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Changes in growth parameters of *Pseudomonas pseudoalcaligenes* after ten months culturing at increasing temperature

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Abstract

In this paper, we report the thermal adaptation of *Pseudomonas pseudoalcaligenes*, characterized as changes in growth parameters. Six clones derived from a single colony of *P. pseudoalcaligenes* were cultured in two different temperature regimes for 10 months, with three clones forming the control group, cultured at a constant temperature, and another three clones forming the high-temperature (HT) group, cultured at increasing temperature (from 41 to 47°C). Three growth parameters were measured: the lag time (λ), which is the period between the time of transfer to a new medium and the time when the cell replication starts; the maximum growth rate (μ_m); and the maximum yield (A). These three parameters are major components of bacterial fitness. The Gompertz and logistic models were used to estimate these three parameters. The two models gave almost identical estimates, but the Gompertz model had R^2 values consistently larger than the logistic model. The HT clones had significantly shorter λ, but higher μ_m and A than the control clones when both were grown at the originally stressful temperature of 45°C, suggesting significant thermal adaptation. Interestingly, the HT clones grew equally well as the control clones at 35°C, i.e. improved performance at 45°C was not associated with a reduced performance at 35°C.

Keywords: Thermal adaptation; Experimental evolution; Growth temperature; *Pseudomonas pseudoalcaligenes*

1. Introduction

Temperature is perhaps one of the most pervasive selection factors affecting all organisms at all levels of biological organizations, and organisms have evolved in and adapted to almost every thermal environment on Earth [1–3]. However, it is often unclear how adaptation is achieved and what phenotypic traits are modified by thermal selection. Even less clear is how rapidly organisms are able to respond to a novel environment.

There are many approaches to studying the evolutionary basis of thermal adaptation, but most of the knowledge on this subject is derived from the phylogeny-based comparative studies of different natural taxa, generally populations or species evolving in different temperature regimes [4–6]. Although it has all the advantages of being able to examine very diverse taxa that have evolved in natural environments with all their complexity, such an approach has the same problem as all comparative studies: the analysis of correlation, not causation. In addition, the interpretation of phylogeny-based studies crucially depends on the assumption of underlying phylogenetic relationships, which are often difficult to recover, even with molecular data [7,8].

Selection studies on the process of organismal adaptation to different thermal environments have several advantages over traditional comparative studies, including the ability to control the experimental conditions and to replicate experiments, thus permitting a more rigorous evaluation of alternative hypotheses [9]. Such experimental studies generate results more feasible to reveal the cause–effect relationship [10,11].

Bacteria are ideal organisms for such selection studies. They have a short generation time, large population size and simple life history, and allow substantial control over environmental variables and over the initial genetic composition of the experimental population. For this reason, most selection studies on thermal adaptation are done

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with one single bacterial species, *Escherichia coli* [9,10,12–21].

Here, we report a study on adaptive responses of a different bacterial species, *Pseudomonas pseudoalcaligenes*, cultured in increasing temperature. *P. pseudoalcaligenes* is a Gram-negative, mesophilic bacterium and does not form spores. It has the common merits of bacteria for long-term selection studies, such as short generation time, easy propagation and maintenance, and asexual reproduction. In addition, results using *P. pseudoalcaligenes* will complement the extensively and comprehensively studied *E. coli*.

It is important, at the very beginning, to emphasize the difference between the two meanings of 'adaptation process': one at the population level and one at the individual level. With respect to temperature, if a population is genetically heterogeneous (with some being more resistant to high temperature than others), then an increase in temperature tends to have two consequences. First, the increased temperature tends to eliminate temperature-sensitive individuals, leading to a reduction of growth rate at the population level. Second, the increased temperature tends to favor temperature-resistant individuals, leading to a change in genetic composition. When the proportion of temperature-resistant genotypes increases, the growth rate of the population gradually increases. This process of 'adaptation' of the population to increased temperature does not require the origin of any novel temperature-resistant genotype.

The second meaning of the adaptation process refers to the process of a population originally devoid of genotypes resistant to high temperature, coming to live in a stressfully high temperature and, therefore, gradually evolving new genotypes resistant to the high temperature. An experimental study of this process typically would start with a genetically homogeneous population, and is perhaps feasible only with bacterial species. The adaptation in the present study pertains to this second meaning of adaptation.

