Growth Pattern and Adenosine Phosphates in Psychrotrophic *Pseudomonas* spp. Grown under Different Conditions

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Growth curves of the psychrotrophic *Pseudomonas* isolates (non-halophiles) showed an increased slope throughout growth (in Type 1 strains) or a decreased slope after the stationary phase of growth (in Type 2 strains). Type 1 and 2 strains had mostly optimum temperatures of 25℃ and below or above, respectively. Adenylate levels in Type 1 strains were high for AMP, moderate for ADP, and low for ATP, and adenosine phosphates in Type 2 strains was occupied by AMP alone during the growth phase with all of them disappearing during the death phase, regardless of the growth conditions (the growth temperatures and the media with altered concentrations of glucose). The AMP occupation in Type 2 strains was suggested to come from the rapid utilization of high-energy bonds due to the rapid growth, since the rapid consumption of adenylate phosphates added to the medium was observed in Type 2 strains. The values of energy charge during growth under the different growth conditions were below 0.49 or 0.01 in Type 1 or 2 strains, respectively. When growing on excess glucose (0.5%), the growth and the final pH values and adenylate concentrations in the cultures were different between Type 1 and 2 strains. These results indicated that the strains of the same Type comprise the same ecological groups, the two Type strains are composed of different ecotypes, and the dominant of AMP are characteristic of the psychrotrophic *Pseudomonas* spp.

Key Words: adenosine phosphates, growth condition, growth pattern, psychrotrophic *Pseudomonas* spp., *Pseudomonas*

Introduction

In the previous paper[1], we reported that growth curves of the psychrotrophic *Vibrio* isolates showed an increased slope throughout growth (in Type 1 strain) or a decreased slope after the stationary phase of growth (in Type 2 strain). The levels of adenylate concentrations in the culture and changes with growth time in adenylate concentrations differed between the two Type strains, regardless of growth at different temperatures. These differences between the two Type strains were regarded to come from different ecotypes. Psychrotrophic *Pseudomonas* strains were also isolated from the same mackerel isolated the psychrotrophic *Vibrio* strains when stored at 0℃[2], which grew at 0℃ with an optimum growth temperature range of 15 to 30℃. Growth curves of the *Pseudomonas* isolates also showed an increased slope throughout growth or a decreased slope after the stationary phase of growth (Fig. 1). This study aimed to investigate whether the psychrotrophic *Pseudomonas* isolates having different growth kinetics are composed of different ecotypes in common with the psychrotrophic *Vibrio* isolates.

Energy charge [(ATP + 1/2 ADP)/(ATP + ADP + AMP)][3] of adenine nucleotide pools in Escherichia coli during growth and starvation was investigated in detail by Chapman et al[4]. They suggested from their results that growth could occur only at energy charge values above about 0.8, that viability is maintained at values between 0.8 and 0.5, and that cells die at values below 0.5. Tabulation of adenylate concentrations previously reported for various organisms and tissues supports the prediction that the energy charge is stabilized near 0.85 in intact metabolizing cells of a wide variety of types. *Saccharomyces cerevisiae* also maintains energy charge values above about 0.8 during prolonged starvation but the values decrease rapidly during starvation and remain viable at those below 0.1 (i.e. energy charge values exhibit generally above about 0.8 during growth and decrease during starvation although a decreased level of the values during starvation alter with the kind of microorganisms)[5]. Incidentally, the energy charge of adenine nucleotide pools was determined by using *Pseudomonas aeruginosa* biofilms. Results indicated that energy charge values were generally low throughout the film, reaching a maximum of only 0.6. Of the adenine nucleotides measured, AMP was the predominant nucleotide[6]. We also reported the predominance of AMP and low energy charge values of adenine nucleotide pools in batch culture throughout growth of the psychrotrophic *Vibrio* isolates[1]. We were interested whether adenylate levels and energy charge values during growth of the psychrotrophic

Pseudomonas isolates are similar to those during growth of the psychrotrophic *Vibrio* isolates.

Adenylate levels and adenylate energy charge values during growth and/or starvation of prokaryotic and/or eukaryotic microorganisms varied with physical and chemical growth conditions[4,5,7-11]. Therefore, adenylate levels and the charge values during growth of the psychrotrophic *Pseudomonas* isolates having different growth kinetics during growth in batch culture were measured at different growth temperatures by using the media with altered concentrations of glucose and with individual adenosine phosphates. In addition, adenylate levels and the charge values during growth of the halophilic-psychrotrophic and the mesophilic *Pseudomonas* isolates were determined for comparison.

