufficient to describe bacterial metabolic complexity (44). Alternatively, by measuring the growth properties of individual strains on specific carbon sources, one can infer phenotypic profiles that provide direct insight into metabolic preferences and growth strategies. These types of measurements are increasingly performed to characterize microbial collections from different biomes (45, 46). Ideally, one would want to analyze phenotypic profiles in conjunctions with the organisms’ genomes in order to obtain insight into the genes and pathways that confer these preferences. Performing these types of
measurements on well-defined synthetic media, in addition to enabling inferences regarding the specific metabolic capacities of microorganisms, could also help inform quantitative models (47, 48).

Here we report the generation and characterization of a collection of 63 heterotrophic bacteria representative of many major marine clades and a systematic analysis of their metabolic preferences. By designing simplified media that capture different fractions of the molecular components of marine DOC, we sought to characterize the metabolic properties of different representative heterotrophs, i.e., how they grow on different conditions that represent different axes of DOC. We next analyzed the phenotypes obtained in order to understand whether these metabolic phenotypes are well captured by phylogeny or other genome-encoded properties. We suggest that our analysis could contribute to helping reduce the complexity of the ocean microbiome to a set of computationally and experimentally tractable variables that can be interrogated with mathematical models and controlled experiments and extended to complex natural communities to explain aspects of their behavior.

RESULTS AND DISCUSSION

Growth of 63 heterotrophs on refactored media provides an atlas of their metabolic preferences. In order to generate a collection of strains representative of major marine lineages common to both the global oligotrophic and temperate oceans, we collected 63 heterotrophic isolates from different sources (Table S1 in supplemental material). Strains were selected via a comprehensive genomic analysis conducted in a recent effort (49), in which over 400 high-quality reference genomes were clustered into functional groups using a trait-based approach focusing on metabolism and microbial interactions. Representative strains were chosen from each functional cluster, applying the additional criteria that they be culturable in standard laboratory conditions (at 26°C in Marine Broth) and have a biosafety level 1 rating. In addition, we also included some nonmarine model strains to serve as a benchmark for our experiments (see Materials and Methods). Overall, the culture collection includes representatives of 5 phyla and 29 families.

We used this collection of heterotrophic bacteria to address, through single strain phenotyping, the question of whether different clades are geared toward efficient degradation or preferred utilization of specific subsets of DOC molecules. Many of the library strains had been reported to grow in undefined complex media, such as Marine Broth. Using the composition of Marine Broth as a scaffold, we refactored yeast extract and peptone (the primary carbon sources in Marine Broth) into eight types of organic carbon (hereafter referred to simply as “carbon classes”): peptides, amino acids, lipids, disaccharides, organic acids, neutral sugars, amino sugars, and acidic sugars (Fig. 1A). For some classes, we selected specific compounds based on their reported presence in marine DOC (50–52). For example, in the amino sugar class, N-acetylglucosamine was chosen because it is a known degradation product of chitin, the primary cell wall component of many marine organisms (53). All refactored media except HMBlips were calibrated to contain the same total mass of carbon-containing compounds (although not necessarily the same number of carbon atoms, see Fig. S4 and Supplementary Text S1), as well as an excess of nitrogen, phosphorus, sulfur, salts, trace metals, and vitamins (see Materials and Methods and Table S2). Together with a negative control lacking added organic carbon, a medium containing all carbon classes, and Marine Broth itself, we tested a total of 11 conditions using growth assays in 96-well plates (Fig. 1B).

All of the 63 marine heterotroph strains grew reproducibly on at least one of the defined media, and optical density (OD) measurements were almost identical across technical replicates and highly consistent across biological replicates (Fig. 2; Fig. S1). For subsequent analyses we focused on the maximal OD (maxOD) observed along the curve of each strain relative to time zero, which we took as a proxy for the efficiency with which each organism can produce biomass on a given carbon category (referred to henceforth also as “productivity”). Note that another important metric, the growth rate at log phase is strongly correlated with maxOD (adjusted $R^2 = 0.71, P < 2.2 \times 10^{-16}$;
Despite containing the same mass of carbon source and supporting roughly similar numbers of strains (Fig. S3), the different media led to very different degrees of productivity. Given that neutral sugars, such as glucose and arabinose, are classically used as preferred carbon sources in bacterial growth experiments, we were surprised to observe that media having peptides and amino acids as the main carbon sources supported the highest amount of biomass growth, while the neutral sugar medium was among the lowest supporters of biomass change (Fig. 1C).

