MICROBIAL ECOLOGY

Microb Ecol (1999) 38:191–200
DOI: 10.1007/s002489900177

Application of Traditional and Phylogenetically Based Comparative Methods to Test for a Trade-off in Bacterial Growth Rate at Low versus High Substrate Concentration

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Received: 5 April 1999; Accepted: 8 July 1999

Abstract

It is often hypothesized that those organisms that are superior competitors for sparse resources fare poorly in competition for abundant resources, and vice versa. If there is indeed such a systematic trade-off, then this has important implications for the choice of bacterial strains in bioremediation and other applications. We studied seven bacterial strains that can grow on either 2,4-dichlorophenoxyacetate (2,4-D) or succinate as a sole source of carbon. Growth rates were measured on each substrate at both low (5 μg/ml) and high (500 μg/ml) concentrations. We used two different methods to test the significance of correlations among growth rates, a traditional method that treats each strain as an independent observation and a newer method that takes into account phylogenetic relationships between strains, thereby avoiding spurious correlations caused by a lack of statistical independence of strains. In both 2,4-D and succinate, we observed significant positive correlations between growth rates measured at high and low substrate concentrations by the traditional comparative method. No significant correlations were detected after adjusting for the phylogenetic relationships among the strains. In neither case did we observe the negative correlation expected from a trade-off between growth rates at high and low substrate levels.

Introduction

It is widely assumed that bacterial species cannot be successful competitors at both low and high resource concentrations, but instead they must pursue one strategy or the other [12, 14, 27]. This view is closely related to the distinction between *r*- and *K*-strategists in animals and plants [29, 32]. When applied to microorganisms, Andrews and Harris [1] expect *r*-strategists to have high maximum growth rates and to require abundant resources to support their rapid growth, whereas *K*-strategies should have lower maximum growth rates and require fewer resources to support their slower growth. In other words, maximum growth rate (μ_{max}) is viewed as the primary determinant of competitiveness when resources are abundant, whereas substrate affinity (K_S) becomes more important when resources are scarce.

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Understanding the nature and generality of trade-offs in bacterial competitiveness at low versus high resource concentrations may have practical application for bioremediation of soils and groundwater contaminated by xenobiotic compounds [14]. That is, successful bioremediation may depend on identifying bacterial strains whose growth parameters are well suited to site-specific conditions in terms of resource concentration. However, prior studies that have looked for possible trade-offs in growth rates as a function of resource concentration have yielded mixed results [14, 22]. Some of these studies employed an experimental approach, in which bacteria were allowed to evolve in the laboratory; the trade-off hypothesis was then tested by comparing the growth properties of ancestral and derived genotypes. Other studies have used a comparative approach, in which growth parameters were estimated in the laboratory for bacterial strains that had been isolated from nature and correlations between these parameters tested.

In recent years, evolutionary biologists have recognized a serious problem with basing inferences about evolutionary adaptations and trade-offs upon the traditional comparative method [16]. The traditional comparative method treats each strain (or species) as an independent observation for purposes of statistical analysis, but in fact certain pairs of strains (or species) are more closely related to one another than are other pairs. Consequently, not all observations are truly independent, creating the opportunity for spurious correlations between various organismal traits. In other words, phylogenetic relatedness tends to produce phenotypic similarity simply because of common ancestry, whether or not the resulting pattern has any particular adaptive significance. For example, consider the universe of all warm-blooded terrestrial vertebrates (i.e., birds and mammals). Treating each species as an independent observation, there is a very strong association between vivipary (as opposed to ovipary) and the possession of hairs (as opposed to feathers). But vivipary and hairiness may each have evolved only once in these combined groups, and so the association between these two traits may reflect a sort of historical accident rather than having any adaptive significance. Fortunately, new methods for comparative analysis have been developed that incorporate phylogenetic relationships between species and thereby allow reliable statistical inferences about associations between traits of interest [3, 4, 10, 13, 16, 23, 37].

For this study, we performed comparative analyses to test the hypothesized trade-off in bacteria between relative performance at high and low resource concentrations, using both the traditional approach (in which each strain is treated as an independent observation) and newer methods that depend on phylogenetically independent contrasts. Seven strains of bacteria isolated from nature were examined, all of which can grow on either 2,4-dichlorophenoxyacetate (2,4-D) or succinate as a sole carbon source. Growth rates of all the strains were measured at both low and high concentrations of each substrate. We will show that the two comparative methods yield different results, although neither analysis provides compelling support for the trade-off hypothesis. We conclude by discussing possible explanations for these results as well as considering an alternative approach to study this issue.