Materials and Methods

Organisms, media, and growth conditions

Non-halophilic *Pseudomonas* spp. (320 strains) used for this experiment were isolated from mackerel when stored at 0℃[2]. All the isolates grew well at 0℃ within two weeks and also at 25℃ within 24 h. They were judged to be psychrotrophs according to the definition of Morita[12]. The halophilic-psychrotrophic *Pseudomonas* isolates (21 strains) from the same mackerel and the mesophilic *Pseudomonas* isolates (40 strains) from mackerel stored at 35℃ were also used for comparison. The mesophiles were no growth at 0℃, had optimum temperatures of 30 to 37℃, and were identified in the same identification methods[2] as the psychrotrophs.

PYBG medium [Bacto peptone (Difco), 0.5%; yeast extract powder (Difco), 0.25%; beef extract (BBL), 0.25%; glucose, 0.1%; in 50% sea water (pH 7.4)] was used for the determination of optimum growth temperature and growth pattern at the optimum temperature. NBG medium [nutrient broth (BBL), 0.8%; glucose, 0.1%; in 25% sea water for non-halophiles or 75% sea water for halophiles (pH 7.4)] was used as a basal medium for the determination of adenylate concentrations in the culture during growth owing to obtain the correct and high values of the concentrations[13]. NBG media with altered concentrations of glucose were used for measuring the effect of glucose in connection with growth pattern and pH and adenylate levels in the culture. NBG media containing 10 nmol/mL of individual adenosine phosphates was used for studying the ability to metabolize the phosphates.

Unless otherwise stated, one loopful of overnight culture grown at an optimum temperature was inoculated into a tubed test medium (5 mL for a test tube of 16 mm in diameter) and incubated at an optimum or the designated temperatures under static conditions.

Determination of growth pattern and pH in the culture during growth

An optimum growth temperature was determined by using a 24 h culture for all the isolates. A growth pattern at an optimum temperature was determined at intervals of 12 h by using all the isolates. The pH of the culture grown on glucose was determined for 10 strains each chosen randomly from the two groups of the isolates that showed an increased growth curve throughout growth and a decreased growth curve after the stationary phase of growth (Fig. 1), and an analytical sample for measuring the culture pH was obtained from each tube culture. Growth was monitored by measuring absorbance at 600 nm.

Determination of adenylate concentrations in the culture during growth

The concentration of adenosine phosphates (AMP, ADP, and ATP) in the culture during growth was determined for five strains each chosen randomly from the two groups of the non-halophilic psychrotrophs having different growth kinetics, the halophilic psychrotrophs, and the mesophiles by the Karl & Holm-Hansen method[14] and the modified Karl & Holm-Hansen method[13]. The part of the culture used as an analytical sample was removed from the homogeneous layer except for the bottom layer of the tubed culture. For measuring adenylate concentrations in the culture during growth at different temperatures, the non-halophilic psychrotrophs were incubated at 30, 20, 10, and 0℃ and the halophiles and the mesophiles at an optimum growth temperature. For measuring the effect of glucose in connection with adenylate levels in the culture, the non-halophilic psychrotrophs were incubated in the media with 0.5, 0.1, and 0% of glucose.

Adenylate concentrations in the supernatant fluid of the culture during growth at an optimum temperature were also determined for five strains each of the two groups of the isolates having different growth kinetics. The supernatant fluid was obtained by centrifugation at 13,000 x g at 4 ℃ for 15 min.

Results

Growth pattern of the non halophilic-psychrotrophic isolates at optimum growth temperatures

Growth curves of the non halophilic-psychrotrophic *Pseudomonas* isolates incubated at an optimum temperature of 25℃ are shown in Fig. 1. The experiment was performed for all the isolates having optimum temperatures of 15 to 30℃, and the results obtained from ten strains

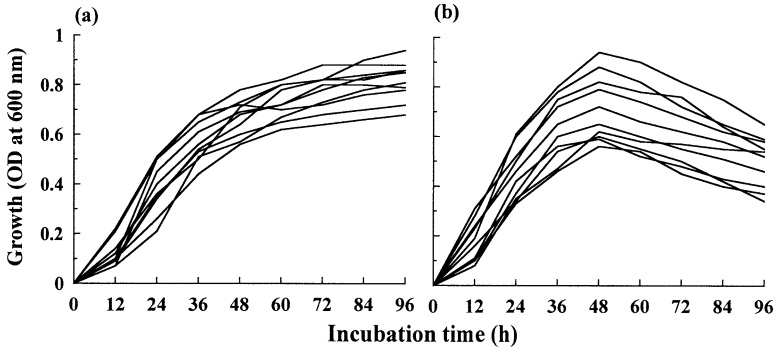

Fig. 1 Growth curves at an optimum temperature of 25℃ in (a) Type 1 strains (the strains where growth curves showed an increased slope throughout growth) and (b) Type 2 strains (the strains where growth curves showed a decreased slope after the stationary phase of growth) of psychrotrophic *Pseudomonas* spp. isolated from mackerel during storage at 0℃. The results obtained from ten strains each of Type 1 and 2 strains are represented.

