# Positive Correlation between Bacterial GC Content and Growth Temperature

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Abstract

Because GC pairs are more stable than AT pairs, GC-rich genomes were proposed to be more adapted to high temperatures than AT-rich genomes. Previous studies consistently showed positive correlations between growth temperature and the GC contents of structural RNA genes. However, for the whole genome sequences and the silent sites of the codons in protein-coding genes, the relationship between GC content and growth temperature is in a long-lasting debate. With a dataset much larger than previous studies (681 bacteria and 155 archaea), our phylogenetic comparative analyses showed positive correlations between optimal growth temperature and GC content both in bacterial and archaeal structural RNA genes and in bacterial whole genome sequences, chromosomal sequences, plasmid sequences, core genes, and accessory genes. However, in the 155 archaea, we did not observe a significant positive correlation of optimal growth temperature with whole-genome GC content or GC content at four-fold degenerate sites. We randomly drew 155 samples from the 681 bacteria for 1000 rounds. In most cases (> 95%), the positive correlations between optimal growth temperature and genomic GC contents became statistically nonsignificant ($P > 0.05$). This result suggested that the small sample sizes might account for the lack of positive correlations between growth temperature and genomic GC content in the 155 archaea and the bacterial samples of previous studies.

*Key words*: GC content, optimal growth temperature, thermal adaptation, phylogenetic generalized least squares (PGLS), resampling analysis.

## Introduction

As guanine (G) strictly pairs with cytosine (C) and adenine (A) pairs with thymine (T) in DNA double helix, the amount of G is equal to C, and that of A is equal to T in the genomes of any cellular organisms. GC content, i.e., the percentage of G + C, is widely used as a measure of genomic nucleotide composition. It is a highly variable trait ranging from 8% to 75% (Basak et al. 2010; Nguyen et al. 2020; Mahajan and Agashe 2021). This genomic trait has been widely studied, and its

evolution has been proposed to be associated with numerous mutational and selective forces driven by genetic, metabolic, and ecological factors (Foerstner et al. 2005; Hildebrand et al. 2010; Mann and Chen 2010; Raghavan et al. 2012; Wu et al. 2012; Agashe and Shankar 2014; Glemin et al. 2014; Šmarda et al. 2014; Reichenberger et al. 2015; Aslam et al. 2019; Dietel et al. 2019; Weissman et al. 2019; Kogay et al. 2020). Among them, the high temperature might be the most long-debating one (Galtier and Lobry 1997; Forsdyke 2021; Meyer 2021). Because G:C pairs have an additional hydrogen bond than A:T pairs, the GC-rich genomes are thermally more stable in high-temperature environments. Bernardi and Bernardi (1986) proposed that high GC content is a thermal adaptation of warm-blooded animals.

As prokaryotes have a much wider thermal distribution than plants and animals, bacterial and archaeal genomes are the best materials to test the thermal adaptation hypothesis. An analysis of 764 prokaryotic species, including mesophilic genera and thermophilic genera, did not find a correlation between whole-genome GC content ($GC_w$) and the optimal growth temperature (Topt) (Galtier and Lobry 1997). However, this study found a significant positive correlation between Topt and the GC content of structural RNA (tRNAs and rRNAs). The rationale of these observations is that the secondary structures of tRNAs and rRNAs are more sensitive to high temperature than the double-strand helix of DNA. In most prokaryotes, protein-coding genes take most of the genome size. Protein structures and functions constrain the GC content evolution at the nonsynonymous sites of the codons. This functional constraint might conceal the hypothetical thermal adaptation. Compared with $GC_w$, the GC content at the third sites of the codons ($GC_3$) is more desirable to test the thermal adaptation hypothesis. Early solitary cases indicated that $GC_3$ might be related to growth temperature. For example, the tyrosy1-tRNA synthetase gene isolated from the thermophile *Bacillus stearothermophilus* has a higher $GC_3$ than the homologous gene in *Escherichia coli*, 68.0% *vs.* 59.4% (Winter et al. 1983). The *leuB* gene isolated from the extreme thermophile *Thermus thermophilus* HB8 has an extremely high $GC_3$, 89.4% (Kagawa et al. 1984). For a general conclusion, Hurst and Merchant (2001) examined the relationship between $GC_3$ and Topt of 29 archaeal species and 72 bacterial species. Unfortunately, they did not find significant correlations between Topt and $GC_3$ or between Topt and $GC_w$. At the same time, they also found a significant positive correlation between the GC content of structural RNAs and the Topt in both archaea and bacteria. As their analysis had accounted for the effect of shared ancestry, they provided more robust evidence against the thermal adaptation hypothesis. Soon afterward, Xia et al. (2002) showed that the growth at increasing temperature (from 37°C to 45°C) for 14,400 generations did not increase but decreased the genomic GC content of the bacterium *Pasteurella multocida*. Furthermore, Lambros et al. (2003) reported a negative correlation between optimal growth temperature and the GC content of protein-coding genes in 550 prokaryotes, despite that the effect of the shared ancestry had not been controlled.