Methods

Bacterial Strains

The seven strains of bacteria used in this study were isolated from either soil or sludge, and all are able to catabolize 2,4-D [37]. Designations and geographical origins of the seven strains are as follows: TFD2 (Michigan), TFD3 (Oregon), TFD6 (Michigan), TFD13 (Michigan), TFD20 (Michigan), TFD41 (Michigan), and TFD43 (Australia). Based on 16S ribosomal DNA sequences (see below), these seven strains all belong to the β subgroup of the Proteobacteria [28]. TFD41 has been assigned to the genus *Ralstonia* [30], which is also the genus to which TFD43 is most closely related [28]. The remaining five strains are most closely related to the genus *Burkholderia* [28]. We also sought to include in our study several 2,4-D degrading strains from the α and γ subgroups of the Proteobacteria in order to increase phylogenetic diversity, but these other strains did not grow reliably in our standard culture medium. To determine phylogenetic branch lengths, the β proteobacterium *Chromobacterium violaceum* was used as an outgroup because of its minimal sequence length variation in comparison with the TFD strain sequences [5].

Culture Medium

Growth rates were measured in acid-washed flasks using the same base mineral medium for all strains and experiments. The base medium contained, per liter, 1.71 g K_2HPO_4, 0.3 g Na_2PO_4, 0.33 g $(NH_4)_2SO_4$, 0.246 g $MgSO_4 \cdot 7H_2O$, 0.12 g $Na_2EDTA \cdot 2H_2O$, 20 mg NaOH, 4 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnSO_4 \cdot H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 30 mg $FeSO_4 \cdot 7H_2O$, 52 mg Na_2SO_4, and 1 mg $NaMoO_4 \cdot 2H_2O$. Succinate or 2,4-D was added to the base medium at a concentration of either 5 or 500 μg/ml.

Estimation of Growth Rates

We initially sought to estimate two growth parameters for each strain: μ_{max} (absolute maximum growth rate) and K_S (resource concentration that supports a growth rate of $\mu_{max}/2$). However, we were unable to obtain reliable estimates of K_S because of technical

difficulties [19]. For example, certain strains showed residual growth (but very low yields) even when we added no carbon to the medium (using acid-washed flasks and double-distilled water); the cells may have used some airborne organic material [11]. Therefore, we opted instead to measure each strain's growth rate at two substrate concentrations, one low (5 μg/ml) and one high (500 μg/ml), in order to test the trade-off hypothesis. We employed small initial inocula, so that the bacteria could grow exponentially (log-linearly) for several generations before they appreciably affected the substrate concentration, even in the low concentration treatment. Of course, at the low substrate concentration the cells encounter fewer substrate molecules than at the high concentration, and their growth rate will be less than at the high concentration if the low concentration is limiting. Also, the lower range of cell densities used in calculating the growth rate exceeded the yield obtained from residual growth when no substrate was added; hence, we could measure substrate-specific and concentration-dependent growth rates despite this residual growth.

Strains were removed from storage at −80°C and inoculated into 10 ml of medium (in 50-ml flasks) containing either 2,4-D or succinate (500 μg/ml). These initial cultures were allowed to grow for 3 days at 25°C while being shaken at 120 rpm. Cells from each initial culture were then transferred into fresh medium containing either 5 or 500 μg/ml of the corresponding substrate and allowed to grow for 4 days; this constituted a preconditioning step to ensure that cells were physiologically acclimated to the relevant medium. Cells from each preconditioning culture were diluted 100-fold into fresh medium with the same substrate and initial concentration to begin the experiment proper. Bacterial cultures were sampled repeatedly at 2 to 8-hr intervals over a period of 12–48 hr. At each time point, the total biovolume (cell number × average cell size) of a culture was measured using a Coulter particle counter [18, 40]. Total biovolume is similar to optical density, insofar as both measures reflect cell size as well as cell number. Culture densities varied at the end of the final preconditioning cycle, resulting in variable inoculation densities. However, for calculating growth rates we selected time points that covered similar ranges of absolute cell density across cultures for both substrate concentration treatments, in order to minimize any density-dependent effects on growth rate. Biovolume measurements were log-transformed, and a growth rate was calculated as the rate of change in biovolume during the period of exponential increase. The mean of three independently estimated growth rates (for each combination of strain, substrate, and concentration) was used in the comparative analyses. Examples of data obtained for strains TFD2 and TFD20 during growth on 500 μg/ml succinate are shown in Fig. 1.