To study the adaptation of organisms to originally stressful temperature, one should ideally monitor the change of fitness during the adaptation process. However, fitness, being commonly known among evolutionary biologists as the propensity of the genotype being represented in future generations, is not particularly amenable to empirical measurements. Instead, we focus on three growth parameters that presumably should be related to fitness, or at least should be major components of bacterial fitness. These parameters are the lag time (λ), which is the period between the time of transfer to a new medium and the time when the cell replication starts; the maximum growth rate (μ_m); and the maximum yield (A). These three parameters characterize the three phases of bacterial growth: the lag phase, the exponential growth phase and the stationary phase, and can be estimated by the re-parameterized Gompertz or the logistic models for bacterial growth [22].

Although previous studies [18,23] have recognized the fact that all three growth parameters are important components of fitness, no study of thermal adaptation has actually fitted growth models and estimated these parameters. One study [23] did estimate λ and μ_m, but no coherent growth model was used to estimate the parameters simultaneously.

The failure to estimate the three basic growth parameters has created much difficulty in interpreting results from previous studies. For example, when a selection study on *E. coli* [12] or a bacteriophage [24] reported thermal adaptation and improved fitness, it is not clear which of the three growth parameters has changed.

This study addresses three questions: (1) Will the Gompertz and the logistic growth model be sufficient to estimate the three growth parameters for *P. pseudoalcaligenes*? (2) Do all, or only some, of the three growth parameters respond to selection under increasing culture temperature? (3) Will fitness gain in one temperature lead to fitness loss in another?

2. Materials and methods

2.1. Bacterial strain

The wild-type strain of *P. pseudoalcaligenes* F331 was originally isolated by the Institute of Microbial Engineering, Fujian Normal University, China, from the soil immersed in hot spring water in Fuzhou, Fujian province, China, and identified as *P. pseudoalcaligenes* by the Institute of Microbiology, the Academy of Sciences of China [25]. The temperature of the hot spring water varies between 45 and 50°C, but the temperature of the immersed soil, from which the strain was isolated, varies widely because of the large variation of ambient temperature: from 0 to 1°C in winter and close to 37°C in summer. The isolated strain had been stored in a glycerol-based medium at −80°C before this experiment. The culture medium used in all experiments was Luria–Bertani (LB) medium (0.5% Bacto-yeast extract, 1% Bacto-tryptone, 0.5% NaCl). Selection experiments were carried out in LB agar medium (i.e. LB medium with 1.5% Bacto-agar), and experiments for determining growth parameters were performed in LB liquid medium. The entire study started with a single colony of the *P. pseudoalcaligenes* F331 strain.

2.2. Determination of the stressful temperature of the ancestral clone

The stressful growth temperatures of the *P. pseudoalcaligenes* strain were determined by culturing this ancestral clone at 28, 35, 41, 43, 44, 45, 46 and 47°C. Bacteria were inoculated from their freezer vial into a 20-ml tube containing 5 ml LB medium and incubated at 35°C for 12 h. This culture was then diluted 1:100 into a 250-ml flask

containing 50 ml fresh LB medium, and incubated while shaking (230 rpm) at the test temperature. Three replicates were carried out for each test temperature. The cell density of a 12-h culture was measured by plate count. Fig. 1 shows that the ancestral clone grew best at 35°C, but poorly at 45°C or higher temperature.

2.3. Long-term selection experiments

A single colony of *P. pseudoalcaligenes* F331 was developed to a baseline clone, and six clones were derived initially from the common baseline clone, which ensures that they are all genetically identical before the selection experiment began. The six clones were assigned to two groups, i.e. the control group (referred to hereafter as Control) and the treatment group (high temperature, referred to hereafter as HT), each with three clones.

The three clones in the control group were independently propagated for 10 months at a constant temperature of 35°C on a 150×15 mm tube slant containing 5 ml LB agar medium. The clones grew quickly at the slants and were transferred twice per day by streaking onto fresh slants. During the experimental period, spreading plates were made and cultured at 35°C once every month for each clone, and one of the fastest-growing colonies (i.e. the biggest) was selected to continue the propagation. In addition, the selected colonies were kept in a glycerol-based suspension in −80°C, after being cultured in LB to stationary phase.