Subsequently, Musto et al. (2004) published a debate-provoking study. As many environmental factors likely influence genomic GC content evolution, closely related species are expected to differ in

fewer environmental factors than distantly related species. The correlation of GC content with growth temperature is less likely disturbed by other factors when the analysis is limited within closely related species. Therefore, Musto et al. (2004) examined the relationship between genomic GC content and Topt with each prokaryotic family. Among the 20 families they studied, the number of families with positive correlations is significantly higher than expected by chance, no matter the effect of the common ancestors was accounted for or not. Meanwhile, they observed a significant positive correlation when considering all independent contrasts from different families together. However, Marashi and Ghalanbor (2004) noticed that most of the significant correlations within each family depend heavily on the presence of a few outlier species. Exclusion of only one species would lead to loss of significant correlations in several families. Basak et al. (2005) pointed out that the correlation is sensitive to the presence or absence of a few outliers in some families because the sample sizes in these families were too small. Using non-parametric correlation analysis that is not sensitive to the presence of outliers, Musto et al. (2005) repeated their analysis and confirmed their previous results. The debate did not end after that. Wang et al. (2006) updated the Topt values for some species and found that the positive correlation between Topt and genomic GC content in two families disappeared. Besides, they suggested that the positive correlation between Topt and genomic GC content in the family Enterobacteriaceae should be explained by the correlation between genome size and optimal temperature. Still, this study did not shake the confidence of Musto et al. (2006) on the correlation between Topt and genomic GC content in prokaryotes. Although Musto and coauthors have rebutted all the criticisms, their studies have not convinced later authors of review articles (Agashe and Shankar 2014; Meyer 2021). For example, Agashe and Shankar (2014) claimed that "*it seems unlikely that genomic GC content is driven by thermal adaptation*" after reviewing the results of Hurst and Merchant (2001) and Xia et al. (2002), but without mentioning the debates on Musto et al. (2004).

As prokaryotic genomes often have many accessory genes that are frequently lost and gained, the genome-wide measures of GC content could roughly reflect the shaping effects of environmental factors in evolution. By contrast, the structural RNA genes ubiquitously exist in prokaryotic genomes, and their GC contents are more comparable in large-scale phylogenetic analyses. Similarly, the core genome or strictly defined orthologous genes could also accurately reflect the historical shaping effect of growth temperature on GC content evolution. Ream et al. (2003) analyzed the GC contents of two genes (*ldh-a* and *α-actin*) across 51 vertebrate species with adaptation temperatures ranging from −1.86°C to approximately 45°C. They did not find any significant positive correlations between living temperature and GC content, whether the GC content is measured by the entire sequences, the third codon position, or the fourfold degenerate sites. However, Zheng and Wu (2010) found a positive correlation between growth temperature and the GC content in the coding regions of four genes across 815 prokaryotic species, including mesophiles, thermophiles, and hyperthermophiles. These four genes shared by all the 815 prokaryotic genomes could be considered strictly defined core genomes.

Using a manually collected dataset of growth temperature and without accounting for the effect of the common ancestors, Sato et al. (2020) recently confirmed the results of Galtier and Lobry (1997). It should be noted that the correlation between Topt and the GC content of structural RNA was consistently observed in much more studies than those mentioned above (Khachane et al. 2005; Kimura et al. 2006; Kimura et al. 2007; Kimura et al. 2013; Sato et al. 2020). By contrast, as reviewed above, the correlation between Topt and genomic GC content, if it exists, depends heavily on the sample size, the families of prokaryotes, the particular sequences, and the methods used to detect it.

Benefit from the manually curated dataset of growth temperature from the database TEMPURA (Sato et al. 2020), we carried out a comprehensive analysis on the relationship between growth temperature and GC content. The present study covers three indexes of growth temperature (maximal growth temperature [Tmax], Topt, and minimal growth temperature [Tmin]) and a series of GC content indexes, including GC content of the whole genome ($GC_w$), GC content of the protein-coding sequences ($GC_p$), GC content at fourfold degenerate sites ($GC_4$), GC content of the genes coding structural RNAs (tRNA, $GC_{tRNA}$; 5S rRNA, $GC_{5S}$; 16S rRNA, $GC_{16S}$; 23S rRNA, $GC_{23S}$) and GC content of non-coding DNA ($GC_{non}$, including intergenic sequences and untranslated regions of mRNA that are generally unannotated in prokaryotic genomes). The whole genome, primary chromosome genome sequences, plasmid genomes, core genes, and accessory genes have been examined separately. Our results consistently support a positive correlation between genomic GC content and growth temperature in bacteria.

## Results

### Strong phylogenetic signals in both GC contents and growth temperatures

A significant force shaping prokaryotic evolution is horizontal gene transfer, which makes the genealogical relationships among bacteria and archaea exhibit a somewhat network-like structure. If bifurcation is not the phylogeny's dominant pattern, most phylogenetic comparative methods will not be necessary for prokaryotic evolutionary studies. We are not sure how much this impression has influenced the researchers in prokaryotic genomic studies, but many papers did not use any phylogenetic comparative methods. Despite the frequent horizontal gene transfers, careful examination of the prokaryotic phylogeny could see a statistical tree (Koonin 2015; DeSalle and Riley 2020; Blais and Archibald 2021). In principle, the necessity of phylogenetic comparative methods depends on the significance of the phylogenetic signal, a measure of the correlation between the evolution of the analyzed trait and the presumed phylogenetic tree. We first measured the phylogenetic signals of the analyzed traits for the 681 bacteria and 155 archaea obtained from the database TEMPURA (Sato et al. 2020). As shown in table 1, all the $\lambda$ values are close to one, which indicates that simple statistical analysis that does not account for common ancestry's effect would lead to inaccurate results (Felsenstein 1985; Symonds and Blomberg 2014).

**Bacterial but not archaeal genomic GC contents correlated with growth temperatures**

We used the phylogenetic generalized least squares (PGLS) regression to examine the relationships between GC contents and growth temperatures. The significant positive and negative slopes of the regressions correspond to significant positive and negative correlations, respectively. Four phylogenetic models, the Brownian motion model (BM), the Ornstein-Uhlenbeck model with an ancestral state to be estimated at the root (OUfixedRoot), the Pagel's lambda model (lambda), and the early burst model (EB), have been applied in the analysis. Their results are qualitatively identical and quantitatively similar. Therefore, we present the BM model results in the main text and deposit other models' results as supplementary tables.