One aspect of the above results that is worth reflecting on is the significance of this pattern in the context of prior observations. It has been suggested in the literature that marine heterotrophic bacteria achieve greater biomass yield and growth rates on amino acids compared to media containing mono or polysaccharides as the sole carbon source (54, 55). The reason for this is unclear, but field studies suggest that nitrogen limitation relieved by the presence of additional nitrogen in amino acids does not explain this discrepancy (55). We did not observe increased growth on the amino sugar-containing medium (among the lowest growing), which contains considerably...
more nitrogen than the nonamino media. Furthermore, a regression analysis showed that there is only a weak relationship between the change in OD and amount of nitrogen in the medium (adjusted $R^2 = 0.079$ and $P = 3.5 \times 10^{-13}$; Fig. S4C). Thus, nitrogen abundance is likely not the main or only reason for the extensive bacterial growth on amino acids, and other explanations could involve the energetic advantage of using preformed amino acids compared to their biosynthesis (56). For a complete discussion on the influence of media stoichiometry on growth, see Supplementary Text S1 and Fig. S4. Note that some strains reproducibly displayed nonnegligible growth on the medium with no carbon added (see Table S7 at https://github.com/segrelab/marine_heterotrophs/ for $P$ values). The high reproducibility between experiments (Fig. S1) likely rules out contamination. Carryover of nutrients from the starter culture is also unlikely to explain this, since these strains grew to a lesser magnitude on other media (unless inhibition by carbon in those other media is a contributing factor). We cannot exclude the possibility that these strains are capable of utilizing compounds other than the added carbon sources (e.g., vitamins [57]) or internal carbon stores for growth (58, 59) or that they possess autotrophic capabilities (60).
Organisms cluster into two main groups and finer structures based on metabolic preferences. We captured each strain’s metabolic phenotype by compiling a growth profile, which consisted of a vector of the maximum change in OD achieved by the strain on each medium. Using a Gaussian mixture model (see Materials and Methods), these 63 growth profiles could be divided into 7 clearly distinct clusters (Fig. 2) The clusters can be described in terms of unique metabolic “signatures,” i.e., distinct sets of carbon classes on which the strains achieved similar biomass growth.

At a very broad level, six clusters seem to partition into two categories of metabolic preferences: one group (clusters 1, 3, and 4) includes organisms that grow robustly on amino acids and relatively poorly on organic acids (Fig. 2A and B); these clusters are enriched in *Gammaproteobacteria* (squared standardized Pearson residual for chi-square test > 4; see methods, Table S3 at https://github.com/segrelab/marine_heterotrophs/). In contrast, clusters 6 and 7 are comprised of organisms that produce significantly more biomass when grown on the organic acid medium compared to the amino acid medium (Fig. 2A and B; Table S4 at https://github.com/segrelab/marine_heterotrophs/); these clusters are strongly enriched in *Alphaproteobacteria* (squared standardized Pearson residual for chi-square test > 4; see methods, Table S3 at https://github.com/segrelab/marine_heterotrophs/). Cluster 5 follows a similar pattern to clusters 6 and 7, but the magnitude of growth is lower and the observed differences are not statistically significant (Fig. 2A and B). The remaining cluster (2) is highly diverse phylogenetically, albeit strongly enriched for *Flavobacteriia* and *Actinobacteria* (squared standardized Pearson residual for chi-square test > 4; see methods, Table S3 at https://github.com/segrelab/marine_heterotrophs/). As a whole, the strains in cluster 2 do not grow robustly on any media type, which may indicate a requirement for growth factors or environmental conditions not represented in the media tested.

In addition to these broad-scale patterns, the strains in several of the clusters show more nuanced differences in their growth phenotypes. Firstly, although the strains in cluster 4 are capable of growing on organic acids, the magnitude of growth is greatly reduced compared to the media containing amino acids (Fig. 2A; Table S4 at https://github.com/segrelab/marine_heterotrophs/). These strains consistently grow to a higher OD on the negative control medium compared to the organic acids, although this difference is not statistically significant (Fig. 2A; Table S4 at https://github.com/segrelab/marine_heterotrophs/); this might suggest that organic acids actually inhibit their growth. This possibility seems apparently inconsistent with the fact that these strains grow well on HMBcmt, which also contains organic acids. However, the concentration of organic acids in HMBorg is approximately 47 mM, 8 times higher than the concentration of organic acids in the HMBcmt. Organic acids are well-known by-products of aerobic growth in some bacteria, and several studies indicate that they inhibit growth in a concentration-dependent manner (61–63). The concentration of acetate alone in HMBorg is 8.7 mM, and in *Escherichia coli*, acetate concentrations as low as 8 mM have been shown to reduce growth by 50% (64); therefore, our results are consistent with a potential concentration-dependent inhibitory effect by organic acids. In contrast, the strongest growth on organic acids is observed for clusters 6 and 7, which also display a limited and varied ability to grow on amino acids. Four of the 18 strains in clusters 5, 6, and 7 grow appreciably on both peptides and amino acids (Fig. 2B); the remaining 14 either grow on one or neither of these two media. Since growth on peptides requires the ability to take up and incorporate amino acids, it is unlikely that these strains lack this capability. Rather, it is possible that some of the amino acids negatively affect growth at the concentrations employed here (65). Overall, except for cluster 2, which seems to include strains with a common pattern of low OD but no specific preference for carbon classes, the heterotrophs’ metabolic functions are thus primed for optimized growth on amino acids or organic acids, but not both.