The HT group was cultured under the same conditions as the control group, but started with a growth temperature of 41°C. Temperature was elevated by 1°C every month to 44°C. When culture temperature was elevated to 44°C, the clones grew slower than before, and clones were transferred daily, rather than twice daily as in the control group, by streaking one loop onto a fresh medium. Also, the culturing temperature was elevated 1°C every 2 months, rather than 1°C every month. Consequently, about 600 transfers have been done for each clone of the control group and about 420 transfers for each clone of the HT group.

The reason for propagating the clones on LB agar medium instead of in liquid medium is that the former provides a more heterogeneous environment than the latter and consequently may allow the existence of more genetic variation [26]. When bacteria grow in a constantly shaken liquid medium, the environment is constantly homogenized and bacterial cells are distributed relatively evenly in the medium. However, when the bacteria grow in the structured environment (agar surface), the discrete distribution of colonies and the local depletion of common resources creates heterogeneity over space, leading to varying intensity of cell-to-cell interference competition [27]. A previous study [28] on a different bacterial species, *Comomonas* sp., showed that selection on agar medium was more effective than in liquid medium.

Each time the culturing temperature was to increase, spreading plates were made and cultured under the increased temperature and the resulting fastest-growing colony (the biggest one) at the increased culture temperature was chosen to continue the HT group. This was done independently for each of the three clones in the HT group. Meanwhile, the chosen colonies were stored in a glycerol-based suspension at −80°C after being cultured in LB to stationary phase.

The ancestral baseline clone and all its derivatives were stored in a glycerol-based suspension at −80°C. Hence, they can be compared at any time. The HT group was compared to the control group and the ancestral clone at the end of 3 months of culturing, but no significant changes in growth characteristics corresponding to increased temperature were observed. The propagation of the clones was continued to the end of 10 months, at which point the differences between the HT and control group were apparent.

While an experimental system with a bacterial species has a number of advantages, such as ease of propagation and manipulation, there is one major problem, i.e. the problem of contamination. As a precaution, the control group and the HT group were always transferred or inoculated in two different laminar flow hoods in two different rooms. Meanwhile, slides were made during every transfer or inoculation, and inspected using light microscopy. The RAPD (random amplification of polymorphic DNA) method, which has been used in our laboratory to probe genomic changes of another bacterial species, *Pasteurella multocida* [29], was used to confirm the absence of external contamination when the experiment finished after 10 months of propagation [30].

2.4. Determination of population density

As outlined in the introduction, this study mainly focused on the three growth parameters characterizing the three growth phases, i.e. the lag phase, the log phase and the stationary phase. The estimation of these growth parameters necessitates the measurement of population density over time. Spectrophotometry was used to estimate population density by first establishing a standard curve for the relationship between the cell density and the optical density (OD). The OD value of the samples was measured at 550 nm in a Pye Unicam PU 8600 UV/VIS spectrophotometer (Philips). To estimate the density of a sample, its OD value was measured and checked against the standard curve to obtain the density.

The standard curve for the density–OD relationship was established as follows. First, the clone was grown in the LB liquid medium to stationary phase. The density was obtained accurately by repeated plate counts, i.e. spreading on plates after dilution (in 0.15 M NaCl) and counting each growing colony as a colony-forming unit (CFU). Once the density of this high-density culture was deter-

mined, a series of dilutions from this culture was made and the OD was obtained for each dilution. The density of each dilution was known. For example, if the original density was D, then the density for a 1/2 dilution was $D/2$. In the OD range of 0.1–1.0, the relationship between the density and the OD is linear with $r = 0.987$, with the relationship of $N = 1712.877 \times OD_{550}$, where N is the density in 'colony-forming units (i.e. viable cells) $\times 10^6$ per milliliter (CFU $\times 10^6$ ml^{-1})'.

The protocol above ignores the possibility that the proportion of dead cells may differ in different growth phases. For example, if a large proportion of cells are dead at the stationary phase but no cell is dead at the exponential growth phase, then the standard curve established by the protocol above would underestimate N at the exponential growth phase. Therefore, samples were taken at different growth phases and separate standard curves of the density–OD relationship were established. These separately established standard curves were almost identical to each other, and so the relationship between N and OD_{550} given in the previous paragraph was used as the standard for determining cell density from OD values.