Consistent with numerous previous studies, we found positive correlations between the GC contents of structural RNA genes and growth temperature in bacteria and archaea (Table 2). We noticed a rank in the slope values, from Tmax, Topt, to Tmin.

Interestingly, we also found positive correlations of Tmax and Topt with various indexes of genomic GC contents, $GC_w$, $GC_p$, $GC_4$, and $GC_{non}$, in bacteria (Table 2). Nevertheless, bacterial Tmin is not correlated with three of the four GC content indexes (Table 2). In archaea, none of the three temperature indexes (Tmax, Topt, or Tmin) have any significant correlations with any of the four genomic GC content indexes (Table 2).

If growth temperature could shape GC contents by the stabilities of RNA secondary structures and DNA double helix, a structural RNA or a DNA double helix that is stable at the Tmax or Topt is, of course, stable at the Tmin. In this logic, it is reasonable to see that the Tmin has weaker or no significant correlations with GC contents.

The difference in the correlations between bacteria and archaea might be attributed to either unknown intrinsic differences between these two domains or the significant difference in the sample size, 681 *vs.* 155.

**Sample sizes matter**

If the lack of significant correlations between genomic DNA and Tmax and Topt in archaea results from the small sample size, the correlations in bacteria will be lost when the sample size of bacteria is reduced to 155. For this reason, we randomly selected 155 bacteria from the 681 bacterial samples for 1000 rounds. The results of resampling analysis confirmed the idea, the sample sizes matter (Table 3). In > 950 rounds, the genomic GC content indexes ($GC_w$, $GC_p$, $GC_4$, and $GC_{non}$) are not correlated with Tmax or Topt ($P > 0.05$). This result could also explain the difference between the present study with Hurst and Merchant (2001), which did not find significant correlations between $GC_w/GC_3$ and Topt by phylogenetic analysis of about 100 prokaryotes. Meanwhile, a few positive correlation cases happen, indicating that significant positive correlations could also be found by chance when the analyzed sample is small.

Besides, the correlations between growth temperature and the GC contents of structural RNA

genes might also be lost occasionally when the sample size is severely reduced (Table 3). In the 1000 rounds of resampling, lacking significant correlations happens in 308 (for Tmax) and 473 (for Topt) rounds for 5S rRNA genes, and 12 (for Tmax) and 21 (for Topt) rounds for tRNA genes. However, in the 16S and 23S rRNA genes, positive correlations were consistently observed in all the 1000 rounds of resampling. We suspected that the tens of times more nucleotides in 16S and 23S rRNA than 5S rRNA make the results of 16S and 23S rRNAs less sensitive to small sample sizes.

In statistics, the rule of thumb boundary between small and large samples is $n = 30$. However, the results in Table 3 indicate that $n = 155$ is a too-small sample in the phylogenetic comparative analyses of the relationship between growth temperature and genomic GC content. Because of the common ancestor, two closely related lineages with highly similar growth temperatures and GC contents should be regarded as nearly one effective sample rather than two independent samples. The effective sample size in phylogenetic comparative studies should be much lower than the census number of the analyzed lineages.

**Qualitative data on growth temperature lead to the same conclusion**

In the ProTraits database (http://protraits.irb.hr/) and the IMG database (https://img.jgi.doe.gov/)(Brbić et al. 2016; Chen et al. 2020), many prokaryotes lack quantitative measures of growth temperature but are qualitatively classified into four categories: psychrophiles/psychrotrophiles, mesophiles, thermophiles, and hyperthermophiles. We constructed a qualitative dataset of prokaryote growth temperature, including data downloaded from these two datasets and the prokaryotes in the TEMPURA database classified into the four categories referring (Sato et al. 2020). By assigning 1, 2, 3, and 4 to the psychrophiles/psychrotrophiles, mesophiles, thermophiles, and hyperthermophiles, respectively, we transformed the qualitatively characters into numerical values. PGLS regression analyses revealed a positive correlation between GC content and growth temperature in bacteria (slope = 0.457, $P = 0.001$), but not in archaea (slope = –0.582, $P = 0.170$). Although this dataset (4696 bacteria and 279 archaea) is much larger than analyzed above (681 bacteria and 155 archaea), it lost much information during the qualitative classification. All the differences in growth temperature within each category disappear.

We also examined whether the contrast in the temperature category is correlated with the contrast in the GC content between terminal tips of the phylogenetic tree by referring (Aslam et al. 2019). In total, 273 pairs of bacteria and 41 pairs of archaea were obtained from the phylogenetic tree (Parks et al. 2020). Pairwise comparison showed significantly higher GC contents in the bacteria with higher ranks in growth temperature (Wilcoxon signed rank test, $P = 0.019$, fig. 1A). Still, no significant differences were observed between archaea with different growth temperature ranks (Wilcoxon signed rank test, $P = 0.446$, fig. 1B).

**Positive correlations observed in genes of both chromosomes and plasmids**

Previous studies showed a significant difference in the GC content between plasmids and chromosomes, with significantly lower GC contents in the plasmids (Rocha and Danchin 2002; Nishida 2012; Dietel et al. 2019). Therefore, we examined the correlations between growth temperatures and GC contents separately in chromosomes and plasmids. The separations of plasmids and chromosomes are arbitrary. We strictly followed the classifications of chromosomes and plasmids of the NCBI genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/). Among the 681 bacteria and 155 archaea analyzed above, 172 bacteria and 42 archaea have plasmid genomes. The bacterial chromosomes also have GC contents ($GC_w$, $GC_p$, $GC_4$, and $GC_{non}$) positively correlated with Tmax and Topt (Table 4). Interestingly, the same pattern was also found in the bacterial plasmids (Table 4) in spite that the correlations of Tmax with $GC_4$ and $GC_{non}$ are just significant at marginal levels ($0.05 < P < 0.1$). All these correlations are not significant in archaea.

