A Positive Correlation between CRISPR-Cas Contents and Cell Cycle Durations Across the Bacteria Domain

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Abstract

CRISPR-Cas systems provide adaptive immunity for prokaryotic cells by recognizing and eliminating the recurrent genetic invaders whose sequences had been captured in a prior infection and stored in the CRISPR arrays as spacers. However, the biological/environmental factors determining the efficiency of this immune system have yet to be fully characterized. Recent studies in cultured bacteria showed that slowing the growth rate of bacterial cells could promote their acquisition of novel spacers. This study examined the relationship between the CRISPR-Cas content and the minimal doubling time across the bacteria and the archaea domains. Every completely sequenced genome could be used to predict a minimal doubling time. With a large dataset of 4142 bacterial samples, we found that the predicted minimal doubling times are positively correlated with spacer number and other parameters of the CRISPR-Cas systems, like array number, *Cas* gene cluster number, and *Cas* gene number. Different datasets gave different results. Weak results were obtained in analyzing bacterial empirical minimal doubling times and the archaea domain. Still, the conclusion of more spacers in slowly-grown prokaryotes was supported. In addition, we found that the minimal doubling times are negatively correlated with the occurrence of prophages, and the spacer numbers per array are negatively associated with the number of prophages. These observations support the existence of an evolutionary trade-off between bacterial growth and adaptive defense against virulent phages.

Keywords

CRISPR-Cas, cell cycle, bacteria, prophage, trade-off.

INTRODUCTION

CRISPR-Cas systems provide adaptive immunity by recognizing and eliminating the recurrent invaders whose genetic information had been captured in a prior infection and stored in the CRISPR arrays as spacers. Accumulating evidence indicates that slowing the host cell growth rate could enhance spacer acquisition, probably because the slow growth affects virus replication more than the cell's response to the invaders. Using a fluorescent CRISPR adaptation reporter method, Amlinger et al. (1) quantified the adaptation efficiency of type II CRISPR in different growth phases of *Escherichia coli*. They found that spacer acquisition occurs predominantly during the late exponential/early stationary phase. In line with this study, McKenzie et al. (2) observed that the *E. coli* cells that adapted and cleared the target DNA earlier grew significantly slower than the population means. The soil-dwelling bacterium *Pseudomonas aeruginosa* can infect the plant and human bodies. It grows slowly in the cooler temperatures of the soil or during plant infection and rapidly under the higher temperature of human bodies. Høyland-Kroghsbo et al. (3) found that decreasing the growth rate could increase the CRISPR adaptation frequency of the bacterium. Recently, Dimitriu et al. (4) found that the bacteriostatic agents that slow down the growth of *P. aeruginosa* could promote the phage-derived acquisition of novel spacers into the CRISPR array. Furthermore, they showed that limited carbon sources associated with slow bacterial growth could also enhance the evolution of CRISPR immunity. The latter result is in line with the previous observations of increased CRISPR-Cas immunity in bacteria grown in a nutrient-limited medium (5). Dormancy could be regarded as a prolonged growth state of the prokaryotic cells. In the response of the type VI CRISPR-Cas system to RNA virus infection, the Cas13 enzymes of the bacteria *Listeria ivanovii* were found to destruct both the RNA virus genomes and the host mRNA molecules, which further induce dormancy of the host bacterial cells (6). In the same study, the immunity of the bacterial cells against the unrelated virus was enhanced

in the type-VI-CRISPR-Cas-system-induced dormant state.

All the above results were obtained from analyzing a few cultured bacteria in laboratories. We could deduce that environmental factors that drive the bacteria to grow slowly in nature would enhance CRISPR-Cas immunity. The present study examines whether there is a universal relationship between growth rate and adaptive immunity across the bacteria domain. The null hypothesis to be tested is that CRISPR-Cas contents are unrelated to the growth rates across the bacteria and archaea domains.