2.5. Estimation of the growth parameters λ, μ_m and A

Many mathematical models have been formulated to characterize the pattern of bacterial growth [22]. Among these models, the Gompertz and the logistic models are the two minimum models containing three parameters and have been demonstrated to fit well to the growth of a variety of bacterial species under a variety of culturing conditions [22,31–33].

Zwietering et al.[22] re-parameterized the Gompertz and the logistic models, respectively, as follows:

$$\ln\frac{N}{N_0} = A e^{-e^{\frac{e\mu_m(\lambda - t)}{A}+1}} \quad (1)$$

$$\ln\frac{N}{N_0} = \frac{A}{1 + e^{\frac{4\mu_m(\lambda - t)}{A}+2}} \quad (2)$$

where N (CFU ml^{-1}) is the population density at time t, N_0 (CFU ml^{-1}) the initial population density; A is the asymptotic value ($A = \ln(N_\infty/N_0)$) in the bacterial growth curve, which characterizes the maximum growth yield of the stationary phase; μ_m (h^{-1}) is the maximum growth rate defined as the tangent in the inflection point, and λ (h) is the lag time. N_0 was determined empirically as described below. The three remaining parameters, A, μ_m, and λ, were estimated by non-linear regression with the NLIN procedure in SAS [34].

In total, 19 clones were available for determining growth parameters at normal (35°C) and stressful temperature (45°C), respectively. These clones were: the common ancestor, designated as Anc; the three Control clones at time 0 (C0-1, C0-2, C0-3), at the end of 3 months (C3-1, C3-2, C3-3) and at the end of 10 months (C10-1, C10-2, C10-3); the three HT clones at time 0 (HT0-1, HT0-2, HT0-3), at the end of 3 months (HT3-1, HT3-2, HT3-3) and at the end of 10 months (HT10-1, HT10-2, HT10-3).

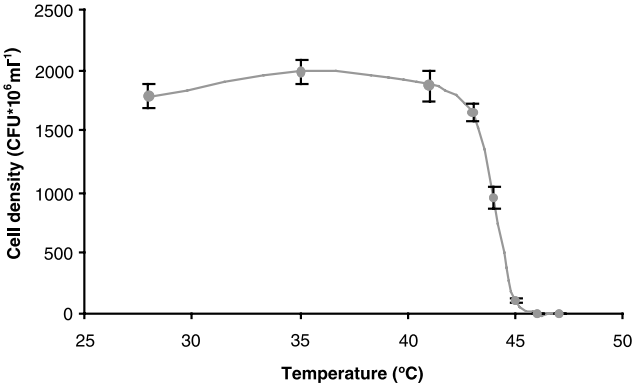

Fig. 1. The cell density (CFU $\times 10^6$ ml^{-1}) of the original *P. pseudoalcaligenes* clone, cultured under different temperatures for 12 h. The cell density was measured by plate count. Each value is the average of three replicates. The error bars are the standard deviations.

The growth parameters of the C0-1, C0-2, and C0-3 clones at 35°C serve as a reference of the performance of the ancestral clone at 35°C before the experiment. The growth parameters of the HT0-1, HT0-2 and HT0-3 clones at 45°C serve as a reference of the ancestral clone at 45°C before the experiment. For the rest of the 19 clones, the growth parameters were determined at both 35°C and 45°C. Thus, a total of 32 sets of growth parameters were determined.

All clones were inoculated from their freezer vials and propagated in 5 ml LB broth at 35°C for 8 h to ensure comparable acclimation to the experimental regime. Then, 0.5 ml of the 8-h culture was transferred into 50 ml of fresh LB medium (with an inoculum at a final concentration of about 10^7 CFU ml^{-1}) in a 250-ml flask and incubated in a shaker (230 rpm) for 14 or 28 h corresponding to the test temperature of 35 and 45°C, respectively. The time is set to 0 at the transfer. To characterize the three growth parameters, samples were taken every 0.5 h during the first 2 h of growth and then at 2-h intervals, and the OD was measured. The initial cell density in the second culture (i.e. N_0) was evaluated by the cell density in the first 8-h culture divided by dilution fold (1% inoculum to the second culture). If the density was too high for reading the OD accurately (i.e. beyond the range of 0.1–1.0), the sample was diluted. The OD of the diluted culture was measured, and then multiplied by the ratio of dilution to obtain the OD for the undiluted sample. Three replicate experiments were run for estimating all the 32 sets of growth parameters.