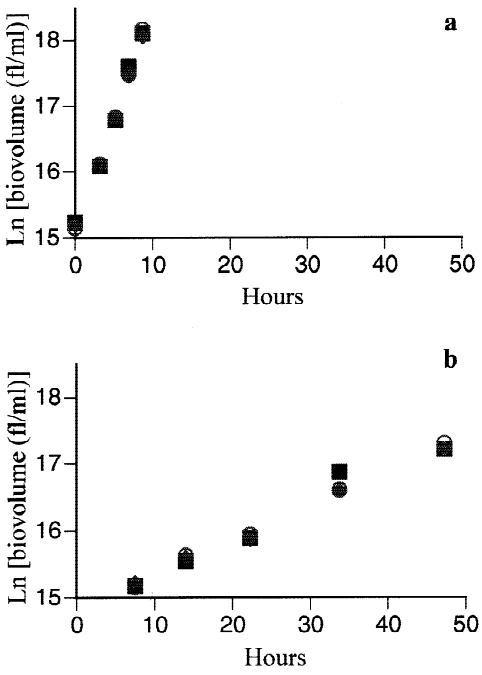

Fig. 1. Exponential growth of strains TFD2 and TFD20. Log-transformed population biovolume (fl biovolume/ml culture) is shown for (a) a fast-growing strain (TFD2), and (b) a slow-growing strain (TFD20) during growth on 500 μg/ml succinate. Population biovolume equals the average cell size (fl) multiplied by the density of cells (per ml). Open circles, closed diamonds, and closed squares represent three independent replicate populations for both TFD2 and TFD20.

16S Ribosomal DNA Sequencing

For each of the seven strains used in this study, the 16S ribosomal RNA (rRNA) encoding genes were amplified by the PCR from genomic DNA using oligodeoxynucleotide primers designed to anneal to conserved regions of the bacterial 16S rRNA gene. We sequenced six of the strains, whereas the 16S rRNA sequence of strain TFD41 was retrieved from GenBank, accession number AF067833. In our sequencing, the forward primer corresponded to positions 8 to 27 of the rRNA gene from *Escherichia coli,* and the reverse primer to positions 1492 to 1510 [9]. Reaction conditions and cycling profiles were as described previously [9]. The amplified product was purified using Millipore 30,000 MWCO columns (Ultrafree-MC; Millipore Corp., Bedford MA) as per the manufacturer's recommendation. Cycle sequencing was performed on a Perkin Elmer 9600 thermal cycler using ABI dye terminator sequencing, and products were analyzed on an ABI 373a DNA sequencer. A collection of 12 primers (MicroSeq 16S rRNA Gene Kit; PE Applied Biosystems, Foster City, CA) provided an average redundancy of 3.0 per nucleotide position. Consensus sequences were deposited in GenBank with the following accession numbers: AF184926 (TFD13), AF184927 (TFD2), AF184928 (TFD20), AF184929 (TFD3), AF184930 (TFD43), and AF184931 (TFD6).

Construction and Scaling of the Phylogeny

The PAUP (Phylogenetic Analysis Using Parsimony) computer program [36] was used to infer the phylogeny of the bacterial strains included in this study, using ~1,400 bp of 16S ribosomal DNA sequence (Fig. 2). A bootstrap analysis ("branch and bound" method, 1,000 iterations) was performed to ascertain confidence in the resulting phylogenetic tree, and the percentage of bootstrap

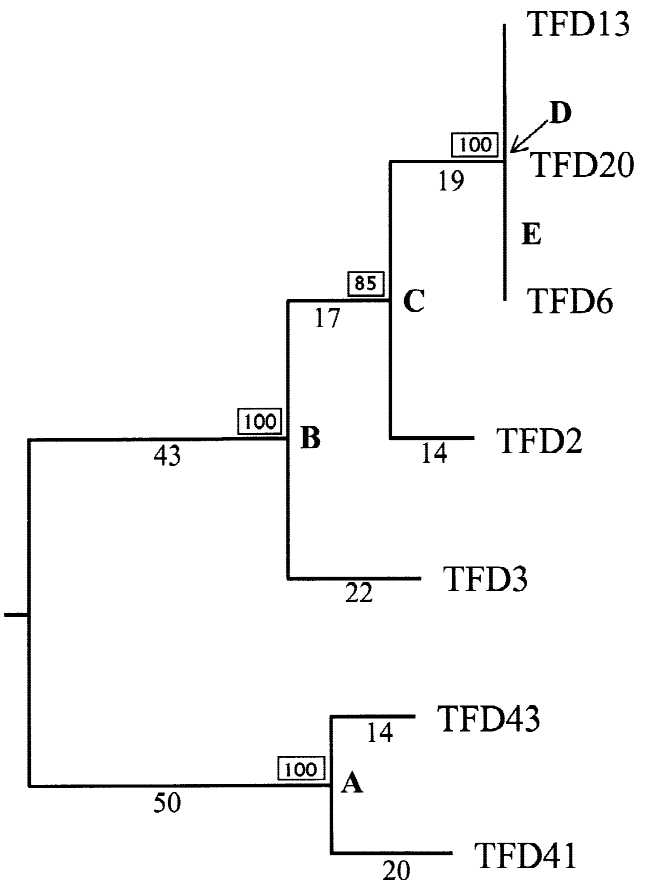

Fig. 2. Phylogenetic relationships of the seven strains used in this study. These relationships were inferred from ~1,400 bp of their 16S ribosomal DNA sequences using the PAUP program [36]. The letters at each node indicate ancestral nodes. A "branch and bound" bootstrap analysis [36] was performed to ascertain confidence in the inferred relationships. The percentage of bootstrap samples supporting each grouping is shown (in box) above the branch at each node, and branch lengths are shown below each branch. Nodes D and E were artificially introduced to provide a bifurcated tree for correlation analyses (see Methods for details).