each of Type 1 and 2 strains (see below) having an optimum temperature of 25℃ are represented since the results were similar among the two Type strains each. Additionally, the greater part of the isolates was occupied by strains having optimum temperatures of 25 and 20℃ (by the former in the main). None of the psychrotrophs were able to grow at 35 ° C or above.

The growth patterns of the psychrotrophic *Pseudomonas* isolates showed an increased slope throughout growth (in Type 1 stain) although the growth shifted from exponential to arithmetic growth, or a decreased slope after the stationary phase of growth (in Type 2 strain). The isolates were almost the same number between Type 1 and 2 strains, were accounted for the more part in Type 2 strain for the isolates having an optimum temperature of 25℃, and were account for the most part in Type 1 strain for those having optimum temperatures of 20 and 15℃ and in Type 2 strain for those having an optimum temperature of 30℃.

The culture pH in the non halophilic-psychrotrophic isolates during growth on glucose

Growth curves and the culture pH in Type 1 and 2 strains during growth on glucose are shown in Figs 2 and 3, respectively. In Type 1 strains (Fig. 2), when growing on 0.1% glucose, growth was very good and growth curves showed an increased slope throughout growth and the initial pH (7.4) in the cultures increased slowly during growth. When growing on 0.5% glucose, growth was inhibited and growth curves showed a decreased slope after the stationary growth phase and the initial pH in the cultures decreased acceleratedly during growth and the final pH reached 4.28

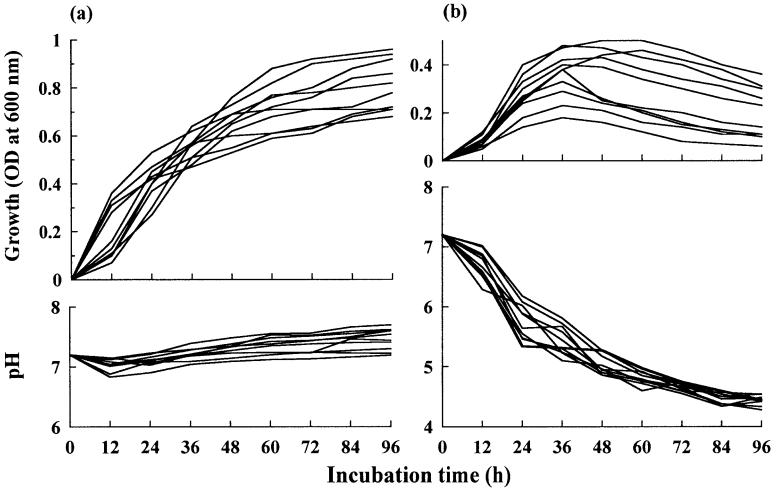

Fig. 2 Growth curves (upper) and pH values (lower) in the cultures in Type 1 strains grown in the media with (a) 0.1 and (b) 0.5% of glucose. The results obtained from ten strains of Type 1 strain are represented.

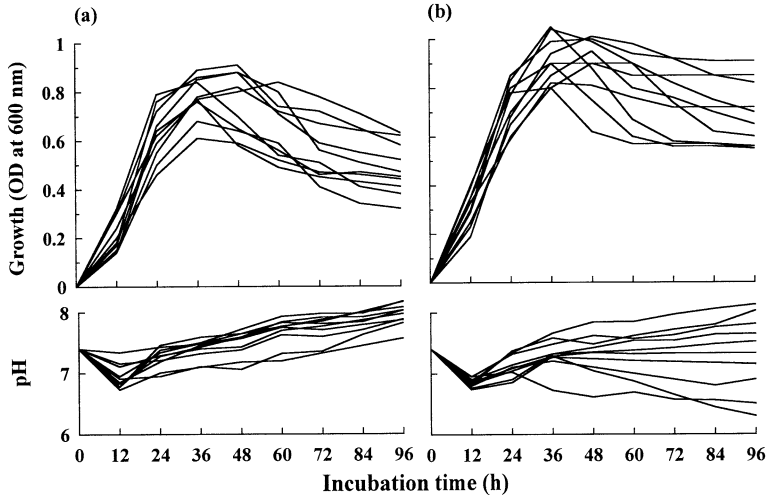

Fig. 3 Growth curves (upper) and pH values (lower) in the cultures in Type 2 strains grown in the media with (a) 0.1 and (b) 0.5% of glucose. The results obtained from ten strains of Type 2 strain are represented.