RESULTS

Construction of the datasets. We constructed three datasets of bacterial minimal doubling times and CRISPR-Cas system contents (Tables S1-S3). The first dataset contains 262 bacterial species with their empirical minimal doubling times retrieved from reference (7). Multiple strains have been sequenced for some bacterial species. In total, 424 genomes were downloaded from the GenBank database (8). These genomes have a wide variation in CRISPR-Cas contents (Table S4), on average, 1.34 CRISPR arrays (ranging from 0 to 12), 43.95 spacers (ranging from 0 to 665), 0.875 *Cas* gene clusters (ranging from 0 to 10), and 5.21 *Cas* genes (ranging from 0 to 49). Among them, 247 genomes do not have any CRISPR-Cas components, 20 have only CRISPR arrays or *Cas* gene clusters, and 157 have both CRISPR arrays and *Cas* gene clusters. For the species with multiple sequenced strains in this dataset, we averaged the multiple CRISPR-Cas content values of each species for further analysis.

The second dataset contains 4142 bacterial genomes with their predicted minimal doubling times obtained from the EGGO database (accessed Nov 26, 2021) (9).

In the third dataset, 508 bacterial genomes, each with only one CRISPR array and one *Cas* gene cluster, were selected from the second dataset.

To control the effects of growth temperature, we also integrated the optimal growth temperatures predicted by the program Tome (version 1.0.0) (10) in the three datasets. Each species' predicted optimal growth temperatures were also averaged in the first dataset.

From the descriptive statistics of the three datasets (Table 1), we can see that the predicted minimal doubling times have a similar median value and a similar range but a lower mean value than the empirical minimal doubling times. Phylogenetic generalized least squares (PGLS) regression analysis showed that the empirical minimal doubling times and the predicted minimal doubling times are significantly correlated (n = 252, slope = 0.228, $P = 1.5 \times 10^{-10}$).

Higher CRISPR-Cas contents in bacteria with long minimal doubling times. We calculated the descriptive statistics for the minimal doubling times of the bacteria with the top 5% highest spacer numbers (Table 1). It appears that the bacteria most rich in spacers grow significantly slower. Previously, Vieira-Silva and Rocha (11) discretized the minimum doubling time (D) into four classes: very fast (D < 1h), fast (1h < D < 2h), intermediate (2h < D < 5h) and slow ($D \geqslant$ 5h). Referring to their classification, our dichotomy between fast and slow was repeated three times, with one h, two h, and five h as the boundary. We compared the slowly-grown bacteria and fast-grown bacteria using the phylogenetic ANOVA method, phyloANOVA (12), and found that the former had higher CRISPR-Cas contents, not just more spacers, than the latter in the second dataset when the show was defined as $D \geq$ 5h and the fast was defined as D < 5h (Table 2). Significant differences were not found in the other two datasets or the phyloANOVA analyses using other definitions of fast and slow in the second dataset. More results with significant differences were obtained when the CRISPR-Cas contents were compared using the Mann-Whitney U test. As ignoring the effects of common ancestry in evolutionary analysis often gave false-positive results (13, 14), we are inclined to accept the results of the phyloANOVA analysis.

For a global relationship between CRISPR-Cas contents and growth rates across the bacterial domains, we performed PGLS regression analysis using CRISPR-Cas contents as the dependent variable and the minimal doubling times as the independent variable. In the first dataset, there is a positive correlation at a marginally significant level (n = 262, slope = 8.683, P = 0.081. Fig. 1A and Table 3) between the empirical minimal doubling times and the spacer numbers. To control the effects of growth temperatures, we performed multiple PGLS regression analyses using optimal growth temperatures as the second independent variable. In this analysis, the empirical minimal doubling times are significantly correlated with spacer numbers (n = 262, slope = 13.145, P = 0.009), *Cas* gene cluster numbers (n = 262, slope = 0.166, P = 0.035), and *Cas* gene numbers (n = 262, slope = 0.985, P = 0.032) (Table 3).