samples supporting each grouping is indicated [36]. Branch lengths were calculated as the minimum number of base changes necessary to obtain the tree generated by PAUP. The estimated branch lengths between ancestral nodes were lengthened slightly using a method that seeks to correct systematic biases in estimating ancestral character states [10]. Growth rates corresponding to each inferred ancestor were calculated as the average of the growth rates for the extant strains derived from the ancestor, weighted by the estimated branch lengths between ancestors and derived strains [10].

The method of scaled independent contrasts for correlation tests employed in this study requires a bifurcated tree. However, strains TFD6, TFD13, and TFD20 showed identical sequences, resulting in a shared terminal branch on the phylogenetic tree. To obtain a bifurcated tree, minimal variation was introduced between TFD6, TFD13, and TFD20 in order to obtain scaled independent contrasts. Specifically, branch lengths of 1 unit each were introduced from an ancestral node (node E in Fig. 2) to strains TFD6 and TFD20, between node E and a node (node D in Fig. 2) ancestral to node E and TFD13, and between node D and TFD13. There are, of course, two other ways of grouping these three strains, but the actual choice is inconsequential for all of our results.

Results

Traditional Comparative Method: Application

The trade-off hypothesis predicts a negative correlation between growth rates at high and low substrate concentrations. We first tested this hypothesis using the traditional comparative method, which treats each strain as an independent observation. Both succinate (Fig. 3a) and 2,4-D (Fig. 3b) showed positive correlations between growth rates measured at 5 and 500 μg/ml. For succinate, this correlation was marginally significant ($r = 0.7747$, $P = 0.0408$; all P values reported are two-tailed), whereas for 2,4-D the correlation was marginally nonsignificant ($r = 0.7431$, $P = 0.0556$). This traditional analysis clearly does not indicate any trade-off between performance at high and low substrate concentrations. Rather, it suggests the opposite trend, whereby some

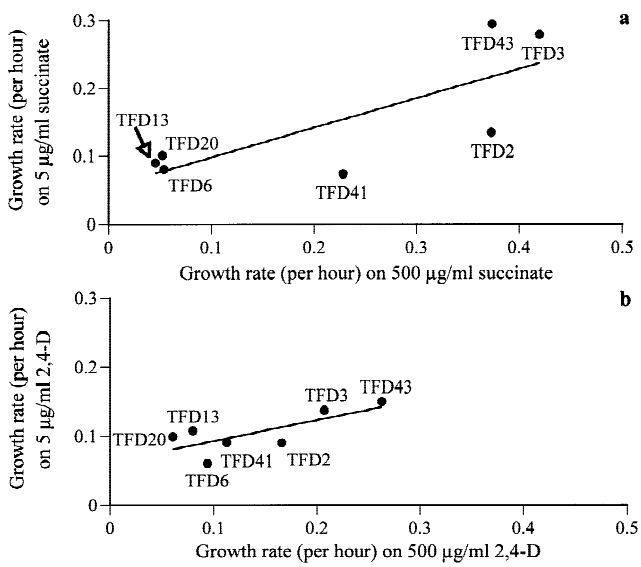

Fig. 3. Correlations using the traditional comparative method. Correlations between growth rates at low and high substrate concentrations are shown for (a) succinate and (b) 2,4-D. Data points are labeled with strain designations. The traditional method treats all strains as independent observations, regardless of their phylogenetic relationships. The correlation is significant for succinate ($r = 0.7747$, $P = 0.0408$). The correlation is marginally nonsignificant for 2,4-D ($r = 0.7431$, $P = 0.0556$).

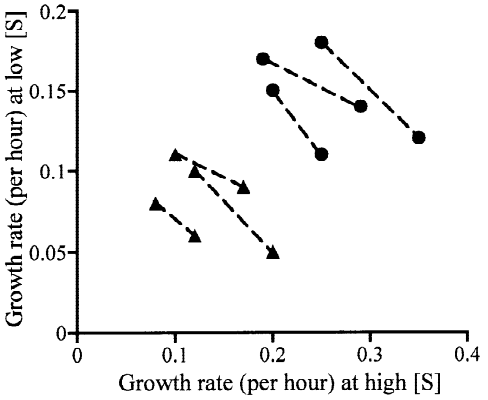

Fig. 4. Hypothetical effects of phylogeny on correlation analyses. Growth rates schematically illustrated at low and high substrate concentrations are positively correlated across two distantly related taxa (triangles and circles), even though several pairs of more closely related strains within each taxon exhibit the negative correlation predicted by the trade-off hypothesis. Lines connect the most closely related pairs. The overall correlation would appear to be positive using the traditional comparative method that treats each species as an independent observation. Newer comparative methods take into account relationships among strains by constructing a series of phylogentically independent contrasts, which could reveal the preponderance of negative associations between the two traits.

strains are superior at both low and high substrate concentrations.

