Enhanced Stress Resistance at Stationary Phase of Oligotrophic Bacteria, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06

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Oligotrophic bacteria, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06, can grow in 10^4 -diluted nutrient broth ($1/10^4$ NB). In the two oligotrophic bacteria, the stationary phase cultures showed enhanced heat, oxidation, and osmotic resistance as compared with cultures at exponential phase, although resistance was less marked than that of *Escherichia coli*. On Southern blotting analysis, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06 genomic DNA hybridized with the sigma factor probes, RpoN-X and RpoD-4.2, and RpoD-4.2, respectively.

Key words: Stress resistance, Oligotrophic bacteria, *Chromobacterium*, *Aeromonas*

Introduction

Microorganisms are capable of growing in various extreme environments, such as under condition of thermal, acidic or osmotic stress³. Bacteria that grow under low-nutrient conditions are known as oligotrophic bacteria¹⁰. Kuznetsov *et al.* defined the oligotrophs as bacteria isolated on organic media containing between 1 and 15 mg of Cl^{-1} ¹⁰. Oligotrophic bacteria utilize a wide variety of carbon compounds to take advantage of whatever substrates are available in low-nutrient environments²⁵. We isolated oligotrophic bacteria from soil. These bacteria grew in a 10^4 -diluted nutrient broth ($1/10^4$ NB, 0.8 mg of organic carbon per liter) with doubling times of 1.5 to 3 days, and reached final yields of 5.2 to 8.6×10^6 colony-forming units (CFU) per ml⁸.

Escherichia coli, a typical copiotrophic bacterium,

did not grow under such conditions, although *E. coli* is capable of surviving various stresses^{13,23}. Starved or stationary *E. coli* cells are more resistant to heat shock, oxidative stress, and osmotic assault than exponential phase cells^{14,24}. A subunit of a putative RNA polymerase, σ^{38} factor encoded by the *rpoS* gene, is a regulator of central importance for gene expression during the transition to stationary phase⁶. *E. coli* responds to glucose starvation by inducing about 30 proteins which confer enhanced starvation resistance⁴. We investigated whether stationary phase cells of two oligotrophic bacteria are more resistant to a variety of environmental assaults than those in exponential phase.

Materials and Methods

Bacterial strains and growth media

Two oligotrophic bacteria, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06, and *Escherichia coli* IFO3301 were used⁸. Nutrient broth (NB) containing 1% meat extract (Wako Pure Chemicals, Osaka), 1%

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polypeptone (Wako Pure Chemicals) and 0.5% NaCl, pH7.0, and 50-fold or 10000-fold (1/50 NB or 1/10⁴ NB) diluted broth were used¹⁹⁾.

Growth conditions

Bacteria were cultured first in 1/50 NB at 27°C for 1 to 3 days without agitation. One ml of the broth was then inoculated into 100 ml of 1/50 NB or 1/10⁴ NB, and cultured at 27°C under static conditions. Growth was measured by colony counting. Samples of 1 ml were pipetted into sterile Petri dishes and mixed with 20 ml of 1/50 NB containing 1.5% agar. The plates were incubated at 27°C for 3 days.

Stress resistance examination

Oligotrophic bacteria and *E. coli* cells grown in 1/50 NB or 1/10⁴ NB at mid-logarithmic and stationary phases were harvested by centrifugation. Aliquots of cultures were diluted to ca. 1 × 10⁶ cells/ml in the same culture medium, and 3 ml was pipetted into ϕ 18 test tubes and used for stress resistance assays. Control experiments with different concentrations of H₂O₂ and NaCl, or various temperatures and exposure periods established that these adaptation doses conferred maximal resistance to challenge by their respective stresses. Levels of stress for *E. coli* were determined by the method described previously^{14,21)}. In the oligotrophic bacteria, the levels of stress were set so that stationary phase cell survival was at least several percent. The levels of stress are indicated in each figure legend. Percent viability was determined by col-

ony counting on 1/50 NB plates, with 100% viability representing the colony counts of the cultures just prior to the stress tests.