In the second dataset, all the CRISPR-Cas content parameters (array number, spacer number, *Cas* gene cluster number, and *Cas* gene number) have significant positive correlations with the predicted minimal doubling times, no matter whether the optimal growth temperatures were controlled or not (slope > 0, P < 0.05 for all cases, Table 3).

The increase of spacer number with the minimal doubling time can be explained by an enhancing effect on spacer acquisition of slow growth as previously observed in the cultured bacteria (1-6). However, slowly grown bacteria may also have more spacers because they have a more significant number of CRISPR arrays, either duplicated or acquired horizontally. The same logic exists for the *Cas* gene number. Therefore, we examined the relationship of minimal doubling times with the spacer number per array and *Cas* gene number per cluster (Here, only genomes with arrays or *Cas* gene clusters were counted. Genomes without CRISPR-Cas systems were not included in the denominator). The spacer numbers per array positively correlate with the empirical minimal doubling times (n = 152, slope = 4.570, P = 0.046. Fig. 1B) and the predicted minimal doubling times (n = 1507, slope = 3.282, P = 0.010). However, the *Cas* gene numbers per cluster are not significantly correlated with the

empirical minimal doubling time (n = 149, slope = –0.003, P = 0.977) or the predicted minimal doubling time (n = 1472, slope = 0.106, P = 0.145). In addition, we also examined the relationship of predicted minimal doubling times with spacer numbers and *Cas* gene numbers in the third dataset, i.e., 508 bacterial genomes, each of which has only one CRISPR array and one *Cas* gene cluster. Only spacer numbers are significantly correlated with the predicted minimal doubling times, regardless of whether optimal growth temperatures were controlled (Table 3).

According to the *Cas* genes (15), 508 bacteria in the third dataset could be classified, including 329 bacteria with class 1 *Cas* gene cluster (304 type I, 21 type III, and four type IV) and 173 bacteria with class 2 *Cas* gene cluster (167 type II, five type V, and one type VI). We performed the PGLS regression analyses for the groups with more than 50 samples, class 1, class 2, type I, and type II. Although the bacteria with type I *Cas* gene cluster is a subset of the bacteria with class 1 *Cas* gene cluster, they did not give consistent correlations (Table 3). In bacteria with class 1 *Cas* gene cluster, the spacer numbers are marginally correlated with the minimal doubling times after controlling the optimal growth temperatures (n = 329, slope = 4.028, P = 0.097). However, in the bacteria with type I *Cas* gene cluster, the *Cas* gene numbers are significantly correlated with the minimal doubling times (n = 304, slope = 0.155, P = 0.041). Significant ($P < 0.05$) or marginally significant ($0.05 \leq P < 0.1$) correlations have not been observed in bacteria with class 2 or type II *Cas* gene clusters. Different types of CRISPR-Cas systems seem not identical in their relationships with minimal doubling times. Future studies with larger samples will be helpful for a conclusion.

CRISPR-Cas content and growth temperature. Previous studies observed that bacterial CRISPR abundance is positively correlated with growth temperatures (16-19), with an abrupt jump at around 45°C (20). We also examined the relationship between the CRISPR-Cas contents and optimal growth temperatures by controlling the minimal doubling time using

167 multiple PGLS regression analyses. The results show that the minimal doubling times and the
168 growth temperatures are independently correlated with CRISPR-Cas contents (Table 3).
169 However, the spacer numbers per array and the *Cas* gene numbers per cluster in the second
170 dataset are not significantly correlated with the optimal growth temperatures ($P > 0.10$ for
171 both cases, Table 3). Similarly, the spacer numbers and the *Cas* gene numbers in the third
172 dataset are not significantly correlated with the optimal growth temperatures ($P > 0.10$ for
173 both cases, Table 3). It seems that the increase of adaptive immunity with growth temperature
174 is mainly achieved by increasing the number of CRISPR arrays and *Cas* gene clusters. By
175 contrast, increasing bacterial adaptive immunity with minimal doubling time seems to be
176 achieved by increasing all aspects of the CRISPR-Cas systems.

