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Morphological Changes of *Pseudomonas pseudoalcaligenes* in Response to Temperature Selection

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Abstract. Adaptation to novel environments usually entails morphological changes. The cell morphology of six experimental populations of *Pseudomonas pseudoalcaligenes* and their common ancestor were examined with scanning electron microscopy (SEM). The six experimental populations were propagated under different temperatures for 10 months: three of them cultured at constant normal temperature (35°C) forming the control group, and the other three cultured at incremental higher temperatures (from 41° to 47°C) as the HT group. SEM showed the deformed and elongated cells in the 6-h cultures of both ancestral and control populations at 45°C, indicating that 45°C is stressful for the ancestral and the control populations. In contrast, the HT populations retained normal cell shape in the 6-h cultures at both 35°C and 45°C. The mean cell volumes of control and HT populations increased 29% and 34%, respectively, relative to the ancestor at their respective thermal regimens, suggestion that the culturing conditions might favor larger cells.

A differential in morphological traits often has adaptive values. One of the most classical examples is Darwin's finches (*Geospiza*) of the Galápagos Islands, in which divergence in the size and shape of the bill represents adaptation to different diets, i.e., finches with larger (especially deeper) bills feed more efficiently on large, hard seeds, whereas the finches with small bills feed more efficiently on small, soft seeds [8].

The morphology of microorganisms also changes in response to variation of the environment. Most thermophilic bacteria have a small and threadlike shape [3]. The threadlike shape has a greater surface-to-volume ratio, improving the speed of material transportation between cells, and the rapid exchange of material with the surrounding environment is also of importance for organisms to develop at elevated temperatures [5]. In mesophilic species, the influence of temperature on the size of the cell is varied among species. It was found that the size of *Salmonella typhimurium* decreases with increasing incubation temperature [5, 6]. However, larger cells were observed in yeast, *Bacillus*, and *E. coli* at higher incubation temperatures [5, 13].

Clearly, the results from just a few species do not allow us to draw any general conclusion, and more studies relating morphology to temperature are needed. We here report a study of morphological changes in *Pseudomonas pseudoalcaligenes* cultured at increasing temperatures. We describe the selection of six populations of *P. pseudoalcaligenes* from a single ancestral clone and propagated separately for 10 months in two distinct thermal environments: the constant normal temperature and incremental higher temperatures. The effects of selection on morphological traits in experimental populations of *P. pseudoalcaligenes* were investigated by scanning electron microscopy (SEM).

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Materials and Methods

Bacterial strains. All of the strains used in this study have been described in a previous study [Shi and Xia, unpublished data, in review]. Briefly, a single clone of *P. pseudoalcaligenes* F331 was used

to generate two experimental groups, each one consisting of three replicate lines. These lines evolved for 10 months in the same culture conditions, except that each group was propagated under a different thermal regimen. One has been cultured at a constant moderate temperature (35°C) and designated as the control group (C1, C2, and C3), the other evolved at gradually increasing temperatures (41°→47°C) and designated as the HT-group (HT1, HT2, and HT3). The six experimental populations (C1, C2, C3, HT1, HT2, HT3) and their common ancestor (A) were stored in 25% glycerol suspension at −80°C and used to examine the cell morphologies.

Cell morphology. As it is difficult to observe small changes in cell morphologies of bacteria under the light microscope, scanning electron microscopy (SEM) was used to examine the minor changes in cell morphology of the populations that have been cultured at different temperatures. SEM is a powerful and effective tool for this application and has proven an important method for studying the deleterious effects of a stressful environment on bacterial cells [11, 15]. However, the preparation of specimens requires special techniques. The procedures for preparing the specimen are gentle enough so that when the specimens are visualized, a three-dimensioned view of the intact surface structure and spatial arrangement of the cells can be observed clearly [11], and the cell dimensions can be directly measured from the electronmicrographs.