3. Results

Two growth models (the Gompertz and the logistic)

were used to quantify the three growth parameters, i.e. the maximum yield (A), the maximum growth rate (μ_m) and the lag time (λ). The parameter estimation was done for the Control and HT clones at three time points: at the beginning of the experiment, after 3 months and after 10 months of the experiment, and at two temperatures: the normal growth temperature of 35°C and the originally stressful temperature of 45°C. The HT clones, propagated at increasing temperature, were expected to gradually diverge from the Control clones in their capacity to grow at 45°C, by changing one or more of the three growth parameters. Below, the performance of the two models is first compared, and then the changes in each of the three growth parameters of the *P. pseudoalcaligenes* strain are examined in response to being cultured at increasing temperature.

3.1. Comparison of the Gompertz and the logistic models

The fit of the model to the experimental data can be measured by the general coefficient of determination (R^2). Because the two models have the same number of parameters (i.e. three), a higher R^2 implies a better model. The Gompertz model had R^2 values consistently higher than the logistic model; however, the estimated growth parameters from the two models were almost identical and the high R^2 values (Table 1) showed that either of the two models was sufficient for modeling the growth of the *P. pseudoalcaligenes* clones. Consequently, the parameters presented below are from the Gompertz model only.

Table 1
Comparison of the fit between the two models of bacterial growth (Gompertz model and logistic model)

Clones	35°C		45°C	
	Gompertz	Logistic	Gompertz	Logistic
Anc	0.9980	0.9962	0.9894	0.9842
C0+HT0	0.9984	0.9968	0.9774	0.9757
	0.9930	0.9907	0.9662	0.9603
	0.9899	0.9863	0.9863	0.9850
C3	0.9973	0.9942	0.9882	0.9818
	0.9964	0.9935	0.9776	0.9722
	0.9953	0.9919	0.9788	0.9717
HT3	0.9966	0.9937	0.9958	0.9899
	0.9950	0.9921	0.9932	0.9893
	0.9963	0.9940	0.9899	0.9846
C10	0.9959	0.9928	0.9935	0.9920
	0.9962	0.9935	0.9907	0.9911
	0.9969	0.9938	0.9909	0.9907
HT10	0.9970	0.9942	0.9980	0.9968
	0.9967	0.9939	0.9976	0.9960
	0.9974	0.9955	0.9982	0.9967

The fit is measured by the coefficients of determination (R^2).
Note: Anc refers to the Ancestor clone.
C0 and HT0 refer to the Control and HT clones at time 0 of the experiment.
C3 and HT3 refer to the Control and HT clones after 3 months of the experiment.
C10 and HT10 refer to the Control and HT clones after 10 months of the experiment.

Table 2
Estimated A values (maximum yield reached). Symbols as in Table 1

35°C[a]			45°C[a]		
Clone ID	A	S.E.[b]	Clone ID	A	S.E.[b]
Anc	4.6477	0.0474	Anc	3.3848	0.0915
C0-1	4.6563	0.0413	HT0-1	3.4637	0.1460
C0-2	4.6569	0.0851	HT0-2	3.6503	0.1870
C0-3	4.6633	0.1076	HT0-3	3.3586	0.0959
C3-1	4.6284	0.0503	C3-1	3.6973	0.1166
C3-2	4.6447	0.0618	C3-2	2.8906	0.1022
C3-3	4.8628	0.0718	C3-3	3.0395	0.1145
HT3-1	4.5758	0.0571	HT3-1	4.0641	0.0653
HT3-2	4.6378	0.0716	HT3-2	3.3227	0.0566
HT3-3	4.8407	0.0661	HT3-3	3.6964	0.0816
C10-1	4.5726	0.0611	C10-1	3.3560	0.0662
C10-2	4.7964	0.0627	C10-2	3.7049	0.0821
C10-3	4.9482	0.0602	C10-3	3.5854	0.0858
HT10-1	4.6342	0.0526	HT10-1	4.6306	0.0358
HT10-2	4.6558	0.0564	HT10-2	4.7223	0.0411
HT10-3	4.9180	0.0571	HT10-3	4.6145	0.0352

[a]Assessment temperature.
[b]Standard error.