177 **More spacers in archaea with long minimal doubling times.** In archaea, the empirical
178 minimal doubling times and the predicted minimal doubling times are not correlated ($n = 100$,
179 slope = 0.055, $P = 0.325$). The method to predict minimal doubling times was mainly based
180 on bacterial data (9). It seems not to work accurately in archaea. Therefore, we only examined
181 the relationship between the empirical minimal doubling times and CRISPR-Cas contents in
182 archaea. Univariate PGLS regression did not find significant correlations ($P > 0.10$).
183 However, after controlling optimal growth temperatures, the empirical minimal doubling
184 times are significantly correlated with the array numbers ($n = 103$, slope = 0.838, $P = 0.027$),
185 spacer numbers ($n = 103$, slope = 23.239, $P = 0.022$), *Cas* gene cluster numbers ($n = 103$,
186 slope = 0.378, $P = 0.005$), and *Cas* gene numbers ($n = 103$, slope = 2.120, $P = 0.017$).
187 Meanwhile, the spacer numbers per array positively correlate with the empirical minimal
188 doubling time ($n = 88$, slope = 7.476, $P = 0.018$).

189 **Relationship between prophage contents, minimal doubling times, and CRISPR-Cas**
190 **contents.** Previously, Touchon et al. found that the frequency of prophage is negatively
191 correlated with the minimal doubling time in bacteria (21). We examined this relationship

using our datasets. Neither prophage numbers nor the presence/absence of prophages are significantly correlated with bacterial empirical minimal doubling times ($P > 0.10$ for both cases). However, both prophage numbers and the presence/absence of prophages are negatively correlated with bacterial predicted minimal doubling times ($n = 4142$, slope = –0.186, $P = 5 \times 10^{-5}$ and $n = 4142$, slope = –0.057, $P = 10^{-5}$, respectively). In the third dataset, the presence/absence of prophages is negatively correlated with the predicted minimal doubling time ($n = 508$, slope = –0.052, $P = 0.040$). Furthermore, we examined the relationship between prophage contents and CRISPR-Cas systems. The spacer numbers per array in the first and second datasets and the spacer numbers in the third dataset consistently correlate negatively with the prophage numbers ($P < 0.05$ for all the cases, Table 4). However, only the spacer numbers of the third dataset are negatively correlated (at a marginally significant level) with the prophage existence. From Table 4, we can also see that prophages have weak or no relationship with the array numbers, the *Cas* gene cluster numbers, or the *Cas* gene numbers.

DISCUSSION

In a few cultured bacterial species, slow growth has been found to enhance spacer acquisition (1-6). Our results showed that the spacer numbers and the other CRISPR-Cas content parameters (array number, *Cas* gene cluster number, and *Cas* gene number) positively correlate with the minimal doubling times in bacteria. In archaea, these positions appear only after controlling the optimal growth temperatures.

Previous authors reporting the enhancing effects of slow growth on spacer acquisition generally explain their observation by the mechanisms of spacer acquisition (1, 3, 4). If the slow growth could enhance spacer acquisition, the CRISPR-Cas systems would be an efficient mechanism to counteract bacteriophages in slowly-grown bacterial cells and so be selected

when the growth rate has been slowed down on an evolutionary scale.