Phylogenetic and metabolic distances correlate poorly with metabolic preference distances. We subsequently asked whether strains that are more closely related to each other phylogenetically are also more similar in their growth profiles across the different media we tested (see Materials and Methods). Regression analysis found an
extremely weak relationship between the two variables (Fig. 3A; adjusted \( R^2 = 0.001 \), \( P = 0.04 \)), indicating that the differences in phylogenetic distances between strains explained an insignificant proportion of the variation in distance between growth profiles.

Subsequently, we asked whether a metric other than phylogenetic distance, e.g., the difference in gene content, would correlate more strongly with phenotypic distance. Specifically, we compared distances between growth profiles to distances between genomes represented as binary sequences of genes (see Materials and Methods). Linear regression revealed an inconsequential association between growth profile distance and genome distance (Fig. 3B; adjusted \( R^2 = 0.004 \), \( P = 0.0005 \)). The same was true when we compared the growth profile distance to the distance between Kyoto Encyclopedia of Genes and Genomes (KEGG) modules, which may better approximate the metabolic distance between strains (Fig. 3C; adjusted \( R^2 = 0.004 \), \( P = 0.0004 \)) (49). Taken together, global genomic data, at least with the metrics used so far, do not seem to capture the differences in metabolic phenotypes observed between strains. One possible explanation for our observations is that the genes key in differentiating growth on various carbon sources are "drowned out" by the noise of uninformative genes contained in the genomes; it is not clear that the genes that matter rise above genes that have no effect in this analysis.

**Genes and pathways associated with growth on specific media reflect different metabolic and ecological strategies.** Given the poor correlation between metabolic distance and gene content or phylogenetic distance, we asked whether a more informative relationship could be revealed by examining whether the presence of individual genes or sets of genes (pathways) is associated with growth on each medium. For each medium, we calculated the correlation (\( r \)) between growth and each gene in the library pangenome (see Materials and Methods and Fig. 4A). Looking at the list of individual significantly correlated genes (Table S5 at [https://github.com/segrelab/marine_heterotrophs/](https://github.com/segrelab/marine_heterotrophs/)), one clear pattern that emerges is that 386 of the 1,076 most strongly correlated genes (absolute value of \( r > 0.5 \)) are associated with growth on the organic acids medium. Conversely, no significant individual genes emerged for growth on the amino acid medium, despite the fact that this medium supports high growth in a number of strains. One possible interpretation of this difference is that while organisms growing on organic acids tend to use a narrow and fairly coherent set of pathways, the organisms that grow on the amino acid medium are utilizing different subsets of the 20 amino acids, and thus different biological pathways.
A concise view of all gene correlation scores can be visualized using principal component PCA: as shown in Fig. 4B, the organic acid medium stands out as the main contributor to the primary PCA axis and is thus the most unique in terms of enriched genes compared to the other media. It is also interesting that the media containing additional nitrogen in the form of amino groups appear to cluster together, suggesting that genes related to the utilization of organic nitrogen partially drive this clustering.

In order to better identify genomic signatures associated with growth phenotypes beyond individual genes, we implemented a gene set enrichment analysis (GSEA) to identify overrepresented pathways. We mapped the set of highly correlated genes to the KEGG database (see Materials and Methods), which resulted in a ranked list of pathways for each medium (Table S6 at https://github.com/segrelab/marine_heterotrophs/); we will refer to these pathways simply as condition-specific "enriched pathways."