to 4.74. In Type 2 strains (Fig. 3), growth was slightly high and the culture pH during growth was narrowly low (above pH 6.30 in the final) on 0.5% glucose than on 0.1% glucose.

Adenylate concentrations in the non halophilic-psychrotrophic isolates during growth at different growth temperatures

Growth curves, adenylate concentrations in the culture, and energy charge in Type 1 and 2 strains during growth at different temperatures are shown Figs 4 and 5, respectively. The experiment was performed for five strains each of Type 1 and 2 strains (also for the psychrotrophic halophiles and the mesophiles), and the results obtained from one strain each in Type 1 and 2 strains are represented here, since the results were similar among five strains of Type 1 or 2 stains (the same applies for Figs 6-11). The energy charge was not represented for Type 2 strain since ADP and ATP concentrations in the culture exceeded the limit (below 0.01 nmol/mL) of the detection (the same applies for Fig. 7).

In Type 1 strain having an optimum temperature of 20℃ (Fig. 4), the concentrations of AMP, ADP, and ATP increased almost parallel with an increment of growth and the levels of the concentrations were high for AMP, moderate for ADP, and low for ATP, independent of growth at the different temperatures. The adenylate concentrations were considerably high when grown at 30℃ although the growth was lower than the optimum temperature (20 ℃) but the concentrations lowered as the growth temperatures (including 10℃) go down than the optimum.

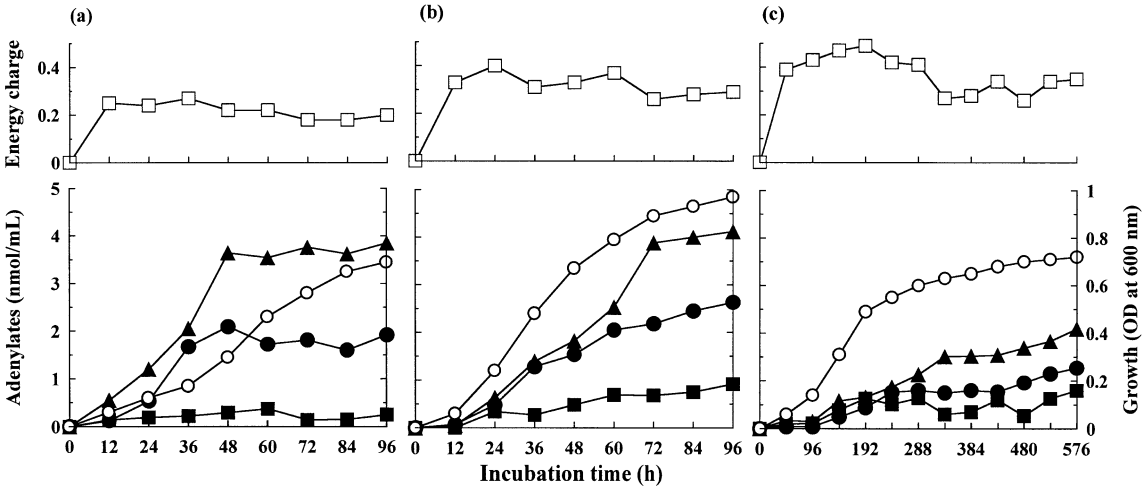

Fig. 4 The concentration of adenosine phosphates in the culture (lower) and adenylate energy charge (upper) during growth at (a) 30, (b) 20, and (c) 0℃ in Type 1 strain (Strain PS1643) having an optimum temperature of 20℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth; (□), energy charge.