However, there are two important considerations that may render this conclusion invalid. First, it is inappropriate to treat each strain as an independent observation, unless one uses phylogenetic information to scale the data in order to meet the assumptions of standard statistical tests. Failure to do so may dramatically alter the apparent correlation between traits. For example, growth rates at high and low substrate concentrations may be positively correlated for one pair of distantly related taxonomic groups, even though several pairs of more closely related strains within each group show the predicted negative correlation (Fig. 4). In the next section, these data are reanalyzed using comparative methods that incorporate information on phylogenetic relationships.

Second, some strains may be fortuitously preadapted to the laboratory environment for reasons that have nothing to do with the competitive relationships between strains in the soil or other natural environments. For example, one strain may grow much faster than another—at both low and high substrate concentrations—simply because the first strain is better suited to the particular combination of base mineral medium, temperature, and other variables used in the laboratory experiments. If one could analyze performance in an environment more similar to that in which the strains had evolved, then one might see the predicted trade-off. In a later section, we present an indirect test of whether certain strains are fortuitously better adapted to the laboratory environment than are other strains.

Phylogenetically Independent Contrasts: Theory

The purpose of using phylogenetic information in comparative studies is to obtain the truly independent data that are required for robust statistical analysis, data that reflect independent evolutionary events. If all of the species in a study shared a single common ancestor from which they had independently evolved for the same length of time, then they could be appropriately treated as statistically independent observations (Fig. 5a). But if the species are hierarchically related (as in most phylogenies), then statistical problems arise because of the relative lack of independence between

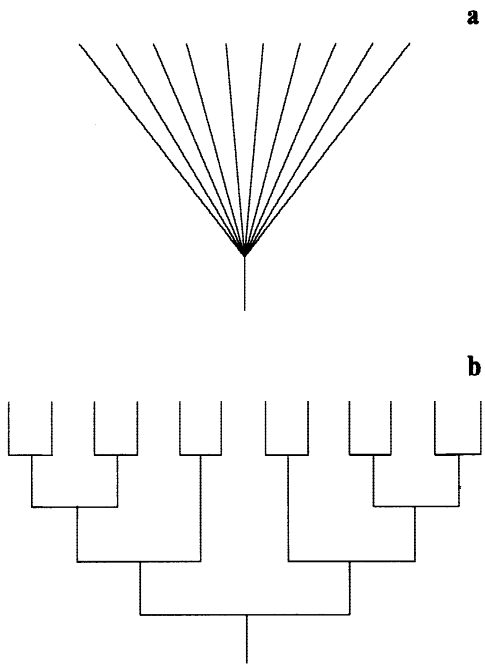

Fig. 5. Equidistant and hierarchical strain phylogenies. Strains (or species) are properly treated as independent observations only if they share a single common ancestor from which they have all evolved for the same length of time. This would require a "star" phylogeny (a), in which all species pairs are equidistant. More generally, pairs of strains (or species) are hierarchically related (b), and newer comparative methods take into account these relationships.

the states of more recently diverged lineages (Fig. 5b). Statistical problems may also arise if evolutionary rates vary among the lineages [16].

Several different methods have been developed in recent years to incorporate phylogenetic information into comparative analyses. Some of the earliest methods relied on making contrasts only between independent pairs of extant species [3, 4, 23, 35], but these methods lacked statistical power because they discarded potentially useful information. More recent methods are preferable because they make use of all the relevant information that is available in the data [10, 13, 16].

Felsenstein's [10] method of independent comparisons makes use of the fact that any two extant species (say, 1 and 2) at adjacent tips on a phylogenetic tree uniquely share a common ancestor. Consequently, any evolutionary change in the traits of interest (say, X and Y) since 1 and 2 split from their most recent common ancestor is independent of changes in X and Y at all other locations in the phylogenetic tree. Hence, the differences in traits X and Y between species 1 and 2 are statistically independent from the differences in X and Y between other such pairs of adjacent species in the tree. For example, (X1–X2) and (Y1–Y2) are independent of (X3–X4) and (Y3–Y4). Moreover, this independence holds for adjacent pairs of nodes at higher levels (i.e., earlier in time) in the tree. By using this method, one can extract $n - 1$ independent contrasts from n extant species, when all of the differences between adjacent tips and nodes are calculated. Statistical tests of the correlation between traits of interest are then made using these $n - 1$ independent contrasts (weighted for expected variance based on branch lengths). This method assumes that the true branching pattern and branch lengths of the phylogeny are known, or at least that they can be estimated with some confidence.