The cell morphology of the tested populations was examined by scanning electron microscopy (SEM). Bacteria were thawed and inoculated into a 20-ml tube containing 5 ml LB (0.5% Bacto-yeast extract, 1% Bacto-tryptone, 0.5% NaCl) broth and were incubated in a shaker (230 rpm) at 35°C for 12 h. Then 0.1 ml of the 12-h bacterial culture was transferred into two 20-ml tubes, each containing 8 ml fresh LB medium, and they were incubated (230 rpm) for another 6 h at 35° and 45°C, respectively. The bacterial cultures after 6 h of incubation under different test temperatures were used for SEM sample preparation.

The protocol for SEM sample preparation was previously described [9]. A 0.2-ml bacterial suspension was filtered through a 0.2-μm GTTP isopore membrane filter (Millipore, Ireland). Bacteria were fixed by immersion in 3% glutaraldehyde with 0.1 M sodium cacodylate buffer overnight and were washed with 0.1 M sodium cacodylate three times. This was followed by post-fixing in 1% OsO_4 in the same buffer for 3 h and rinsing with the same buffer and deionized water three times for each treatment. The samples were dehydrated by an ethanol series of 40, 50, 60, 70, 80, 85, 90, 95, and 100% ethanol and stored in 100% ethanol. Specimens in 100% ethanol were critical-point dried in a CO_2 atmosphere (BAL-TEC CPD 030 Critical Point Dryer) and mounted on aluminum stubs and gold-coated for 5 min in a BAL-TEC SCD 005 Sputter Coater and examined under a STEREO SCAN 440 scanning electron microscope.

Cell volume. Cell dimensions were measured directly from the SEM photographs to calculate the cell volumes by the following equation:

$$V\ (\mu m^3) = \frac{\pi}{4} W^2 L + \frac{\pi}{3} W^2 R$$

W and L represented the width and length, respectively, of the central part of the cylindrical cell, and R was the equatorial radius of the spheroid caps at both ends of the cylinder. Average cellular volumes were calculated by using 30 individual bacteria per population. Cells showing division, deformation/rupture, or elongation into a string were not included. The mean cell size of all tested populations was measured in cultures of both 35° and 45°C.

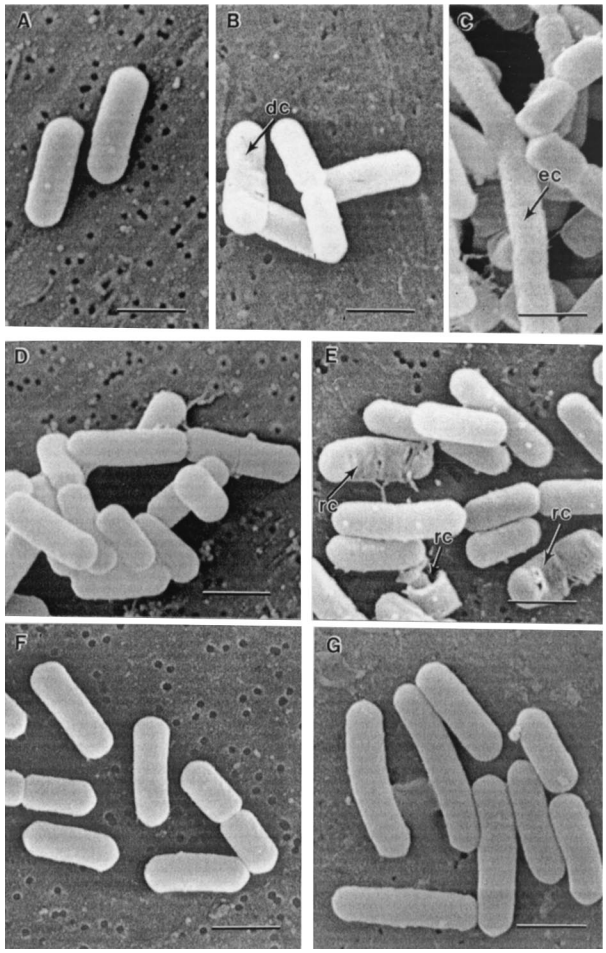

Fig. 1. Cell morphologies of 6-h cultures from different test populations at different culture temperatures. (A) Ancestor at 35°C. (B), (C) Ancestor at 45°C. (D) Control populations at 35°C. (E) Control populations at 45°C. (F) HT populations at 35°C. (G) HT populations at 45°C. dc: deformed cell; ec: elongated cell; rc: ruptured cell; Bar = 1 μm. Magnification, ×15,000.