Furthermore, we could put the CRISPR-Cas-enhancing effect of slow growth into an ecological context. Studies on natural bacterial communities support the existence of trade-offs between intraspecific competition ability (faster growth) and invulnerability to predation (including virus resistance) (22-25). With the limited resources available in natural environments, the bacteria that allocate more resources to defense have to grow slowly, and the bacteria with high competition ability allocate less amount of resources to virus resistance. The trade-off between faster growth and defense is destined if maintaining and expressing the CRISPR-Cas system exert a significant metabolic burden. This metabolic burden has been observed in *Streptococcus thermophilus* by comparing a strain that constitutively expresses the Cas protein and a strain with a non-functional mutant of Cas (26). However, acquiring a few spacers was not associated with fitness costs (26). Besides the metabolic burden, autoimmunity induced by self- and prophage-targeting spacers is another widely-cited cost of CRISPR-Cas systems (27, 28). A trade-off between fast growth and defense using CRISPR-Cas systems might be mediated by the autoimmunity targeting prophages. In the present study, we found evidence for the mutually exclusive effects between prophage and CRISPR-Cas immunity across the bacterial domain (Table 3). Because prophages are more frequent in bacteria with small minimal doubling times (21), there should be a selective force against CRISPR–Cas systems in fast-grown bacteria (29, 30).

In this study, the three bacterial datasets did not always give consistent results. We suggest that the differences in the results should be attributed to the differences in the accuracy and the datasets' sample size. In a previous study, we emphasized that phylogenetic comparative studies generally required larger samples than typical statistical analyses (31). Here, we want to briefly discuss the empirical and predicted data's advantages and disadvantages. At first glance, the empirical minimal doubling times seem superior to the

predicted minimal doubling times because the prediction methods could not achieve an accuracy of 100%. As the wide-spread diversity among the strains designated as one bacterial species (32), the ideal empirical data should include different parameters (e.g., minimal doubling time, optimal growth temperature, and CRISPR-Cas contents) collected from the same strain. Unfortunately, this is not always the case. In many cases, a phenotype value is assigned to a bacterial species without mentioning the strain identity, so the values of different phenotypes might come from different strains. By contrast, all the predicted phenotype values of a strain come from the genome sequences of the same strain. Potential errors resulting from polymorphism within each species could be eliminated if all the parameters were predicted/annotated from genome sequences.

MATERIALS AND METHODS

The datasets of prokaryotic empirical minimal doubling times and predicted minimal doubling times were retrieved from reference (7) and the EGGO database (accessed Nov 26, 2021) (9), respectively. To avoid the outlier effects on the statistical results, we only retained the minimal doubling time shorter than 60h. Because the data on minimal doubling times are highly skewed, they were log-transformed using base *e*. To cover more prokaryotes, we used the optimal growth temperatures predicted by the program Tome (version 1.0.0) (10). The phylogenetic trees and taxonomic information of bacteria and archaea were retrieved from the Genome Taxonomy Database (GTDB, accessed Apr 8, 2022) (33). Because each prokaryotic species rather than each strain has one empirical minimal doubling time value, phylogenetic comparative analysis of the empirical minimal doubling times requires a species tree. From the GTDB, we constructed a species tree using the representative genome of each species. The prokaryotic genome assembly files were downloaded from the GenBank database (accessed Apr 26, 2021) (8). The CRISPR-Cas systems of the prokaryotic genomes were

annotated using CRISPRCasFinder (version 4.2.20) (34). Using VirSorter 2.2.3 (35), prophages were found in bacteria but not archaeal genomes. To avoid underestimation of the CRISPR-Cas systems, partially assembled genomes have been discarded, and only those with assembly levels of “complete genome” or “chromosome” were retained in our analyses.

We estimated the phylogenetic signals (λ) of the analyzed characters using the R (Version 4.0.2) package phytools (Version 0.7-47) (36). All the analyzed characters exhibit significant phylogenetic signals, indicating that phylogenetic comparative methods are required in the analyses to control the effect of common ancestry. The correlations between different characters were estimated by the PGLS regression using the R (Version 4.0.2) package phylolm (version 2.6.2) (37). Pagel’s λ model has been applied in this study.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.674 MB.

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Table 1. Descriptive statistics for the minimal doubling times of all the bacteria in our datasets and the top 5% CRISPR-spacer-rich bacteria.