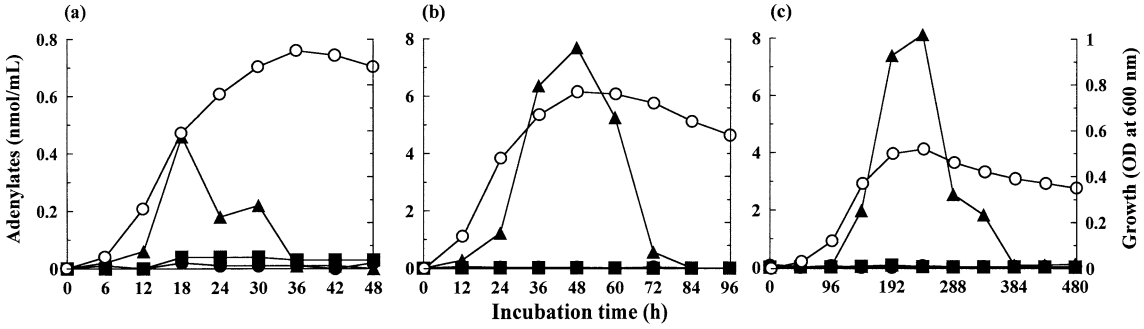

Fig. 5 The concentration of adenosine phosphates in the culture during growth at (a) 30, (b) 20, and (c) 0℃ in Type 2 strain (Strain PS1907) having an optimum temperature of 30℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth.

The charge values ranged from 1.8 to 4.9 during growth at different temperatures and declined with an increment of growth time and growth temperature.

In Type 2 strain having an optimum temperature of 30℃ (Fig. 5), AMP concentrations rose rapidly during the logarithmic growth phase, reached a maximum during the logarithmic growth phase to the stationary growth phase, and then fell abruptly to practically zero during the stationary growth phase to the death phase: that is, changes with growth time in AMP concentration differed among the growth temperatures (the existence of AMP during the growth phase was elongated as the growth temperature lower). AMP concentration in the culture grown at an optimum temperature of 30℃ was very low compared to that grown at 20℃ and below although the growth was highest at 30℃. The ADP and ATP in the culture were hardly detected throughout growth at different temperatures. Therefore, the charge values also were practically zero throughout growth at different temperatures.

Adenylate concentrations in the non halophilic-psychrotrophic isolates during growth on glucose

Growth curves, adenylate concentrations in the culture, and energy charge in Type 1 and 2 strains during growth on glucose are shown Figs 6 and 7, respectively. In both Type 1 and 2 strains, changes with growth time in adenylate concentrations showed a similar trend as the results from the isolates grown at different temperatures, independent of the concentration of glucose in the medium. In Type 1 strain (Fig. 6), however, adenylate concentrations were very low in the culture grown on 0.5% glucose compared to those grown on 0.1 and 0% glucose. As mentioned above, the growth also was very low in the medium with 0.5% glucose than in that with 0.1% glucose. Moreover, there was small difference between ADP and ATP levels in both the cultures grown on 0.5 and 0% glucose. The charge values ranged from 0.13 to 0.42 and were highest when grown on 0.1% glucose. On the other hand, in Type 2 strain (Fig. 7), AMP concentration was very low in the culture grown on 0 % glucose than in those grown on 0.5 and 0.1% glucose although the marked variations of

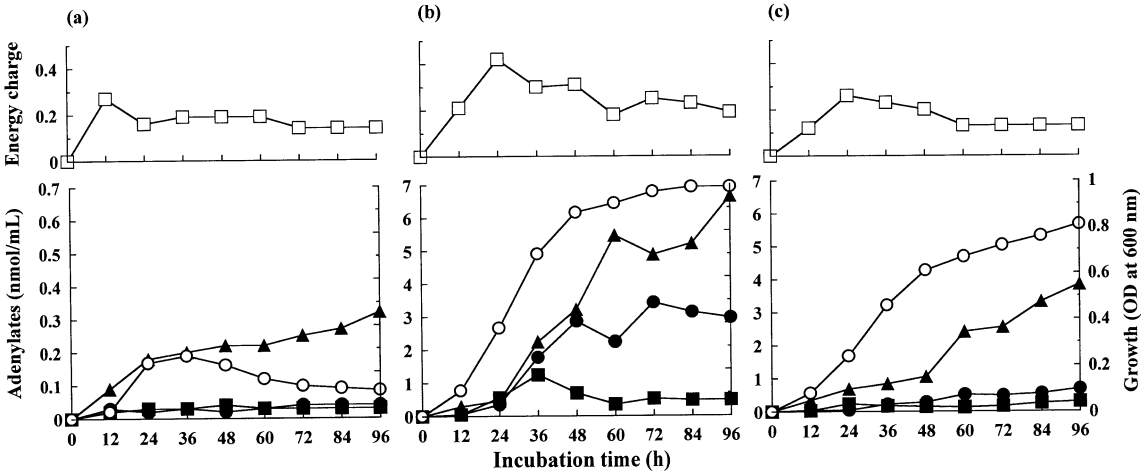

Fig. 6 The concentration of adenosine phosphates in the culture (lower) and adenylate energy charge (upper) during growth in the media with (a) 0.5, (b) 0.1, and (c) 0% of glucose in Type 1 strain (Strain PS1725) having an optimum temperature of 20℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth; (□), energy charge.