Isolation of genomic DNA

E. coli and oligotrophic bacteria were grown in 2 liters of 1/50 NB. Total genomic DNA was isolated by the method of Avery and Kaiser¹⁾. Cells were lysed with TNE buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA-2Na, and 0.2 M NaCl) containing 1% SDS at 60°C for 10 min. After three extractions with phenol, the genomic DNA was precipitated with ethanol.

Southern hybridization with σ factor probes

Aliquots of about 5 μ g of genomic DNA were digested completely with suitable restriction endonucleases, electrophoresed in 0.7% agarose gels, and transferred onto nylon membranes, GeneScreen Plus (NEN Research Products, USA). Hybridization was carried out using a hybridization buffer (0.5M sodium phosphate buffer, pH 7.2, 7% SDS, and 1 mM EDTA-2Na) for 12 h at 52°C with digoxigenin (DIG)-11-dUTP labeled probes. The sequences of the oligonucleotide probes are shown in Table 1. After hybridization, the membranes were washed three times in 40 mM sodium phosphate buffer (pH 7.2) containing 1% SDS at 52°C for 40 min. Hybridizing DNA bands were detected using a DIG DNA detection kit (Boehringer Mannheim Biochemicals, Mannheim, Germany).

Table 1. Sequences of oligonucleotide probes used for Southern analysis

RpoD-4.2 (20 mer) ^a	TGRCGRATACGYTCRCGRGT
RpoH-2.4 (23 mer) ^b	GTYGCRACCTTTRACRATRCGCCA
RpoN-X (26 mer) ^c	GGRCTRTGCAGRTAYTTYTGCGTGGT
FliA-2.4 (20 mer) ^d	ACSGASCSSGSGSACCCARTC
FliA-4.2 (20 mer) ^d	TGGATCTGSSASACSCGSSA

R=A or G, Y=C or T, S=C or G

^a The sequence of the RpoD-4.2 probe was highly conserved in σ^{70} family¹²⁾.

^b Heat shock sigmas from *E. coli* RpoH (σ^{32}) and *Citrobacter freundii* HtpR have consensus sequences in region 2.4¹²⁾.

^c RpoN-X probe was designed from conserved amino acid sequences of *E. coli* RpoN, *S. typhimurium* NtrA and *K. pneumoniae* NtrA^{16,17,20)}.

^d FliA-2.4 and -4.2 were designed from *Salmonella typhimurium* FliA^{12,18)}.

Results

Growth of oligotrophic bacteria

The optimized culture medium for the growth of the two oligotrophic bacteria was 1/25 to 1/50 NB, while these strains did not grow on 1/5 NB⁸). Figure 1 shows the growth curves of *E. coli* and the oligotrophic bacteria *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06 in 1/50 NB and 1/10⁴ NB. In 1/50 NB, *Chromobacterium* sp. Y95, *Aeromonas* sp. Z06 and *E. coli* entered stationary phase at 48 h, 72 h, and 12 h, respectively. The optical densities of the oligotrophic bacteria at stationary phase were about 2-fold higher than that of *E. coli*. In 1/10⁴ NB, the oligotrophic bacteria reached

Fig. 1. Time course of growth of *Chromobacterium* sp. Y95, *Aeromonas* sp. Z06, and *E. coli* on 1/50 NB (A) and 1/10⁴ NB (B). *Chromobacterium* sp. Y95 (■), *Aeromonas* sp. Z06 (▲), and *E. coli* (●).

stationary phase about 24 h later than in 1/50 NB cultures. The cell density of the oligotrophic bacteria in 1/10⁴ NB increased by about 10⁵ fold during 4 days of cultivation, and reached final yields of 5 to 8 × 10⁶ colony-forming units (CFU) per ml. *E. coli* did not multiply in 1/10⁴ NB.