We next asked whether the enriched pathways are indicative of the medium under which the enrichment was identified. In other words, are the growth media enriched for pathways that metabolize the class of carbon substrate they contain? In several cases, growth on the various media was associated with the KEGG pathways describing the metabolism of the specific compounds they contained. For example, the sugar-based media (HMBoligo, HMBntrl, HMBacdsug) are depleted of pathways involved in the metabolism of amino acids and enriched for pathways involved in galactose, starch and sucrose metabolism, and interconversions between the pentose monosaccharides and glucuronate, a degradation product of alginate (Fig. 5A) (52). The media lacking sugars are not enriched for these sugar degradation pathways; instead, they share an association with the glyoxylate and dicarboxylate metabolism pathway, which can replenish sugars from amino acid precursors. In other cases, growth on a specific category of carbons is associated with pathways that can be involved in the utilization of those compounds but deviate from the most basic expectation. For example, organisms growing on organic acids are enriched for specific
portions of the ethylmalonyl-CoA pathway (EMC) (Fig. 5A; Fig. S5A), a well-described method for the assimilation of two-carbon compounds and biosynthesis of carbohydrates from fatty acids (66). Notably, the EMC pathway is an alternative to the glyoxylate shunt (67, 68), which is used to feed anaplerotic reactions of the TCA during growth on C2 substrates, such as acetate (Fig. 5B). A key enzyme in glyoxylate pathway, isocitrate lyase, is known to be absent in certain marine bacteria, such as *Rhodobacter sphaeroides*, which nonetheless possess the ability to grow on acetate as a sole carbon source (69).

Not all pathways found to be enriched can be easily associated with degradation of the carbon sources included in the medium: these pathways seem to have no direct relationship to the molecular processes associated with the utilization of the corresponding media components. For example, growth on organic acids is also correlated with the KEGG pathway for porphyrin metabolism, specifically the complete pathways for vitamin B12/cobalamin biosynthesis as well as bacteriochlorophyll a and b (Fig. 5B). On the other hand, the folate biosynthesis pathway is enriched for growth on peptides, lipids, and the complete medium (Fig. 5A). In addition, growth on peptides (and lipids) is associated with pathways for motility, including chemotaxis and flagellar assembly, as well as for biofilm formation (Fig. 5A).

**Conclusions.** By defining a suite of carbon compound classes that together make up biomass and asking which marine bacteria grow on each class, we generated a map of the broad-scale metabolic preferences of these bacteria. This map is important because heterotrophs process much of the dissolved oceanic carbon, in ways that may depend

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**FIG 5** Growth on each medium is associated with specific biological pathways, as determined by a gene set enrichment analysis (see Materials and Methods). (A) The set of highly correlated genes for each medium was mapped to the KEGG pathway database; for each medium, pathways that are significantly overrepresented in the highly correlated genes are colored light blue (black indicates a lack of statistical enrichment). KEGG pathways are annotated with colors corresponding to their BRITE database classification. (B) One of the interesting pathways that emerges from this analysis is the ethylmalonyl-CoA pathway (EMC) pathway, which appears in KEGG as part of the Glyoxylate and Dicarboxylate Metabolism pathway (asterisk in panel A). The EMC pathway is strongly enriched for growth on organic acids. This pathway is a multifunctional pathway, known to be usable for organic acid assimilation as an alternative to the glyoxylate shunt.
on the emergent metabolic properties of microbial communities to which they belong. Obtaining a clear picture of the carbon utilization capabilities of individual strains in relationship to their taxonomic signature seems, therefore, an essential step toward understanding how heterotrophic bacteria impact carbon cycling in the marine environment. Understanding individual strain traits will further allow researchers to know if it is feasible and reasonable to use a few representative taxa and metabolic processes as a general effective description of heterotrophic metabolism in the oceans, e.g., for the purpose of implementing mechanistic models of communities and global ocean biogeochemical cycles. At the same time, irrespective of the details of the phylogeny-metabolism relationship, one can view the broad-scale approach presented here as a valuable effort toward building more informed dynamical models of ecological processes. In particular, we suggest that partitioning heterotrophs into clusters based on their preferred utilization of combinations of DOC fractions may add to existing observations of successions and inform ecological models at desired levels of resolution. This work might help identify whether the broad-scale metabolic preferences suggested to explain microbial succession patterns in the marine environment (30) agree with the growth preferences of heterotrophic bacteria and perhaps predict succession patterns based on heterotrophic growth. Our results suggest that these preferences are partially predictable based on phylogeny but that extensive intraclade variability requires alternative approaches to group bacteria based on their metabolic role in the oceans. For example, bacteria belonging to cluster 4 (including some *Alteromonas* and *Marinoacter* strains) would be highly competitive across a wide range of organic matter, as they grow fairly well on amino acids in addition to lipids, disaccharides, and (to some extent) amino sugars. In contrast, those belonging to cluster 1 seem to be more specifically tuned to amino acids.