Of course, one cannot know the actual values of the traits at the ancestral nodes, but these can be estimated by assuming a particular model of evolutionary change. Felsenstein's method assumes a model of evolutionary change that is equivalent to random Brownian motion. In such a process, each trait has an equal likelihood of a positive or negative change of equal magnitude over any given period of time. Hence, the expected mean change is zero, and the expected variance around this mean is directly proportional to the elapsed time (estimated from the branch lengths). The value of any trait at each ancestral node is therefore estimated simply as the midpoint of its descendant lineages scaled by the distances of those lineages from the node.

Various modifications of these methods have been developed that assume different models of evolutionary change [13, 16]. Martins and Garland [26] ran computer simulations to examine the effect of these different assumptions on the statistical validity of the resulting evolutionary inference. Although they found that these assumptions had some effect on statistical validity, fortunately the effects were usually minor. More importantly, Martins and Garland found that all of the methods that incorporate phylogenetic relatedness (but which assume different models of evolutionary change) performed much better than did the traditional approach, even when the assumed model of evolution is incorrect. Therefore, in practice, methods that use phylogenetically independent contrasts will yield more reliable inferences than traditional comparative analyses, even when the assumptions that underlie the phylogenetic method are violated.

Phylogenetically Independent Contrasts: Application

We used Felsenstein's method of independent contrasts (see above) to reexamine the correlation between bacterial growth rates at high and low substrate concentrations, using the phylogenetic relationships among the seven strains depicted in Fig. 2.

In succinate, the significant positive correlation between growth rates at high and low concentrations, based on the traditional comparative method, is not supported by the method of phylogenetically independent contrasts (Fig. 6a: $r = 0.2487$, $P = 0.6347$). In 2,4-D, the phylogenetic method again gives no indication of a significant correlation (Fig. 6b: $r = 0.3458$, $P = 0.5019$), whereas the traditional method suggested a positive correlation. Thus, by employing phylogenetically independent contrasts, comparative data that seemed to flatly contradict the trade-off hypothesis now appear to be inconclusive. These changed results, although inconclusive with respect to the trade-off hypothesis, suffice to show that phylogenetic considerations are important for comparative studies in microbial ecology.

An Indirect Test of the Preadaptation Hypothesis

Phylogenetically independent contrasts provide no support for the hypothesized trade-off between relative growth rates at high and low substrate concentrations. A possible explanation for the failure of this hypothesis is that certain strains may be fortuitously preadapted to growth in the laboratory. Thus, one strain may grow faster than another simply because the first strain prefers the particular combination of mineral medium, temperature, and other variables used in

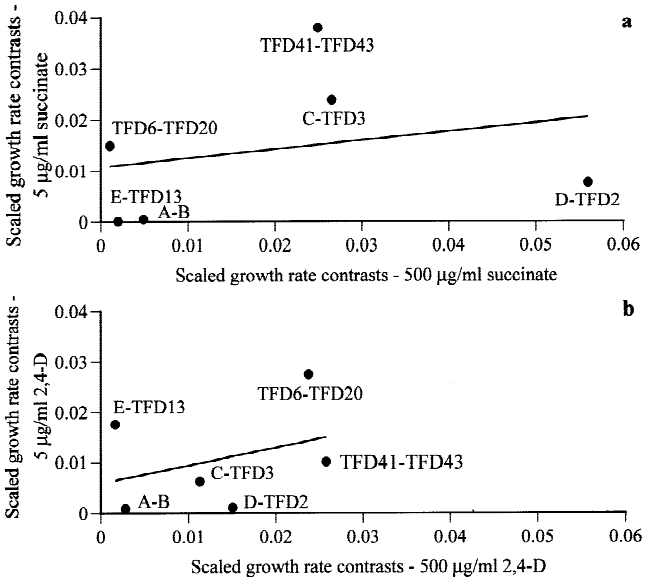

Fig. 6. Correlations using new comparative methods. Correlations between growth rates at low and high substrate concentrations are shown for (a) succinate and (b) 2,4-D. These new methods take into account phylogenetic relationships among strains, in order to construct a series of evolutionarily independent contrasts. Data points are labeled with the appropriate strain pairs, node pairs, or strain–node combinations that were used for the phylogenetically independent contrasts. The suggested correlations are weak and nonsignificant for both succinate ($r = 0.2487$, $P = 0.6347$) and 2,4-D ($r = 0.3458$, $P = 0.5019$), whereas the traditional comparative method suggested strong positive correlations (Fig. 3).

laboratory experiments at both high and low substrate concentrations. A direct test of this hypothesis is beyond the scope of this study, as it would require the ability to perform competition experiments between strains at high and low substrate concentrations *in situ* in the environments from which the strains were isolated. However, an indirect test can be performed by asking whether growth rates on succinate and 2,4-D are positively correlated. That is, all other aspects of the growth conditions (medium composition, temperature, etc.) are held constant, so that those strains that are fortuitously preadapted to these conditions should benefit regardless of the substrate that is provided.