Thermal and heat-induced thermal resistance

Aliquots of *E. coli*, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06, cultured to mid-logarithmic and stationary phase on 1/50 NB and 1/10⁴ NB, were subjected to thermal stress. Stationary cells of the oligotrophic bacteria were more resistant to high tem-

Fig. 2. Resistance to heat shock of *E. coli*, *Chromobacterium* sp. Y95, and *Aeromonas* sp. Z06. Exponential (○ or △) and stationary (● or ▲) phase cells were prepared from 1/50 NB (solid lines) and 1/10⁴ NB cultures (dashed lines). *E. coli* (A) was challenged at 52°C, and *Chromobacterium* sp. Y95 (B) and *Aeromonas* sp. Z06 (C) were challenged at 47°C.

perature (47°C) than those in exponential phase (Fig. 2). As shown in Fig. 2, after a 4-min challenge at 47°C, 60% of stationary phase *Chromobacterium* sp. Y95 cells on 1/50 NB retained viability, while viability of those in log phase was only 0.9%. Stationary cells of 1/10⁴ NB cultures also showed thermal resistance.

The log phase cells of *Chromobacterium* sp. Y95 developed thermal resistance following exposure to 37°C for 1 h. The pre-heated log phase cells remained 18% viable after a 4-min challenge at 47°C (Fig. 3). This viability was about 20-fold higher than that of non-pre-heated log phase cells.

In *E. coli*, stationary cells exhibited marked resistance to heat shock, compared with those at exponential phase (Fig. 2). Also, exponential phase cells exhibited markedly enhanced survival at 52°C following prior exposure to 39°C for 30 min (Fig. 3).

Resistance to oxidative and osmotic shock

Stationary cells of the oligotrophic bacteria were

Fig. 3. Heat-induced thermal resistance of *E. coli* and *Chromobacterium* sp. Y95.

Aliquots of exponential cultures were untreated (○) or pre-heated (●) before thermal challenge and tested for viability. *E. coli* cells (A) were pre-heated at 39°C for 30 min before challenge at 52°C. *Chromobacterium* sp. Y95 cells (B) were pre-heated at 37°C for 1 h before heating at 47°C.

also more resistant to challenge with H₂O₂ and osmotic shock than those in exponential phase (Fig. 4 and 5). Stationary cells of both 1/50 NB and 1/10⁴ NB cultures showed resistance to osmotic or H₂O₂ stress, although this resistance was less marked than that of *E. coli*. Also, the NaCl and H₂O₂ concentrations at which stress was induced were low compared to *E. coli*, indicating that the oligotrophic bacteria were susceptible to low levels of stress. For example, if stress levels for *E. coli* were loaded, the oligotrophic bacteria died out completely. Although the stationary cells of the oligotrophic cultures were less resistant than those of *E. coli*, they showed enhanced resistance

Fig. 4. Oxidative resistance of exponential and stationary phase cells.

Exponential (○ or △) and stationary (● or ▲) phase cells were prepared from 1/50 NB culture (solid lines) and 1/10⁴ NB culture (dashed lines). *E. coli* (A), *Chromobacterium* sp. Y95 (B), and *Aeromonas* sp. Z06 (C) were challenged with 50 mM, 2.0 mM, and 0.8 mM H₂O₂, respectively.

Fig. 5. Osmotic resistance of exponential and stationary phase cells.

Exponential ($\circ$ or $\triangle$) and stationary ($\bullet$ or $\blacktriangle$) phase cells were prepared from $1/50$ NB (solid lines) and $1/10^4$ NB cultures (dashed lines). *E. coli* (A), *Chromobacterium* sp. Y95 (B), and *Aeromonas* sp. Z06 (C) were challenged with 2.4 M, 0.03 M, and 0.2 M NaCl, respectively.

to heat, osmotic, and oxidative stresses as compared with the respective mid-logarithmic phase cells.