3.2. The maximum yield (A)

The Control and HT clones did not differ significantly from each other at the end of 10 months (t-test, $t = 0.256$, DF = 4, $P = 0.8106$), or from the ancestral clone, in the maximum yield (A) when cultured at the normal temperature of 35°C (Table 2). When cultured at the originally stressful temperature of 45°C, however, the HT10 clones had a mean A value significantly higher than the three C10 clones (t-test, $t = 10.2755$, DF = 4, $P = 0.0005$) or the three HT0 clones (paired-sample t-test, $t = 21.9399$, DF = 2, $P = 0.0021$). Thus, culturing the clones at increasing temperature for 10 months allows the clones to reach a significantly higher maximum yield at the originally stressful temperature of 45°C.

All clones, except for the three HT10 clones, had A values significantly lower when cultured at 45°C than when cultured at 35°C (Table 2). It is interesting to note that the three HT10 clones had a high mean A value at both 35°C and 45°C. Thus, as far as the maximum yield was concerned, a gain at 45°C did not imply a loss at 35°C.

3.3. The maximum growth rate (μ_m)

When the clones were cultured at 35°C, the μ_m value did not differ much between the Control or HT clones, with $t = 1.1023$, DF = 4, $P = 0.3322$ for C3 and HT3 clones, and $t = 0.4639$, DF = 4, $P = 0.6668$ for C10 and HT10 clones, based on data in Table 3. When the clones were cultured at 45°C, however, the mean μ_m value differed significantly between the Control and the HT clones, being 0.2667 for

Table 3
Estimated μ_m values (maximum growth rate, h^{-1})

35°C[a]			45°C[a]		
Clone ID	μ_m	S.E.[b]	Clone ID	μ_m	S.E.[b]
Anc	1.1230	0.0487	Anc	0.2794	0.0200
C0-1	1.1970	0.0474	HT0-1	0.2667	0.0276
C0-2	1.2819	0.1137	HT0-2	0.2707	0.0321
C0-3	1.0921	0.1053	HT0-3	0.3261	0.0287
C3-1	1.6078	0.1080	C3-1	0.2596	0.0180
C3-2	1.2274	0.0764	C3-2	0.2940	0.0322
C3-3	1.4047	0.1054	C3-3	0.2466	0.0228
HT3-1	1.3442	0.0865	HT3-1	0.3563	0.0153
HT3-2	1.3159	0.1001	HT3-2	0.4715	0.0320
HT3-3	1.1793	0.0696	HT3-3	0.4454	0.0342
C10-1	1.5887	0.1296	C10-1	0.3126	0.0181
C10-2	1.5627	0.1122	C10-2	0.4218	0.0332
C10-3	1.4038	0.0858	C10-3	0.3127	0.0202
HT10-1	1.6578	0.1172	HT10-1	1.5407	0.0839
HT10-2	1.5112	0.1020	HT10-2	1.3706	0.0769
HT10-3	1.1693	0.0571	HT10-3	1.2379	0.0575

Symbols as in Table 1.
[a]Assessment temperature.
[b]Standard error.

the three C3 clones and 0.4244 for the three HT3 clones ($t = 4.1898$, DF = 4, $P = 0.01381$), and being 0.3490 for the three C10 clones and 1.3831 for the three HT10 clones ($t = 10.8977$, DF = 4, $P = 0.0004$). Thus, culturing the clones at increasing temperature allowed the clones to become increasingly adaptive to growing at 45°C (Table 3).

Apart from the difference in μ_m between the Control and the HT clones, a clear adaptation to the culture medium of the clones was also observed. For the μ_m values quantified at 35°C, the average μ_m value was 1.1735 for the ancestral clone and the three C0 clones, 1.4133 for the three C3 clones and 1.5184 for the three C10 clones. The difference among the three means was statistically significant, based on a one-way analysis of variance ($F = 6.8076$, $DF_{between} = 2$, $DF_{within} = 7$, $P = 0.0228$). Thus, the μ_m value steadily increased with culturing time.