To that end, we used both the traditional method and the method of phylogenetically independent contrasts to calculate the correlation between growth rates on succinate and 2,4-D. The traditional method indicates strong correlations in growth rate across substrates at both high and low substrate concentrations (5 μg/ml; $r = 0.8746$, $P = 0.0100$; 500 μg/ml: $r = 0.8921$, $P = 0.0069$), and these results are consistent with the preadaptation hypothesis. Using the phylogenetically based method, however, neither correlation is supported (5 μg/ml: $r = 0.08136$, $P = 0.8782$; 500 μg/ml: $r = 0.0662$, $P = 0.6227$). This analysis therefore suggests that there are indeed differences in preadaptation to the laboratory conditions among the seven strains studied here, but that most of this variation in preadaptedness is due to differences between clusters of closely related strains rather than to independently evolved differences between individual strains (Figs. 3 and 6). These results serve to reemphasize the large effect that phylogenetic considerations can have in correlation analyses.

Discussion

This study had two overlapping goals. The first of these goals was to examine whether bacterial strains that are good competitors at high substrate concentrations tend to be inferior competitors at low substrate concentrations, and vice versa. This trade-off hypothesis, if valid, has important implications for understanding the structure of microbial communities, as well as for identifying strains that are most likely to be useful in applications such as bioremediation. Previous tests of this hypothesis have yielded mixed results [14, 17, 19, 20, 21, 22, 27, 38, 39, 41], and the results of our study must be added to those that do not support this hypothesis. This lack of support for the trade-off hypothesis is consistent with recent discoveries in several microbes of both high- and low-affinity uptake systems for a variety of specific nutrients [6, 24, 31, 34, 42]. If such dual systems are common in natural populations of bacteria, this would argue against the necessity of a strong trade-off. However, there are important caveats to this negative interpretation of our results that will be considered below.

The second goal of our study was to illustrate the application of recent advances in the comparative method to microbial ecology [10, 15, 16]. The traditional comparative approach has been to view each species or strain as an independent observation when one calculates correlations between two traits, such as growth rates at high and low substrate concentrations. This approach implicitly ignores the phylogenetic relationships among species or strains. However, there is a general lack of statistical independence of observations with respect to evolutionary hypotheses because of these phylogenetic relationships. As a consequence, the traditional comparative method may often generate spurious correlations [26]. A newer comparative method explicitly takes into account these phylogenetic relationships and

thereby avoids this problem. In this study, we found that correlations between growth rates at high and low substrate concentrations were weaker when the phylogenetic relationships were taken into account than when they were ignored (contrast Figs. 3 and 6).

These recent advances solve certain statistical problems with the comparative approach as it was formerly applied. Nonetheless, there remain vexing problems in determining the validity of inferences based on the comparative method. We will now discuss two such problems. First, it is typically assumed that phylogenetic relationships among organisms can be determined using essentially any set of molecular or other data capable of resolving evolutionary relatedness. The presumption is that any one gene has the same genealogy (at the level of species and higher taxa) as do all other genes. But with bacteria, this assumption may be violated, especially for those traits that are encoded by plasmids or other mobile genetic elements. (See also Avise [2] for a more general discussion of this problem in the context of populations with Mendelian genetic systems.) Phylogenetic relationships among the strains used in this study were inferred from chromosomal sequences encoding the 16S ribosomal subunit. However, *tfdA* and other genes that encode the ability to catabolize 2,4-D are often plasmid-borne [7], so that the phylogenetic relationships used for independent contrasts may have been inappropriate for testing the correlation between growth rates at high and low concentrations of 2,4-D.

Indeed, McGowan et al. [28] recently showed that the phylogeny of bacteria that degrade 2,4-D obtained using the 16S ribosomal gene is different from the phylogeny of the *tfdA* genes themselves, which implies horizontal gene transfer (see also [8]). When evaluating the potential trade-off between growth rates at high and low concentrations of 2,4-D, it is unclear whether the *tfdA* or 16S ribosomal gene phylogeny is more appropriate, because it is unclear *a priori* which genes might mediate this hypothetical trade-off. Therefore, in addition to scaling the 2,4-D growth rate data using the 16S ribosomal gene phylogeny, we performed a comparable scaling using the *tfdA* gene phylogeny [28] for five of the same seven TFD strains (*tfdA* sequences were unavailable for the other two strains). Using phylogenetically unscaled growth rate estimates for the five strains, there is a significant positive correlation between growth rates at 5 and 500 μg/ml 2,4-D ($r = 0.9466$, $P = 0.0147$). Scaling based on the *tfdA* gene phylogeny for these five strains reduced the correlation coefficient of this relationship and made it statistically insignificant ($r = 0.8104$, $P = 0.0962$). These results using the *tfdA* gene phylogeny are qualitatively similar to those based on the 16S ribosomal gene phylogeny. This similarity suggests that the failure of the trade-off hypothesis is probably not due to effects of horizontal gene transfer. The fact that we failed to find any evidence of a negative correlation between growth rates at high and low succinate concentrations further indicates that possible complications due to horizontal gene transfer were not generally responsible for the failure of the trade-off hypothesis.