Results

Changes in cell morphology. The cell morphology of the ancestral strain after 6 h of incubation at 35°C and 45°C was examined by scanning electronic microscopy (SEM). The cell morphology of the 35°C culture was apparently normal (Fig. 1A), but deformed (dc, Fig. 1B) and elongated cells (Fig. 1C) were observed in the 45°C culture. The elongated cells (ec) in the 45°C culture suggested that high temperature might have prevented completion of reproduction. The cell morphology of the 6-h cultures at the exponential growth phase deviated from that of the normal exponential phase. The presence of destruction and elongation of cells in the exponential phase provided evidence that 45°C was stressful to the ancestral strain.

Table 1. The mean cell sizes of the test populations. Mean value ± std. error.[a] C1, C2, C3 and HT1, HT2, HT3 represent the three control populations and HT populations, respectively, and A represents the ancestor. Ta, assay temperature

Ta	Populations	Width (μm)	Length (μm)	Radius (μm)	Volume (μm^3)[b]
35°C	A	0.482 ± 0.0047	0.982 ± 0.0214	0.182 ± 0.0027	0.225 ± 0.0077
	C1	0.495 ± 0.0036	1.145 ± 0.0416	0.198 ± 0.0016	0.272 ± 0.0093
	C2	0.516 ± 0.0058	1.218 ± 0.0363	0.189 ± 0.0023	0.310 ± 0.0124
	C3	0.519 ± 0.0069	1.103 ± 0.0299	0.184 ± 0.0032	0.288 ± 0.0108
	HT1	0.483 ± 0.0097	1.078 ± 0.0356	0.191 ± 0.0018	0.247 ± 0.0135
	HT2	0.490 ± 0.0077	1.284 ± 0.0383	0.193 ± 0.0020	0.296 ± 0.0157
	HT3	0.511 ± 0.0078	1.304 ± 0.0574	0.189 ± 0.0032	0.328 ± 0.0207
45°C	A	0.512 ± 0.0080	1.024 ± 0.0346	0.183 ± 0.0039	0.262 ± 0.0106
	C1	0.504 ± 0.0054	1.182 ± 0.0489	0.199 ± 0.0016	0.291 ± 0.0121
	C2	0.501 ± 0.0025	1.202 ± 0.0502	0.187 ± 0.0025	0.287 ± 0.0110
	C3	0.517 ± 0.0063	1.178 ± 0.0461	0.184 ± 0.0026	0.300 ± 0.0120
	HT1	0.496 ± 0.0038	1.288 ± 0.0653	0.213 ± 0.0035	0.304 ± 0.0137
	HT2	0.511 ± 0.0059	1.488 ± 0.0540	0.181 ± 0.0036	0.355 ± 0.0140
	HT3	0.514 ± 0.0049	1.629 ± 0.0501	0.194 ± 0.0035	0.392 ± 0.0123

[a] Each mean value was the average of 30 cells' size based on the scanning electron microscopy photos.
[b] The cell volume was calculated by the equation: $V(\mu m^3)=\pi W^2 L/4+\pi W^2 R/3$, where the W, L were the width and length, respectively, of the cylindrical central part of the cell, and R was the equatorial radius of the spheroid caps at both ends of the cylinder.

The cell morphology of control populations was similar to that of the ancestor at the two test temperatures (Fig. 1D and E). Cells from the 45°C culture showed high-temperature lesions and loss of the characteristic rod shape, indicating 45°C was also stressful for the control populations. However, for the HT populations, the cell morphology of the 45°C culture was quite similar to that of the 35°C culture (Fig. 1F and G), indicating thermal adaptation by the HT populations after 10 months of propagation at high temperature.