For the μ_m values estimated at 45°C, the average μ_m value did not differ between the three HT0 clones plus the ancestral clone and the three C3 clones (t-test, $t = 0.9456$, DF = 5, $P = 0.3878$). However, these clones pooled together had a mean μ_m value (= 0.2776) that was significantly lower than that for the three C10 groups (= 0.3490), with $t = -2.6713$, DF = 8 and $P = 0.0283$. This is consistent with the results of the μ_m values quantified at the culturing temperature of 35°C. In short, the 10 months of culturing time at a constant temperature of 35°C produced clones that grew faster than the ancestral clones at both 35 and 45°C. This suggests the importance of having the Control clones. If Control clones had not been used, the HT10 clones might have been compared with the HT0 clones or the ancestral clone, and all differences might have been attributed to culturing at the increasing temperature. Such an attribution clearly would be erroneous, because even if temperature has no effect, the HT10 clones, after evolving for 10 months in the culture medium, would be different from the original clones at the beginning of the experiment.

Consistent with this observation, the maximum yield (A) examined in the previous section also increased with culturing time, with the mean A value being 4.6561 for the ancestral clone and the three C0 clones, 4.7120 for the three C3 clones and 4.7724 for the three C10 clones. The difference, however, was not statistically significant. This suggested that μ_m could change more readily with environmental changes than A.

All clones, except for the three HT10 clones, had μ_m values significantly lower when cultured at 45°C than when cultured at 35°C (Table 3). It is interesting to note that the three HT10 clones had a high mean μ_m value at both 35 and 45°C. Thus, as far as μ_m was concerned, a gain at 45°C did not imply a loss at 35°C, consistent with the estimated A values.

3.4. The lag time (λ)

Culturing the clones at increasing temperature had a significant effect on the lag time (λ; Table 4). After 3 months of culturing at increasing temperature, the three HT3 clones, growing at 45°C, had a shorter mean lag time (= 1.8055) than the three C3 clones (= 2.6216), although the difference was not statistically significant. The difference in the mean λ value between the C10 (mean $\lambda = 3.0209$) and HT10 (mean $\lambda = 1.3147$) clones was significant ($t = 7.6958$, DF = 4, $P = 0.0015$).

All clones, except for the three HT10 clones, had λ values lower when growing at 35°C than when growing at 45°C. The three HT10 clones, growing at 45°C, had λ values as low as those of the clones growing at the

Table 4
Estimated λ values (lag time, h)

35°C[a]			45°C[a]		
Clone ID	λ	S.E.[b]	Clone ID	λ	S.E.[b]
Anc	1.2725	0.0802	Anc	3.7736	0.4084
C0-1	1.2560	0.0677	HT0-1	4.6272	0.6059
C0-2	1.0586	0.1297	HT0-2	2.9834	0.7353
C0-3	1.1118	0.1760	HT0-3	4.3040	0.4437
C3-1	1.0482	0.0715	C3-1	3.5406	0.4402
C3-2	1.0500	0.0959	C3-2	2.3359	0.5348
C3-3	1.0582	0.1028	C3-3	1.9882	0.5359
HT3-1	1.0705	0.0864	HT3-1	2.2541	0.2362
HT3-2	1.1382	0.1094	HT3-2	1.5125	0.2338
HT3-3	1.1165	0.1028	HT3-3	1.6498	0.3130
C10-1	1.1127	0.0876	C10-1	3.1353	0.3059
C10-2	1.2593	0.0886	C10-2	3.3287	0.3526
C10-3	1.0247	0.0851	C10-3	2.5988	0.3583
HT10-1	1.1652	0.0736	HT10-1	1.3725	0.0697
HT10-2	1.1752	0.0812	HT10-2	1.2416	0.0827
HT10-3	1.3661	0.0946	HT10-3	1.3301	0.0788

Symbols as in Table 1.
[a]Assessment temperature.
[b]Standard error.

temperature of 35°C. Thus, as far as the λ value was concerned, a reduction of the lag time at 45°C did not imply an increase of the lag time at 35°C.