Datasets	*n*	Median	Mean	Standard deviation	Maximum	Minimum
The first dataset						
All the bacteria	262	2.66	6.71	8.70	52.80	0.23
Top 5%	13	6.50	11.57	10.62	36.94	0.92
The second dataset						
All the bacteria	4142	2.30	3.41	3.24	54.17	0.12
Top 5%	207	6.571	6.66	4.24	18.84	0.19
The third dataset						
All the bacteria	508	2.25	3.60	4.16	54.17	0.14
Top 5%	25	2.88	4.51	4.85	19.99	0.58

Please see the beginning of the Result section for the definition of the three datasets.

Table 2. Slowly-grown bacteria have higher CRISPR-Cas contents than fast-grown bacteria.

	F value	*P*
Array number	178.0	0.018
Spacer number	293.5	0.002
Cas gene cluster number	341.4	0.002
Cas gene number	365.7	0.001

The comparison was performed using phylANOVA with the *P* values adjusted by the Benjamini-Hochberg (BH) procedure. The slowly-grown (n = 891) was defined as the minimal doubling times ≥ 5h and the fast-grown (n = 3251) was defined as the minimal doubling times < 5h. Similar comparisons using one and two hours as the cutoffs did not reveal significant differences.

Table 3. Correlations of the predicted minimal doubling times (*D*) with CRISPR-Cas contents.

		D		*D* with OPT controlled		OPT with *D* controlled	
	N	Slope	*P*	Slope	*P*	Slope	*P*
The first dataset							
Array numbers	262	0.035	0.791	0.138	0.285	**0.056**	**4×10^{-5}**
Spacer numbers	262	8.683	0.081	**13.145**	**0.009**	**2.187**	**4×10^{-4}**
Spacer numbers per array	152	**4.570**	**0.046**	**5.047**	**0.033**	0.183	0.439
Cas gene cluster numbers	262	0.096	0.235	**0.166**	**0.035**	**0.039**	**3×10^{-6}**
Cas gene numbers	262	0.505	0.286	**0.985**	**0.032**	**0.261**	**10^{-7}**
Cas gene numbers per cluster	149	–0.003	0.977	0.066	0.552	**0.025**	**0.010**
The second dataset							
Array numbers	4142	**0.168**	**0.002**	**0.151**	**0.005**	**0.056**	**10^{-14}**
Spacer numbers	4142	**5.326**	**0.003**	**5.212**	**0.003**	**1.456**	**2×10^{-8}**
Spacer numbers per array	1507	**3.282**	**0.010**	**3.408**	**0.008**	–0.101	0.434
Cas gene cluster numbers	4142	**0.091**	**0.001**	**0.085**	**0.002**	**0.033**	**10^{-17}**

Cas gene numbers	4142	**0.562**	**9×10^{-4}**	**0.528**	**0.001**	**0.203**	**3×10^{-18}**
Cas gene numbers per cluster	1077	0.106	0.145	0.105	0.153	6×10^{-4}	0.931
The third dataset							
Spacer numbers	508	**4.351**	**0.029**	**4.588**	**0.021**	–0.421	0.115
Cas gene numbers	508	0.177	0.107	0.172	0.118	0.010	0.515
Bacteria with class 1 *Cas* genes							
Spacer numbers	329	3.89	0.109	4.028	0.097	–0.241	0.461
Cas gene numbers	329	0.058	0.449	0.044	0.559	**0.023**	**0.025**
Bacteria with type I *Cas* genes							
Spacer numbers	304	3.580	0.167	3.901	0.134	–0.405	0.243
Cas gene numbers	304	**0.155**	**0.041**	0.141	0.060	0.016	0.113

OPT, optimal growth temperature. Please see the beginning of the Result section for the definition of the datasets. The correlations were examined using multiple PGLS regression analyses, with the positive sign of the regression slope to indicate the positive correlation. Significant, marginally significant, and nonsignificant results were shown in bold, regular, and grey, respectively