In the two previous studies comparing the GC content between plasmids and chromosomes (Rocha and Danchin 2002; Nishida 2012), the common ancestor effect was not accounted for. By the way, we performed a phylogenetic paired t-test (Lindenfors et al. 2010) and confirmed the pattern of lower GC content in plasmids (Table S8).

**Positive correlations observed in both core genes and accessory genes**

To correspond to the previous gene-centered studies (Zheng and Wu 2010), we examined the correlations in bacterial core genes, i.e., genes present in all the bacteria. With the increase in the number of bacteria, the number of core genes decreases rapidly. With a trade-off between the number of core genes and the number of bacteria, we selected 28 core genes present in 420 genomes, mostly ribosomal protein genes. Significant positive correlations have been found between GC contents ($GC_p$ and $GC_4$) and growth temperatures, Tmax, and Topt (Table 5).

At the opposite side of the core genes, the accessory genes present in one or a few bacteria. When we define the accessory genes as the genes present in less than 5% of the analyzed bacterial genomes, on average, each bacterium has 152 accessory genes. Positive correlations were observed between GC contents ($GC_p$ and $GC_4$) and growth temperatures (Tmax and Topt), although the values of significance are slightly larger than those in core genes (Table 5). Similar patterns were observed when we increased the threshold in defining accessory genes to 10% ($P < 0.05$ for all cases).

By the way, we compared the GC content between bacterial core genes and accessory genes using a phylogenetic paired t-test (Lindenfors et al. 2010). Unlike the previous analysis of 36 prokaryotes that did not account for the effect of common ancestors (Bohlin et al. 2017), we did not observe significant differences in GC content between the core genes and the accessory genes (Table S16). We also compared the chromosomal accessory genes and plasmid accessory genes. The accessory genes on chromosomes have significantly higher GC contents than those on plasmids (Table S17).

**Discussion**

The GC pairs are thermally more stable than AT pairs in both DNA double helix and structural RNAs. However, this difference is not necessarily a strong enough force to shape the evolution of GC content. As RNA structures are more sensitive to the elevation of temperature than DNA double helix, the growth temperature is stronger in shaping the GC content evolution of the structural RNA genes than in shaping the genomic GC content evolution. Positive correlations between growth temperature and the GC content of structural RNA genes have been repeatedly observed in various prokaryotic studies (Galtier and Lobry 1997; Hurst and Merchant 2001; Khachane et al. 2005; Kimura et al. 2006; Kimura et al. 2007; Kimura et al. 2013; Sato et al. 2020). However, there was a long debate on the correlation between growth temperature and genomic GC content. Benefit from a new manual-curated dataset of prokaryotic growth temperature (Sato et al. 2020), we performed a phylogenetic comparative analysis with a much larger sample than previous studies (Hurst and Merchant 2001; Musto et al. 2004). In 681 bacteria, the genomic GC contents, no matter $GC_w$, $GC_p$, $GC_4$, or $GC_{non}$, are positively correlated with growth temperatures, Tmax and Topt. However, in 155 archaea, there are no significant correlations. Then, we resampled 155 bacteria from the 682 bacteria for 1000 rounds. In most cases, the significant positive correlations between genomic GC contents and growth temperatures disappeared. The resampling analysis indicates that the small sample sizes of the previous analyses (Hurst and Merchant 2001) might lead to the lack of significant correlations. It is effortless to increase the sample size several times if accurate phylogenetic relationships are not considered in the analysis. As shown in Table 1, we found that both growth temperatures and GC contents exhibit strong phylogenetic signals. Overlooking the effect of common ancestors would severely affect the accuracy of the results (Felsenstein 1985).

Our resampling analysis indicates that the lack of significant correlations in archaea might result from the small number of effective samples. We hope to repeat the present study in the future with a larger sample of archaeal genomes. However, it should also be kept in mind that the possibility of no correlation between GC content and growth temperature has not been convincingly excluded. Some intrinsic differences between bacteria and archaea might produce a sharp difference in the correlation between GC content and growth temperature. Most prokaryotes have negatively supercoiled DNA, whereas the prokaryotes that grow at temperatures higher than 80°C (mostly archaea) generally have their genomic DNA positively supercoiled with a particular enzyme, reverse gyrase (Vettone et al. 2014). A high level of supercoiling might stabilize the DNA double helix at high temperatures and relieve the high GC content requirement.

A recent study suggests that sequential amino acid substitutions are involved in the thermal adaptation in the archaeal order *Methanococcales* and revealed arginine as the most favored amino acid (Lecocq et al. 2020). As six GC-rich codons encode the arginine, the thermal adaptation at the proteomic level would affect the evolution of genomic GC content. As the 4-fold degenerate sites are free from the evolutionary forces coming from the natural selection acting on protein sequences, our observations of similar correlations of $GC_w$, $GC_p$, and $GC_4$ with growth temperature indicate that the

nucleotide composition evolved independently in bacterial adaptation to high temperature.