While some of the associations between pathways and carbon class can be easily attributed to a direct enrichment of the corresponding metabolic utilization processes, other enrichment patterns point to processes (including nonmetabolic ones) for which the biological connection is not obvious. We hypothesize that these “cryptic” associations between carbon classes and enriched KEGG pathways carry information about environmental adaptations, suggesting a connection between the metabolic preferences of marine heterotrophs and ecologically relevant roles they play within a microbial community. For example, we found growth on peptides to be correlated with genes whose function relates to biofilm formation, chemotaxis, and motility. Since previous studies have found these traits to be significantly enriched in microbial communities sampled from marine particles (28, 70–73), we speculate that the ability to grow robustly on peptides may be associated with a specific temporal role in the colonization of particles in marine environments. Peptide assimilation begins with the largely nonspecific extracellular cleavage of protein fragments (74, 75), enabling microbes to thrive on a wide variety of substrates. Labile organic matter, such as peptides, is turned over rapidly in seawater (76), and the ability to sense, target, and adhere to these substrates would confer a competitive advantage in the early colonization of “fresh” proteinaceous matter. Several studies have identified specific phases of algal blooms characterized by pronounced increases in peptide consumption associated with characteristic phylogenetic lineages of marine heterotrophs (30, 31, 77); similar observations have also been mirrored in mesocosm experiments (28). Our findings support the notion that a preference for peptides may be a defining feature of bacteria that thrive in specific phases of succession on marine particles or during algal blooms.

A second example is that of bacteria growing on organic acids, which we found to be strongly enriched for the ethylmalonyl-CoA pathway, possibly reflecting the importance of this pathway in the photoheterotrophic capabilities shared by many of these bacteria (60, 78). These same bacteria also display strong enrichment for vitamin B12 production (a defining characteristic of phytoplankton-heterotrophs interactions [79]), corroborating the previously suggested role of these bacteria as key partners of eukaryotic phytoplankton. These findings are also consistent with genomic and experimental evidence that photoheterotrophy is a widespread metabolic strategy among
certain Alphaproteobacteria clades (60, 78), such as the Rhodobacterales from our library. Previous studies have indicated that these strains lack the genes for carbon fixation and are therefore unable to grow autotrophically but use light to supplement their energetic requirements (60, 80). This agrees with our observation that these strains did not grow in the medium lacking carbon sources. It also suggests that the ethylmalonyl-CoA pathway could play a dual role in organic acid assimilation (as supported by the enrichment mentioned above) and CO₂ fixation, as documented in other phototrophs (81). Taken together, these cryptic associations can be interpreted as correlated adaptations with a potential ecological significance, similar to recently identified linked trait clusters (49).

While the phenotypic matrix shown in Fig. 2 is generally thought in terms of its columns, representing the growth profiles of the different organisms, one can also reflect on the relevance of its rows, which display the type of communities that each DOC fraction is able to support. Notably, different carbon sources seem to support very different numbers of taxa: for example, amino acids support many different species, while amino sugars only support appreciable growth in a narrow category of bacteria. The broad utilization of amino acids by many organisms may simply reflect the fact that they may be able to directly import and use amino acids as building blocks, funneling them directly into biomass. Alternatively, it is possible that distinct sets of organisms preferentially use different individual amino acids, in a way that our current setup (where all amino acids are mixed into a single carbon fraction) would not be able to dissect. One could apply to carbon classes a categorization similar to the one used to describe microbial species as generalists or specialists. In the same way as a species that can grow on multiple nutrients is thought of as a generalist, a carbon source that can support multiple clades could be called “versatile.” A carbon fraction that only supports a small number of clades (similar to a specialist organism) could be thought of as nonversatile, or “exclusive.” In the future it will be interesting to study the distributions of versatility in different environments and their ecological implications, e.g., with consumer-resource models that use statistical ensembles of random matrices to parameterize ecological models (82).

This laboratory study only provides a glimpse of the myriad factors likely to influence heterotrophic metabolic activity in the ocean, as the complete picture of microbial growth encompasses much more than the quantity and structure of carbon sources. Prior work has indicated that the elemental stoichiometry of DOC has a strong influence on microbial community function (83). While our refactored media are designed so as to contain the same amount of carbon, we cannot exclude the possibility that the elemental ratios of the defined media affect growth beyond the effect of the specific carbon sources. A compelling next step would be to examine the effect of nutrient availability on heterotroph carbon source preference. The landscape of organic matter in the ocean is far more complex than could be represented in laboratory experiments, and biotic interactions greatly influence metabolic activity. However, some of the trends we observed have been reported by previous studies, supporting the ecological relevance of our data set (30, 84). While we do not expect the exact patterns observed in our experiments to play out in nature, our study provides a framework to continue investigating the role that heterotrophic bacteria play in carbon cycling.