Southern blotting analysis using sigma factor probes

Bacteria synthesize a number of different sigma factors which serve to mediate differential gene expression under a variety of conditions^{7,26}. As shown in Figure 6, the RpoD-4.2 probe hybridized with *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06 genomic DNA. Most genes expressed in exponentially growing cells are transcribed by the *rpoD* gene product, holoenzyme $E\sigma^{70}$. Region 4.2 of σ^{70} is highly conserved among the primary sigma factors, and par-

Fig. 6. Southern blotting analysis of the genomic DNA from *E. coli*, *Chromobacterium* sp. Y95, and *Aeromonas* sp. Z06 with RpoD-4.2 and RpoN-X probes.

ticipates in recognition of the -35 promoter sequence¹².

Chromobacterium sp. Y95 genomic DNA also hybridized with the RpoN-X probe (Fig. 6). The *rpoN* gene encodes σ^{54} , which is required for transcription of genes expressed depending on the availability of nitrogen sources¹⁵. Structurally, σ^{54} is different from other σ subunits, lacking most of the conserved sigma domains.

The other probes, RpoH and FliA, did not hybridize with genomic DNA of either oligotrophic bacterium^{9,11,18}. The results of multiple sequence alignment of sigma factors genes suggested that many of these factors form a homologous protein family^{2,5}. Four general regions of similarity have been defined, and most bacterial sigma factors share at least two of these conserved regions. *E. coli* RpoS and RpoH, and *Citrobacter freundii* HtpR play a role in starvation-induced oxidative and heat resistance, and contain sequences similar to the rpoD-4.2 probe^{12,17}.

Discussion

In natural environments, bacteria often encounter conditions inappropriate for growth. Many bacteria have evolved highly sophisticated mechanisms to survive under such conditions. During starvation, *E. coli*

cells decrease their overall metabolic rate and proceed to a more resistant state²³). *E. coli* recognizes 1/10⁴ NB medium as inappropriate propitious for growth, and the adenylate energy charge was dropped to about 0.5 after 72 h of cultivation⁸). Starved *E. coli* cells can develop multiple stress resistance and survive long periods of starvation. After 60 days of culture in ultrapure water, 9% of *E. coli* cells retained viability⁸).

We isolated oligotrophic bacteria, which were tentatively defined as bacteria which multiplied at least 10-fold over 3 days of culture in 1/10⁴ NB when inoculated at about 10³ cells/ml, from soil⁸). These strains, Y95 and Z06, also grew in 1/10⁵ NB, and reached about 1 to 10 × 10⁵ cells/ml after 3 days of cultivation (data not shown). The length of the Y95 cells grown in 1/10⁴ NB were 20 to 30% shorter than that of cells grown in 1/50 NB⁸). In stationary phase cells, the two oligotrophic bacteria grown in 1/10⁴ NB exhibited enhanced resistance compared with those in exponential phase. These observations indicated that the two oligotrophic bacteria retained the ability to multiply and showed enhanced stress resistance under low-nutrient conditions. To adapt to a low-nutrient soil environment, these features are advantageous for the oligotrophic bacteria. However, the two oligotrophic bacteria were more susceptible to lower concentrations of NaCl or H₂O₂ compared with *E. coli*. Although *E. coli* did not multiply in 1/10⁴ NB, this bacterium showed a strong capacity to adapt to various stresses.

In eubacteria, sigma factors are transcription factors that operate global switches in gene expression by conferring promoter-specific transcription initiation on bacterial RNA polymerase⁵). Stress protection is controlled by these sigma factors which are expressed in response to various stress conditions. On Southern blots, *Chromobacterium* sp. Y95 and *Aeromonas* sp. Z06 genomic DNA hybridized with the sigma factor probes, RpoN-X and RpoD-4.2, and RpoD-4.2, respectively, and multiple hybridizing bands were seen on the blots. These results suggest that there is a family of sigma factors which regulate various mechanisms of stress resistance in the two oligotrophic bacteria.

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