Adaptation of the clones to the medium did not seem to lower λ when we compare the λ values among the Control clones. The three Control clones at the end of the 10 months of culturing did not have a lower λ value than these clones at the beginning of the selection experiment (paired-sample t-test, $t = 0.0929$, DF = 2, $P = 0.9344$). This is different from the growth parameter μ_m, which showed significant difference between the Control clones at the end of the 10 months and the Control clones at the beginning of the selection experiment.

4. Discussion

Rapid cell growth and replication are crucial to the survival of bacterial species. The cell growth and replication of bacterial species are mainly correlated to the three growth parameters, namely the maximum yield (A), the maximum rate of increase (μ_m) and the lag time (λ). Previous studies [12,13,15,17,18] have shown that *E. coli* cultured in a novel temperature for thousands of generations is more competitive than the ancestral strain. Most likely, some or all of the three growth parameters might have changed during the adaptation process. However, these studies have not examined which growth parameter responds to selection under increased temperature.

In this study, we have shown that all three parameters responded to selection under increasing temperature in the *P. pseudoalcaligenes* strain. The original strain grew poorly at 45°C, with low A values, low μ values and high λ values relative to the corresponding growth parameters at 35°C. However, after being cultured at increasing temperature for 10 months, the clones grew well at 45°C, with the growth parameters comparable to those at 35°C (Tables 2–4).

In one study [23], the three parameters were not estimated simultaneously. Separate estimation without a coherent model would confound the estimation and produce biased estimates. Although some kind of adaptation in bacterial strains is generally observed after the bacteria have been cultured for a long time under novel temperature, there is no experiment that is rigorous enough to conclude that the adaptation results from the improvement of all three parameters. This is significant, because without this experiment, there are many possible scenarios. For example, only one parameter improves, but the other two remain the same (three possibilities); one parameter improves, but two others get worse and the improvement outweighs the deterioration of the other two, so that the overall growth is still increased (three possibilities); two parameters improve, but the other remains the same (also three possibilities); two parameters improve and the other gets worse, but the improvement in the two parameters offsets the deterioration in the other (also three possibilities). Hence, the results in the present study extend the study of thermal adaptation in *E. coli*.

Most studies on thermal adaptation have been carried out with implicit or explicit references to the evolutionary capabilities of natural populations in responding to global warming [35]. In *E. coli* [12], it takes just about 1 month of culturing in increasing temperature to produce adaptation comparable to that achieved in *P. pseudoalcaligenes* after culturing for 10 months. This implies that different species, even within the eubacterial kingdom, could differ dramatically in their response time to changing temperatures. The relatively slow response of *P. pseudoalcaligenes* documented in our study may imply a much longer period for eukaryotes to evolve new adaptation to changing temperature.

Exactly what genetic changes had occurred to allow the HT clones to grow well at 45°C is unknown. Several hypotheses can be proposed. It is well documented that bacteria adapted to different thermal environments may alter the fatty acid composition of the cell membrane [36–38]. Adaptation to high temperature may enhance the ratio of saturated to unsaturated fatty acids in the cell membranes [39,40]. The membrane fluidity of the organism is altered effectively by changes in fatty acid composition. By such means, organisms can regulate the activity of vital membrane-bound enzymes and transport systems [38], and adjust the uptake of nutrients, so as to improve the capacity of the HT clones to grow at high temperature.

One thing that is particularly worth noting is that the HT10 clones, adapted to growing at 45°C, did not show decreased growth at 35°C, as evidenced by the three parameters quantified at both temperatures (Tables 2–4). The clones simply widened the temperature range for growth.

Trade-off is a common phenomenon in evolution. Increased reproductive output typically is associated with a reduction in longevity. A species adapted to cold temperature generally cannot survive and reproduce well in a hot climate. All these observations very naturally lead to the hypothesis of 'Jack of all trades, master of none'. The present results, together with those of other experimental selection studies [18], suggest that this hypothesis may not be general. However, this present study has the defect of not comparing the performance of the groups at temperatures lower than 35°C, and the data, consequently, are insufficient to reject the hypothesis.

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