**MATERIALS AND METHODS**

Selection and construction of strain library. We assembled a library of 63 heterotrophic isolates representing major marine lineages common to both the global oligotrophic and temperate oceans (Table S1). The representative strains were determined by a comprehensive genome analysis presented in Zoccarato et al. (49), in which 473 high-quality marine microbial genomes were analyzed using a workflow aimed at detecting traits rather than the presence of individual genes. Based on the occurrence patterns of all traits, the genomes were clustered into 47 genome functional clusters (GFCs) expected to represent groups of organisms with defined ecologies and life histories. We selected representative strains from each GFC based on their availability from strain repositories or individual lab collections, applying the additional criteria that they be culturable in standard laboratory conditions (at 26°C in Marine Broth), be nonpathogenic with a BSL1 rating, and be heterotrophic. In addition, we also included five nonmarine strains. Two *E. coli* strains were chosen to serve as a benchmark for our
experiments: E. coli Seattle 1946 (25922) is a well-studied strain commonly used for quality control in microbial phenotyping assays; the origin of E. coli strain MRE600 is not well documented but it is believed to have been recovered from an environmental water sample. The remaining three strains were chosen because they were commercially available representatives of the GFCs that emerged as part of the original genomic analysis: Cronobacter universalis (crono) was isolated from fresh water; Shewanella dentrifricans OS217 (shawden) was isolated from brackish water; and Exiguobacterium oxitolerans T-2-2 (exig) was isolated from a water sample found at a fish processing plant. Strains were obtained from the sources listed in Table S1. Samples were streaked on marine agar, and single colonies were subsequently picked and inoculated into Marine Broth. Stocks were prepared from liquid cultures in 50% glycerol and stored at −80°C.

**Construction of refactored media.** As a first step in our analysis we compared strains according to their growth phenotypes with the goal of determining to what extent heterotrophic bacteria can utilize available marine carbon sources. We therefore took a well-known marine culture medium (Difco Marine Broth), on which all strains were experimentally determined to grow, and selected a core set of macromolecular compounds likely to comprise the undefined components of Marine Broth: yeast extract and peptone. Eight media containing different classes of carbon sources were developed, each with multiple individual components: peptides, amino acids, lipids, organic acids, disaccharides, monosaccharides, amino sugars, and acidic sugars (Table S2). A medium containing all eight classes and a negative control lacking added carbon were developed as well. In addition, nitrogen, phosphorus, and sulfur sources, as well as salts and vitamins, were added in excess to all of the refactored media. The exact quantities and components of each medium are detailed in Table S2.

Since the different media were built to approximately reproduce fractions of the Marine Broth medium, they were not uniform in their content of specific components. In particular, 8 of the 10 refactored media have the same total mass of carbon-containing sources, but not necessarily the same number of carbon atoms (Table S8 at https://github.com/segrelab/marine_heterotrophs/). The number of carbon atoms could not be calculated for difcoMB because the exact composition of the medium is unknown; therefore, we chose to standardize the mass of total carbon source to imitate the components of difcoMB as closely as possible, although it resulted in varying amounts of carbon and nitrogen atoms across the different media (Table S8 at https://github.com/segrelab/marine_heterotrophs/ and Fig. S4A). Similarly, the number of carbon and nitrogen atoms for the medium containing peptides (and therefore the complete medium) could only be estimated because an exact molecular formula for Casamino Acids (a hydrolysate of casein) is not available. The negative control medium (HMB–) has 0 added carbon source, and HMBlips has a total of 2.2 g/L of carbon source compared to 5 g/L for the other media due to stalling issues in the lipid mixture; this combined with the much greater molecular weight of the lipid components contributes to the low number of carbon atoms in the medium (Table S8 at https://github.com/segrelab/marine_heterotrophs/ and Fig. S4A). Note that the HMBlips medium was prepared with a commercially available lipid mixture (Sigma Lipid Mixture 1), which, in addition to the lipids themselves, contains pluronic F-68 as an anti-foaming agent and Tween 80 as an emulsifier. We cannot rule out the possibility that these molecules may be metabolized by some of the bacteria, partially contributing to the growth observed on HMBlips.