As the frequent gain and loss of plasmids, the plasmid DNAs could be regarded as accessory genomes. Because of the high turnover rates of plasmids and accessory genes in prokaryotic evolution, we could regard them as new immigrants, as opposed to the natives for the chromosomes and core genes. Although the core genes and even the ribosomal RNA genes may occasionally be transferred across different prokaryotic lineages (Tian et al. 2015; Sato and Miyazaki 2017), the fitness cost of inter-species replacement of homologous sequences (Bershtein et al. 2015) restricts the frequency of the core genes. Genes performing essential informational tasks in the cell are less frequently transferred across lineages (Jain et al. 1999; Kacar et al. 2017). Our phylogenetic correlation analysis showed that positive correlations between GC contents and growth temperatures exist in chromosomes and core genes and exist in plasmids and accessory genes. Also, there is no sharp difference in the correlations between the new immigrants and the natives.

In large-scale analyses of horizontal gene transfer in prokaryotes, GC-content similarity between donor and recipient was found to be one factor, or one of the factors, governing the compatibility of the new immigrants in new hosts (Popa et al. 2011; Porse et al. 2018). The effect of promoter GC content on the expression of the new immigrants was suggested to be the underlying mechanism governing the compatibility (Gomes et al. 2020). Here, we suggest that the temperature-associated structural stabilities, including the stability of DNA double helix, the stability of the transient DNA-RNA duplex during transcription, and maybe the stability of the possible secondary structures of mature mRNA (Basak et al. 2010), might be another nonexclusive factor governing the compatibility. The new immigrants compatible with the host have a GC content adapted to the host's growth temperature.

A previous serial transfer experiment seems to be contradictory to our results. Increased genomic GC content was not observed in the bacterium *P. multocida* after 14,400 generations of increasing temperature from 37°C to 45°C (Xia et al. 2002). Although we observed a positive correlation between genomic GC content and growth temperature, we do not think a small increment in GC content, resulting from either a GC-biased mutator or integration of a GC-rich exogenous sequence, would bring a great advantage to the host organism. Most likely, it is just a slight advantage. According to the population genetic theory, the slightly beneficial mutants will be efficiently selected only when they are in a large population. The experimental evolution generally involves severe, periodic reductions in population size, and the bottleneck effect dramatically reduces the fixation probability of beneficial mutations (Wahl et al. 2002). As we see, large-scale statistical analysis has the advantage of revealing slightly beneficial traits.

Musto et al. (2004) emphasize that only when closely related species are compared, the growth temperature is likely to be the only influencing factor in GC content evolution. Our pairwise comparison of neighboring branches with different ranks of growth temperature (fig. 1) gave the same conclusion as our PGLS analyses. We agree that many factors would influence GC content evolution,

and the positive relationship between growth temperature and GC content is a statistically significant result. In the 273 pairs of bacteria, there are 153 pairs where high growth temperature ranks have higher GC contents and 119 pairs with the opposite pattern.

Finally, we should remark that what we observed are just correlations between GC content and growth temperature, which imply rather than prove the causal effects between the two variables. Besides the thermal adaptation hypothesis (Bernardi and Bernardi 1986), we should be open to other intricate explanations for the observed correlations.

## Materials and Methods

We downloaded the prokaryote growth temperatures from the database TEMPURA (Sato et al. 2020). This database contains 8,639 manual curated prokaryotes (549 archaea and 8090 bacteria). Using the links to the NCBI Taxonomy database (Federhen 2012) and the taxonomy IDs provided by TEMPURA for each prokaryotic strain, we obtained 1110 prokaryotes whose genome assembly levels were labeled as"complete" from the NCBI database (Sayers et al. 2021). Among them, we found the phylogenetic information for 682 bacteria and 156 archaea from GTDB (Genome Taxonomy Database) (Parks et al. 2020). The sequences of these genomes were downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/. To avoid annotation bias resulting from different methods, all the genomes were re-annotated using the DFAST, version 1.2.11, with its default parameters (Tanizawa et al. 2018). In total, we obtained the annotations for 681 bacterial genomes and 155 archaeal genomes. The GC contents of these prokaryotes were calculated from their genome sequences.

We also constructed a large dataset according to their growth temperature qualitatively. First, we divided the 836 prokaryotes mentioned above into four categories according to their growth temperature referring to (Sato et al. 2020): psychrophiles/psychrotrophiles (Topt < 20°C), mesophiles (20 ≤ Topt < 45°C), thermophiles (45 ≤ Topt < 80°C), and hyperthermophiles (80°C ≤ Topt). Then, we downloaded the lists of prokaryotes labeled with psychrophiles/psychrotrophiles, mesophiles, thermophiles, or hyperthermophiles from the ProTraits database (http://protraits.irb.hr/) and the IMG database (https://img.jgi.doe.gov/) (Brbić et al. 2016; Chen et al. 2020). After discarding the overlapping items, the conflicting items, and the items lacking phylogenetic information in the GTDB database (Parks et al. 2020), we obtained a new dataset including 4696 bacteria and 279 archaea (Table S18). The whole-genome GC contents of these prokaryotes were downloaded directly from the NCBI genome database (https://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt).

As the contrasts between different pairs of terminal tips of the phylogenetic tree are independent of each other, pairwise comparisons between pairs of terminal tips could control the effect of common ancestors. Referring to (Aslam et al. 2019), we wrote a script to select pairs of closely related bacteria with different ranks of growth temperature (psychrophiles/psychrotrophiles, mesophiles, thermophiles, and hyperthermophiles). In cases where two or more neighboring tips with the same rank were used to pair with bacteria with another rank, we used the average value of their GC contents to represent the

GC content of their internal node.

The phylogenetic signals (λ) of both GC contents and growth temperatures were estimated using the phylosig function of the R (Version 4.0.3) package phytools (Version 0.7-70) (Revell 2012). The PGLS regression was performed using the R (Version 4.0.3) package phylolm (version 2.6.2) (Ho and Ane 2014).