407 Table 4. Correlations between the prophage contents and CRISPR-Cas contents.

		Prophage number		Prophage existence	
	N	Slope	*P*	Slope	*P*
In the first dataset					
Array numbers	262	–0.025	0.606	0.003	0.783
Spacer numbers	262	**–0.002**	**0.037**	-3×10^{-4}	0.313
Spacer numbers per array	262	**–0.010**	**0.007**	–0.001	0.268
Cas gene cluster numbers	262	–0.062	0.422	–0.010	0.633
Cas gene numbers	262	–0.013	0.332	–0.003	0.321
Cas gene numbers per cluster	262	–0.085	0.316	**–0.054**	**0.029**
In the second dataset					
Array numbers	4142	0.017	0.256	–0.002	0.621
Spacer numbers	4142	-3×10^{-4}	0.421	-10^{-4}	0.216
Spacer numbers per array	4142	**–0.005**	**0.002**	–0.001	0.160
Cas gene cluster numbers	4142	0.039	0.169	–0.001	0.856
Cas gene numbers	4142	0.005	0.341	–0.001	0.608
Cas gene numbers per cluster	4142	–0.028	0.284	–0.005	0.448
In the third dataset					
Spacer numbers	508	**–0.005**	**0.009**	**–0.001**	**0.093**
Cas gene numbers	508	–0.016	0.672	0.002	0.837

408 The correlations were examined using PGLS regression analyses, with the negative sign of
409 the regression slope to indicate the negative correlation. The statistically significant (and
410 marginally significant) cases were shown in bold. Prophage existence: 0 and 1 were
411 designated as absence and presence in the PGLS analysis. Please see the beginning of the

Result section for the definition of the three datasets.

Figure legend
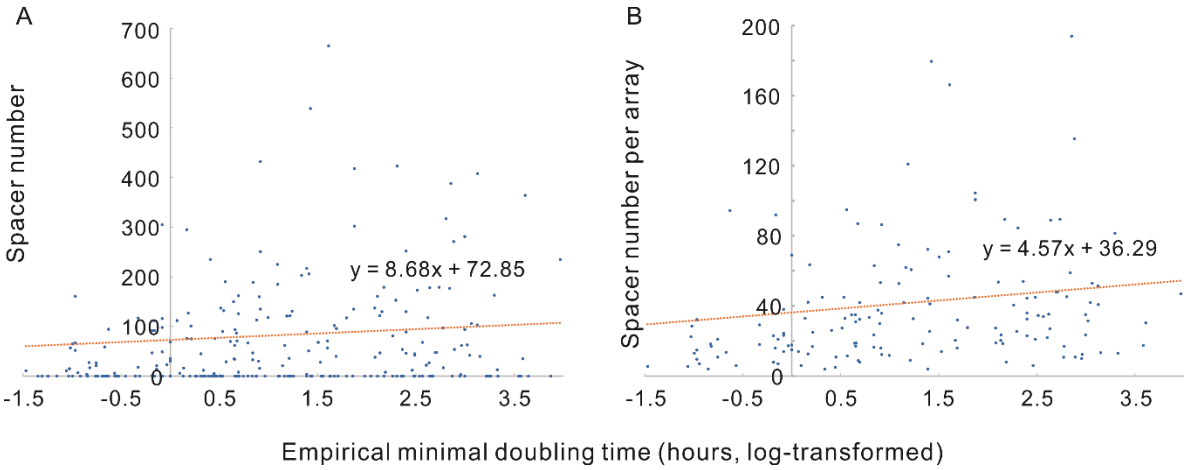
Fig. 1. Correlations of empirical minimal doubling times with CRISPR spacer numbers (A) and spacer numbers per array (B) in bacteria. The correlations were examined using PGLS regression analyses, with the positive sign of the regression slope to indicate the positive correlation. (A), $n = 262$, $P = 0.081$. (B), $n = 152$, $P = 0.046$.