**Growth assay.** Using sterile technique, strains were streaked from frozen stocks (maintained at −80°C) onto marine agar plates and placed in a 26°C incubator. Cultures were grown 72 h, then single colonies were picked and inoculated in 2 mL Marine Broth in 5-mL falcon tubes. Liquid cultures were grown with shaking (200 rpm) at 26°C and ambient light for 48 h. Negative controls without added bacteria were used to verify the absence of contamination. Starter cultures of 1.5 mL were then inoculated into each well of a 96-well plate containing 149 μL of medium, in triplicate. Only the interior wells were utilized; edge wells contained Marine Broth without added bacterial cultures to reduce evaporation and check for contamination. All combinations of strains and media were tested. Plates were wrapped with parafilm and incubated at 26°C and ambient light without shaking for 246 h. Negative controls for each medium (without added bacteria) were included in addition to edge wells. Plates were removed from the incubator individually, the lids were checked for condensation, and the optical density (600 nm) was measured in a Biotek Synergy HT plate reader (software version 3.05.11) at 26°C approximately every 24 h. Plates were shaken prior to the read at 0 h only. Results (in the form of time series of OD$_{600}$ for each well) were downloaded from the plate reader software as excel files and analyzed using the R statistical programming language (85).

**Growth profiles.** The heterotrophs selected for the library displayed widely divergent growth dynamics, and we chose to focus on their potential to generate biomass regardless of growth rate. As described below, we used the growth curves to estimate, for each strain, a growth profile across media, capturing the maximal change in optical density achieved by that strain at any time point during the growth assay. The three technical replicates for each organism ($i$) and condition ($j$) were highly similar to each other (Fig. S1) and were averaged at each time point to produce an average growth curve OD$_{ij}(t)$. We next identified for all pairs $ij$, the maximum value ($\text{max}_i$($\text{OD}_{ij}(t)$)) reached by $\text{OD}_{ij}(t)$ throughout the growth time course. The normalized version of this maximum value across all strains, $\text{maxOD}_{ij}(i=1, \ldots, 63; j=1, \ldots, 10)$ is used for subsequent comparative analyses of heterotroph phenotypes. Following a Kruskal-Wallis test for each medium, the nonparametric post hoc Dunn’s test was used to determine significant growth between the test and negative control without added bacteria for each medium. The Benjamini-Hochberg correction for multiple testing was applied, and cases with $P$ < 0.05 were ascribed the unchanged $\text{maxOD}_{ij}$ value. Conversely, $\text{maxOD}_{ij}$ values for strain/medium pairs that did not experience positive growth were set to 0.
Note that we chose to use optical density as a proxy for strain growth because it best captures the total biomass yield, which is most relevant for global stoichiometry in the ocean. In other words, we were interested in the ability of each organism to produce biomass on a given carbon category. Other growth metrics, such as growth rate and the time to reach the maximum OD, constitute important variables that affect meaningful ecological phenotypes, like competition for limited resources, but were not thoroughly analyzed in this study. Given that several strains display diauxic shifts and other nonlinearities during the active growth phase, these other metrics may be difficult to estimate as individual parameters and would ideally require higher time resolution. We have, however, explicitly asked whether there is any systematic relationship between maximum OD and initial growth rate. As shown in Fig. S2, the two quantities are strongly correlated (adjusted $R^2 = 0.71$, $P < 2.2 \times 10^{-16}$).

Unsupervised clustering of growth profiles. The growth profiles (maxOD) of all 63 strains were clustered using a Gaussian mixture model implemented in the R mclust package (86), a contributed R package for model-based clustering, classification, and density estimation based on finite normal mixture modeling, abundantly used to analyze biological data sets (e.g., references 87–89). Model-based clustering approaches, such as gaussian mixture modeling, provide a probabilistic alternative to traditional methods, such as k-means and hierarchical clustering; the challenges of selecting the “correct” number of clusters and clustering method are resolved by statistical model selection rather than heuristic methods (90, 91). mclust provides functions for parameter estimation via the expectation maximization algorithm for normal mixture models with a variety of covariance structures and functions for simulation from these models. Initialization is performed using the partitions obtained from agglomerative hierarchical clustering. By default, mclust applies 14 models and identifies the one that best characterizes the data. mclust achieves this by computing, for each model, the Bayesian information criterion (BIC), which has been shown to work well in model-based clustering (92, 93). Specifically, BIC was used within mclust to identify the optimal covariance parameters (in our case VEI), as well as the optimal number of clusters (in our case, 7). See Fig. S6 for a comparison of BIC values obtained for all models.

A chi-square test of independence was performed to examine the relationship between taxonomic class and cluster number. The relationship between these variables was significant ($P = 0.0005$). The squared standardized Pearson residuals were then examined to indicate which classes of bacteria contributed significantly to the lack of fit between the observed data and null model (the taxonomic class and cluster assignment are independent); values greater than 4 were considered statistically significant at a critical alpha value of 0.05 (Table S3 at https://github.com/segrelab/marine_heterotrophs/).