To avoid false-positive results that might happen in multiple correlation analyses of the same dataset, we controlled the false discovery rate by the Benjamini-Hochberg (BH) procedure using the p.adjust function in R (Version 4.0.3).

**Supplementary Material**

Supplementary data are submitted along with the main text.

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**Author Contributions**

DKN conceived the study and wrote the manuscript. EZH, XRL, ZLL, and JG performed the data analysis. All authors read, improved, and approved the final manuscript.

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Table 1. The phylogenetic signals of the variables analyzed in this study.

| Traits | Bacteria | | | Archaea | | |
|---|---|---|---|---|---|---|
| | *n* | Pagel′s λ | *P* | *n* | Pagel′s λ | *P* |
| Tmax | 681 | 0.957 | $3.5\times10^{-178}$ | 155 | 1.000 | $1.5\times10^{-72}$ |
| Topt, | 681 | 0.950 | $1.1\times10^{-196}$ | 155 | 0.988 | $5.7\times10^{-70}$ |
| Tmin | 681 | 0.933 | $6.6\times10^{-152}$ | 155 | 0.966 | $1.9\times10^{-53}$ |
| $GC_w$ | 681 | 1.000 | $2.6\times10^{-294}$ | 155 | 1.000 | $5.8\times10^{-60}$ |
| $GC_p$ | 681 | 1.000 | $4.7\times10^{-292}$ | 155 | 1.000 | $2.4\times10^{-59}$ |
| $GC_4$ | 681 | 1.000 | $4.8\times10^{-238}$ | 155 | 1.000 | $5.1\times10^{-53}$ |
| $GC_{non}$ | 681 | 1.000 | $8.6\times10^{-305}$ | 155 | 1.000 | $2.0\times10^{-65}$ |
| $GC_{tRNA}$ | 681 | 0.998 | $6.0\times10^{-275}$ | 155 | 1.000 | $2.1\times10^{-91}$ |
| $GC_{5S}$ | 646 | 1.000 | $7.0\times10^{-178}$ | 130 | 1.000 | $9.1\times10^{-51}$ |
| $GC_{16S}$ | 681 | 0.999 | $8.7\times10^{-250}$ | 155 | 0.996 | $3.7\times10^{-86}$ |
| $GC_{23S}$ | 681 | 1.000 | $2.1\times10^{-245}$ | 155 | 1.000 | $6.8\times10^{-83}$ |

Tmax, Topt, and Tmin represent maximal, optimal, and minimal growth temperature, respectively; $GC_w$, $GC_p$, $GC_4$, $GC_{tRNA}$, $GC_{5S}$, $GC_{16S}$, $GC_{23S}$, and $GC_{non}$ represent the GC contents of the whole genome, the protein-coding sequences, the fourfold degenerate sites, the genes coding tRNAs, the genes coding 5S rRNA, the genes coding 16S rRNA, the genes coding 23S rRNA, and the non-coding DNA (including intergenic sequences and untranslated regions of mRNA), respectively. The phylogenetic signals of the chromosomal genes, the plasmid genes, the core genes, and the accessory genes are also very close to one and deposited in supplementary Table S1-S4.

Table 2. PGLS analysis of GC contents and growth temperatures.