Changes in cell size. The mean cellular volumes increased substantially in both control and HT populations (Table 1) during selection. The average cell sizes of control and HT populations increased by 29% and 34%, respectively, compared with the ancestor at their respective culturing temperature regimens. However, the cell volumes of the populations from the two experimental groups exhibited different plasticity when exposed to different temperatures. The mean cell volume of the control populations at 35°C showed no significant difference from that of 45°C cultures (paired sample t-test, DF = 2, t = −0.2053, p = 0.8564), whereas the mean cell volume of the 45°C cultures of the HT populations was significantly larger than that of the 35°C cultures (paired sample t-test, DF = 2, t = 28.8231, p = 0.0012). Interestingly, the variation in cell volume of the experimental populations when exposed to different temperatures was largely owing to the variation in cell length, according to the results in Table 1. Despite the discrepant plasticity of the cell size between the two groups, the selection appears to favor larger cells, on the basis of the greatly enhanced cellular volume in both control and HT groups compared with the ancestor.

Discussion

Morphological variation is the most visible component of biodiversity, and morphological changes of organisms in a novel environment are the most visible indicator of organismal adaptation. The present study was designed to examine the cell morphologies of *P. pseudoalcaligenes* in response to elevated culturing temperatures. The visible structural feature of the cell morphology is of importance for microorganisms to survive and duplicate at different temperatures.

Changes in cell morphology. The cell morphology under SEM provided strong evidence that 45°C is a stressful temperature for the ancestral and control populations, characterized by the destruction and elongation of cells in the exponential phase culture. Although it is impossible to ascertain from this study the cause of the destruction of bacteria and retardation of the cell division, it is clear that 45°C represents a hostile environment to both ancestral and control populations. A recently proposed bacterial suicidal response hypothesis [2, 7] provides a possible explanation for both the destruction and cessation of cell division in the control and ancestral cultures under conditions of high temperature. This hypothesis [2] predicts that, when a stressful treatment is imposed on an exponentially growing bacterial population, growth is abruptly impeded while metabolism continues

unaffected. The imbalance between anabolism and catabolism results in a burst of free-radical production that causes significant damage to intracellular components, including DNA and proteins, and it is the free-radical burst rather than the stress itself that leads to destruction of the cell [1]. When DNA is damaged, the SOS response is induced [17], and one of the factors of the SOS response is the expression of SulA protein, which stops cell division [16].

The cell morphology of HT populations under SEM with the characteristic rod shape and normal cell division supports the suggestion that the HT group adapted to the initially stressful temperature of 45°C after 10 months of propagation at incremental higher temperatures.

Changes in cell size. Although it is hard to ascertain whether the cell size per se is the selection target or a correlated response to selection based on some other traits, selection seems to favor larger cells, from the results of our study. The average cell volumes in both the control and HT groups increased substantially at their corresponding selection environment over the 10 months. A previous study on *E. coli* also showed that cell size increased rapidly during the first 2,000 generations of long-term adaptation to the moderate temperature of 37°C [12]. The changes in cell size of both the control and HT populations may be due to the environmental change (i.e., introduction of the study organism into the experimental environment) that radically perturbed the adaptive landscape.

We also found that the HT populations have higher plasticity of cell size than that of control populations facing the temperature variation. Such a difference between the two groups may result from the different environments in which the two groups evolved. In the control group, replicate populations were propagated at a constant temperature, but populations of HT group were incubated under incremental temperatures. Adaptation to changing environments often produces conspicuous phenotypic variation [4]; furthermore, a high temperature has been demonstrated to promote the mutation rate that would give rise to genetic and phenotypic variation [10]. Increased cell size was also observed in other bacteria when exposed to the metalloid oxyanions chromate, selenate, and arsenate etc. as a protection system for bacteria facing a stressful environment [14]. The physiological basis of the enhanced plasticity in cell size of HT population was unclear. That may also be a protection system developed during long-term culturing under incremental temperatures, enabling the bacteria to tolerate high temperatures.

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