Relationship between growth profiles and phylogenetic/gene content distance. We constructed a phylogenetic tree for all the strains in our collection using protein sequences of 206 single-copy homologous genes. The homologous genes were obtained by first conducting an all-against-all comparison of translated nucleotide sequences using BLAST (94). The BLAST pairs were then filtered for reciprocal hits and clustered using MCL (95). The sequences in each cluster were aligned with Muscle (96) and trimmed with Gblocks (97), and HMMER (98) was used to build hidden Markov models (HMMs) for each cluster. We then searched the genomes for each HMM, and the orthologs were selected based on their presence in all genomes (multiple copies were ignored). The homologous sequences for each strain were concatenated and aligned with Muscle, cleaned with Gblocks, and the final maximum-likelihood tree was inferred using FastTree (99). The cophenetic distance ($P_{ca}$) between all pairs of strains was calculated using the cophenetic.phylo function of the R ape package (100).

The gene content distance ($C_{ga}$) and the metabolic trait distance (KEGG module distance, $M_{ga}$) for all pairs of strains were calculated as described in Zoccarato et al. (49), using the Jaccard distance. All of the genomes used in this study were reannotated with KEGG orthology (KO) identification numbers by Zoccarato et al. For Fig. 3B, we created the library pan-genome by matching the genes based on their KO identifiers, ignoring duplicate genes within the same genome. We built the matrix by first taking the nonredundant union of genes for all strains in the library, and then we organized the matrix so that the rows represented strains, the columns represented the genes, and the presence or absence of genes in each strain was given a binary encoding. The gene content distance ($C_{ga}$) was subsequently determined by calculating the Jaccard distance between binary vectors for all pairs of strains. The metabolic trait distance ($M_{ga}$) was calculated in an identical way using binary vectors for each strain encoding the presence or absence of KEGG modules; the methodology for determining the presence of modules within each genome is detailed in reference (49) In this case, the final matrix columns are KEGG modules, which represent pathways, structural complexes (e.g., transmembrane pumps or ribosomes), functional sets (e.g., aminoacyl-tRNA synthases or nucleotide sugar biosynthesis), or signaling modules (e.g., phenotypic markers such as pathogenicity).

The distance between each pair of growth profiles ($G_{ga}$) was calculated using the Euclidean distance between vectors of continuous numbers corresponding to the MaxOD values. We chose the Euclidean distance due to its suitability for microbial culture data sets with the same number of variables measured on similar scales (101–104) and because we were interested in the geometric proximity of the growth values (105). For each pair of strains, the Euclidean distance between growth profiles was plotted against the cophenetic distance, gene content distance, and KEGG module distance. Linear regression was performed using the lm function of the R base package (85).

Correlations between growth and individual gene presence/absence. We created the library pan-genome by taking the nonredundant union of all genes across strains. The point biserial correlation coefficient ($r$) was calculated between growth on each medium and the presence (binary) of the 6,255 individual genes in the library pan-genome. $P$ values for $r$ were adjusted using the Benjamini-Hochberg procedure and then filtered for values less than 0.05. The resulting set of $r$ were considered the values
for highly correlated genes. The matrix of correlation values, \( r \), was subjected to principal-component analysis using the princomp function of the \text{R} base package (85).

Pathway mapping/enrichment. Enriched pathways were obtained by filtering the pangenome for significantly correlated genes (adjusted \( P < 0.05 \)) and mapping the set for each medium to the KEGG database using the \text{R} clusterProfiler package (106). \( P \) values were calculated by the hypergeometric distribution and corrected for multiple testing using the Benjamini-Hochberg procedure. Enrichment was considered statistically significant for adjusted \( P < 0.05 \).

Data availability statement. All raw data sets and scripts used to generate the figures presented in this article are available at https://github.com/segrelab/marine_heterotrophs.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

\text{TEXT S1}, DOCX file, 0.01 MB.
\text{FIG S1}, TIF file, 1 MB.
\text{FIG S2}, TIF file, 0.4 MB.
\text{FIG S3}, TIF file, 0.3 MB.
\text{FIG S4}, TIF file, 2.2 MB.
\text{FIG S5}, TIF file, 2.5 MB.
\text{FIG S6}, TIF file, 0.6 MB.
\text{TABLE S1}, XLSX file, 0.01 MB.
\text{TABLE S2}, XLSX file, 0.02 MB.

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Author contributions were as follows: E. Forchielli, D. Sher, and D. Segrè designed the study, E. Forchielli performed the experiments and the statistical analysis of the data, E. Forchielli and D. Segrè wrote a first version of the manuscript, and all authors refined the final version. All authors have read and approved the final version of the manuscript.

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