| | Bacteria | | | Archaea | | |
|---|---|---|---|---|---|---|
| | Slope | $P$ | $P_{BH}$ | Slope | $P$ | $P_{BH}$ |
| $GC_w$-Tmax | $7.1\times10^{-4}$ | $7.1\times10^{-4}$ | $9.4\times10^{-4}$ | $6.6\times10^{-4}$ | 0.115 | 0.153 |
| $GC_w$-Topt | $5.7\times10^{-4}$ | 0.009 | 0.011 | $3.3\times10^{-4}$ | 0.377 | 0.503 |
| $GC_w$-Tmin | $2.8\times10^{-4}$ | 0.156 | 0.226 | $5.2\times10^{-4}$ | 0.126 | 0.168 |
| $GC_p$-Tmax | $6.6\times10^{-4}$ | 0.002 | 0.002 | $5.6\times10^{-4}$ | 0.183 | 0.209 |
| $GC_p$-Topt | $5.3\times10^{-4}$ | 0.015 | 0.016 | $2.4\times10^{-4}$ | 0.522 | 0.597 |
| $GC_p$-Tmin | $2.5\times10^{-4}$ | 0.202 | 0.231 | $4.6\times10^{-4}$ | 0.180 | 0.205 |
| $GC_4$-Tmax | 0.001 | 0.003 | 0.003 | $9.9\times10^{-4}$ | 0.321 | 0.321 |
| $GC_4$-Topt | 0.001 | 0.016 | 0.016 | $2.2\times10^{-4}$ | 0.806 | 0.806 |
| $GC_4$-Tmin | $5.5\times10^{-4}$ | 0.239 | 0.239 | $6.9\times10^{-4}$ | 0.393 | 0.393 |
| $GC_{non}$-Tmax | $8.0\times10^{-4}$ | $1.7\times10^{-4}$ | $2.8\times10^{-4}$ | $9.1\times10^{-4}$ | 0.025 | 0.041 |
| $GC_{non}$-Topt | $6.4\times10^{-4}$ | 0.004 | 0.006 | $6.4\times10^{-4}$ | 0.080 | 0.129 |
| $GC_{non}$-Tmin | $2.7\times10^{-4}$ | 0.170 | 0.226 | $6.5\times10^{-4}$ | 0.048 | 0.077 |
| $GC_{tRNA}$-Tmax | $4.1\times10^{-4}$ | $2.2\times10^{-16}$ | $5.9\times10^{-16}$ | $7.1\times10^{-4}$ | $1.8\times10^{-11}$ | $7.2\times10^{-11}$ |
| $GC_{tRNA}$-Topt | $3.9\times10^{-4}$ | $2.6\times10^{-14}$ | $6.9\times10^{-14}$ | $5.0\times10^{-4}$ | $2.5\times10^{-7}$ | $6.7\times10^{-7}$ |
| $GC_{tRNA}$-Tmin | $1.5\times10^{-4}$ | $9.1\times10^{-4}$ | 0.002 | $4.2\times10^{-4}$ | $1.8\times10^{-6}$ | $4.7\times10^{-6}$ |
| $GC_{5S}$-Tmax | $5.5\times10^{-4}$ | $1.2\times10^{-6}$ | $2.4\times10^{-6}$ | 0.001 | $1.9\times10^{-5}$ | $3.9\times10^{-5}$ |
| $GC_{5S}$-Topt | $4.4\times10^{-4}$ | $1.4\times10^{-4}$ | $2.9\times10^{-4}$ | $8.9\times10^{-4}$ | $1.6\times10^{-4}$ | $3.2\times10^{-4}$ |
| $GC_{5S}$-Tmin | $3.5\times10^{-4}$ | 0.001 | 0.002 | $6.1\times10^{-4}$ | 0.005 | 0.010 |
| $GC_{16S}$-Tmax | $5.4\times10^{-4}$ | $2.2\times10^{-16}$ | $5.9\times10^{-16}$ | $8.2\times10^{-4}$ | $3.9\times10^{-11}$ | $1.0\times10^{-10}$ |
| $GC_{16S}$-Topt | $5.2\times10^{-4}$ | $2.2\times10^{-16}$ | $8.8\times10^{-16}$ | $7.2\times10^{-4}$ | $1.1\times10^{-10}$ | $4.5\times10^{-10}$ |
| $GC_{16S}$-Tmin | $4.6\times10^{-4}$ | $2.2\times10^{-16}$ | $8.8\times10^{-16}$ | $5.5\times10^{-4}$ | $8.5\times10^{-8}$ | $3.4\times10^{-7}$ |
| $GC_{23S}$-Tmax | $6.6\times10^{-4}$ | $2.2\times10^{-16}$ | $5.9\times10^{-16}$ | 0.001 | $2.2\times10^{-16}$ | $1.8\times10^{-15}$ |
| $GC_{23S}$-Topt | $6.5\times10^{-4}$ | $2.2\times10^{-16}$ | $8.8\times10^{-16}$ | 0.001 | $1.2\times10^{-14}$ | $9.5\times10^{-14}$ |
| $GC_{23S}$-Tmin | $4.9\times10^{-4}$ | $2.2\times10^{-16}$ | $8.8\times10^{-16}$ | $8.3\times10^{-4}$ | $8.0\times10^{-11}$ | $6.4\times10^{-10}$ |

GC contents were the dependent variables, and growth temperatures were the independent variables. The results in this table were obtained using the Brownian motion model. Similar results obtained from three other models are deposited in supplementary Table S5-S7. $P_{BH}$, Benjamini-Hochberg adjusted $P$ value. Please see Table 1 for the meanings of the other abbreviations.

Table 3. The appearance of correlations in 1000 rounds of resampling analyses.

| | Significantly Negative ($P < 0.05$) | Not Significant ($P > 0.05$) | Significantly Positive ($P < 0.05$) |
|---|---|---|---|
| $GC_w$-Tmax | 0 | 974 | 26 |
| $GC_w$-Topt | 0 | 991 | 9 |
| $GC_p$-Tmax | 0 | 976 | 24 |
| $GC_p$-Topt | 0 | 993 | 7 |
| $GC_4$-Tmax | 0 | 962 | 38 |
| $GC_4$-Topt | 0 | 992 | 8 |
| $GC_{non}$-Tmax | 0 | 974 | 26 |
| $GC_{non}$-Topt | 0 | 992 | 8 |
| $GC_{tRNA}$-Tmax | 0 | 12 | 988 |
| $GC_{tRNA}$-Topt | 0 | 21 | 979 |
| $GC_{5S}$-Tmax | 0 | 308 | 692 |
| $GC_{5S}$-Topt | 0 | 473 | 527 |
| $GC_{16S}$-Tmax | 0 | 0 | 1000 |
| $GC_{16S}$-Topt | 0 | 0 | 1000 |
| $GC_{23S}$-Tmax | 0 | 0 | 1000 |
| $GC_{23S}$-Topt | 0 | 0 | 1000 |

In each round of resampling, 155 samples were randomly drawn from the 681 bacteria. PGLS regression analysis were performed for each round. GC contents were the dependent variables, and growth temperatures were the independent variables. The results in this table were obtained using the Brownian motion model. Please see Table 1 for the meanings of the other abbreviations. The datasets for each round of resampling are deposited in Supplementary Data S1.