The second caveat arises because the hypothesized trade-off between performances at high and low substrate concentrations may be masked by fortuitous differences among strains in the extent of their preadaptation to laboratory growth conditions. Imagine, for example, that some strains are adapted to habitats that are similar in temperature, mineral composition, and so on to the conditions used in our laboratory experiments, whereas other strains prefer dissimilar conditions. Certain strains might have a consistent advantage over other strains regardless of substrate concentration, and these differences would therefore promote a positive correlation between growth rates measured at low and high substrate concentrations. This effect could therefore obscure or even override any negative correlation due to the hypothesized trade-off.

Several evolutionary studies with higher organisms have shown this systematic bias against detecting trade-offs. These studies have relied on contrasting the results of comparative studies in which the performance capabilities of natural isolates are measured under novel conditions (i.e., dissimilar to those under which they evolved) with the results of experimental evolutionary studies. In these experimental studies, populations are allowed to evolve in the laboratory, so that the organisms are adapted to the same conditions in which their performance capabilities will be measured. For example, to test the predictions of the theory of *r*- and *K*-selection, Mueller and Ayala [29] allowed replicated experimental populations of *Drosophila melanogaster* to evolve for eight generations in the laboratory at either high or low population density. They then estimated the per capita growth rates of the derived populations at different population densities, and they compared these responses across the selected populations. They observed the predicted trade-off, such that the populations selected at low density grew more slowly at high density than did those selected at high density, and vice versa. However, this same trade-off was *not* detected when Mueller and Ayala [29] performed a comparative study using 25 strains of *D. melanogaster* isolated from nature, presumably because of fortuitous differences among these strains in their preadaptation to the laboratory envi-

ronment. A direct demonstration of the confounding effect of fortuitous preadaptation to novel environments (from the standpoint of an organism's evolutionary history) was made by Service and Rose [33], also using *D. melanogaster*. By means of an evolution experiment, the authors demonstrated a life-history trade-off in the flies between early fecundity and longevity, when the assays of fecundity and longevity were performed in the same culture medium that had been used during the experimental evolution. However, when these same traits were measured in a novel culture medium, the trade-off was largely obscured. It is clear from these studies that the power of the comparative method suffers when performance traits are measured under conditions that differ substantially from those that prevailed during the organisms' evolutionary history. Evolution experiments performed in the laboratory allow one to circumvent this problem, not by eliminating the differences between nature and the laboratory, but rather by using populations that have a defined history of adaptation to the laboratory environment in which the performance assays are conducted.

Thus, two of the authors of this paper also have performed evolution experiments to examine possible trade-offs between growth rates at low and high substrate concentrations [41]. The former was achieved by evolving bacterial populations in chemostat culture (wherein cells are perpetually resource limited), and the latter by evolving populations in serial batch culture (wherein cells periodically experience a surfeit of resources). We then measured the competitive performance of the derived strains, at both low and high substrate concentrations, to ascertain whether or not trade-offs occurred during their evolution under the two regimes. Overall, the results of this experimental study did not support the trade-off hypothesis, and several individual strains evolved in a manner contradictory to it. If the trade-off hypothesis were generally true, then evolved lines showing increased fitness in their selective regime should have shown decreased fitness in the alternative regime. In fact, among the strains that adapted significantly to their selective regime (batch or chemostat culture), more strains showed significant fitness increases in the alternative regime than decreases. These experiments clearly show that bacteria are not always subject to a trade-off between performance under sparse versus abundant resource conditions. This finding is consistent with the results of the comparative study described here.

In conclusion, the hypothesis of a trade-off in bacterial fitness between low and high substrate concentrations is difficult to test. This hypothesis is not supported by either the results of this comparative study or the results of a parallel experimental study [41]. Nonetheless, the trade-off hypothesis is intuitively appealing and may still apply to certain microorganisms under specific ecological and evolutionary conditions [41]. Therefore, this hypothesis may still have considerable importance for the field of microbial ecology and warrants further study by strategies that seek to avoid the inferential difficulties described here.

Acknowledgments

We thank W. Holben and N. Tonso for providing the bacterial strains used in this study, T. Marsh for providing the 16S rRNA gene sequence for strain TFD41, and C. McGowan for sharing data on plasmid phylogeny. We also thank L. Forney, P. Rainey, L. Snyder, J. Tiedje, M. Travisano, and anonymous reviewers for helpful comments on this manuscript. This research was supported by the NSF Center for Microbial Ecology (DEB-912006).

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