Table 4. PGLS analysis of GC contents and growth temperatures in chromosomes and plasmids.

| | Plasmid | | | Chromosome | | |
|---|---|---|---|---|---|---|
| | Slope | $P$ | $P_{BH}$ | Slope | $P$ | $P_{BH}$ |
| $GC_w$-Tmax | 0.001 | 0.009 | 0.043 | $9.6\times10^{-4}$ | 0.029 | 0.043 |
| $GC_w$-Topt | 0.001 | 0.005 | 0.031 | $9.6\times10^{-4}$ | 0.023 | 0.031 |
| $GC_p$-Tmax | 0.001 | 0.016 | 0.043 | $9.1\times10^{-4}$ | 0.038 | 0.046 |
| $GC_p$-Topt | 0.001 | 0.010 | 0.031 | $9.2\times10^{-4}$ | 0.031 | 0.034 |
| $GC_4$-Tmax | 0.002 | 0.072 | 0.072 | 0.002 | 0.027 | 0.043 |
| $GC_4$-Topt | 0.002 | 0.044 | 0.044 | 0.002 | 0.017 | 0.031 |
| $GC_{non}$-Tmax | $8.3\times10^{-4}$ | 0.055 | 0.060 | 0.001 | 0.021 | 0.043 |
| $GC_{non}$-Topt | $9.3\times10^{-4}$ | 0.025 | 0.031 | 0.001 | 0.021 | 0.031 |

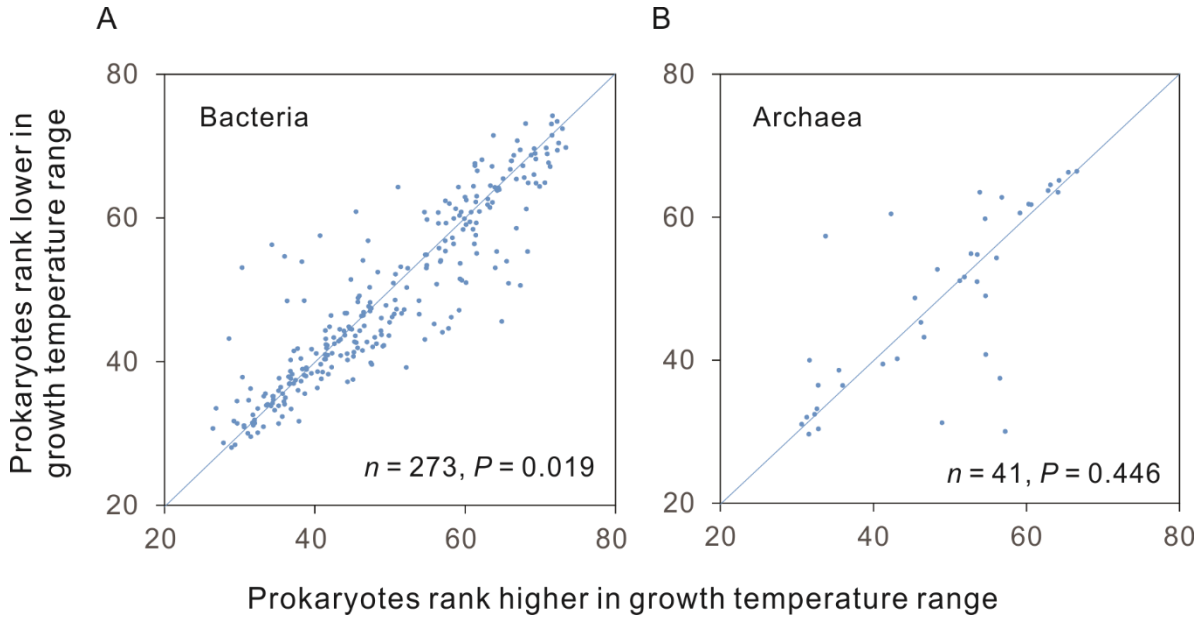
GC contents were the dependent variables, and growth temperatures were the independent variables. The results in this table were obtained using the Brownian motion model. Similar results obtained from three other models are deposited in supplementary Table S10-S12. $P_{BH}$, Benjamini-Hochberg adjusted $P$ value. Please see Table 1 for the meanings of the other abbreviations.

Table 5. PGLS analysis of GC contents and growth temperatures in core genes and accessory genes

| | Core Genes | | | Accessory Genes | | |
|---|---|---|---|---|---|---|
| | Slope | $P$ | $P_{BH}$ | Slope | $P$ | $P_{BH}$ |
| $GC_p$-Tmax | $7.6\times10^{-4}$ | $9.6\times10^{-4}$ | 0.002 | $9.0\times10^{-4}$ | 0.001 | 0.002 |
| $GC_p$-Topt | $6.4\times10^{-4}$ | 0.007 | 0.025 | $6.3\times10^{-4}$ | 0.026 | 0.030 |
| $GC_4$-Tmax | 0.002 | $6.3\times10^{-4}$ | 0.002 | 0.002 | 0.003 | 0.003 |
| $GC_4$-Topt | 0.002 | 0.004 | 0.025 | 0.002 | 0.019 | 0.030 |

GC contents were the dependent variables, and growth temperatures were the independent variables. The results in this table were obtained using the Brownian motion model. Similar results obtained from three other models are deposited in supplementary Table S13-S15. $P_{BH}$, Benjamini-Hochberg adjusted $P$ value. Please see Table 1 for the meanings of the other abbreviations.

Figure legend

Figure 1.

Pairwise comparison of the GC contents between closely related prokaryotes with different growth temperature ranges. Both bacteria (*A*) and archaea (*B*) were classified into four ranks according to their growth temperature, from low to high: psychrophiles/psychrotrophiles, mesophiles, thermophiles, and hyperthermophiles. The diagonal line represents cases in which prokaryotes with different ranks have the same GC contents. Points above the line (153 pairs of bacteria and 17 pairs of archaea) represent cases in which prokaryotes with higher ranks have higher GC contents than their paired relatives, while points below the line (119 pairs of bacteria and 24 pairs of archaea) indicate the reverse. All the *p* values were calculated using two-tailed Wilcoxon signed-rank tests. The exact values of the GC contents are shown in supplementary Table S9.