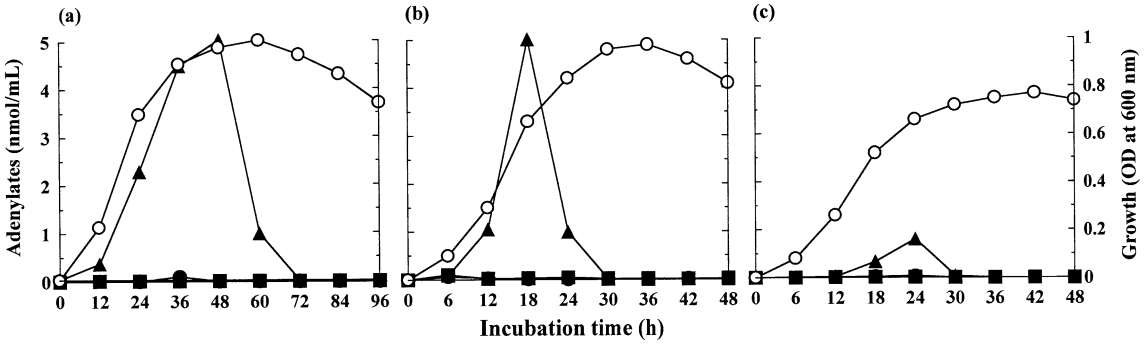

Fig. 7 The concentration of adenosine phosphates in the culture during growth in the media with (a) 0.5, (b) 0.1, and (c) 0% of glucose in Type 2 strain (Strain PS1143) having an optimum temperature of 30℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth.

growth were not observed between the absence and the presence of glucose in the medium.

Effect of individual adenosine phosphates in growth medium in the non halophilic-psychrotrophic isolates

Growth curves and adenylate concentrations in the culture in Type 1 and 2 strains during growth in the presence of individual adenosine phosphates are shown in Figs 8 and 9, respectively. In Type 1 strain (Fig. 8), when growing in the presence of AMP, adenylate concentrations including AMP increased with an increment of growth: that is, the added AMP was hardly utilized by Type 1 strain. When growing in the presence of ADP or ATP, the added ADP and ATP decreased directly with growth and slowly during growth, respectively: that is, the added ADP and ATP were utilized by Type 1 strain. In addition, the decrease tendencies of the added ADP and ATP during growth were different among the tested strains. The levels of adenylate concentrations were different between the cultures grown in the presence and absence of ATP: that is, AMP level was lowest in the culture grown in the presence of ATP as represented here but highest in that grown in

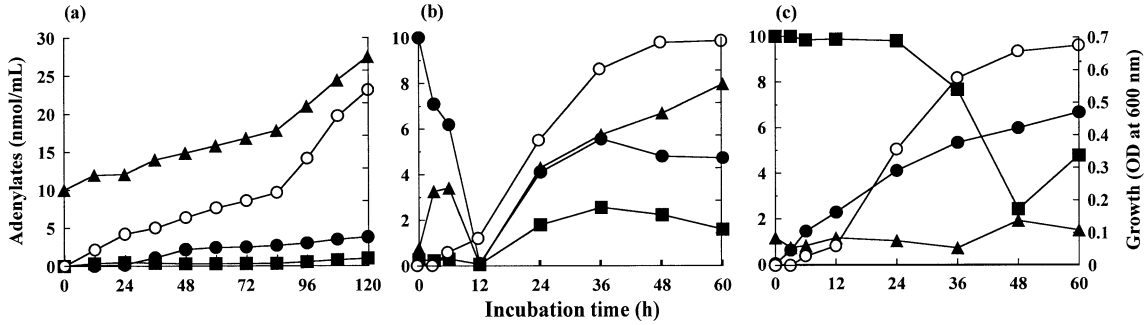

Fig. 8 The concentration of adenosine phosphates in the culture during growth in the media containing 10 nmol/mL of (a) AMP, (b) ADP, or (c) ATP in Type 1 strain (Strain PS237) having an optimum temperature of 20℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth.

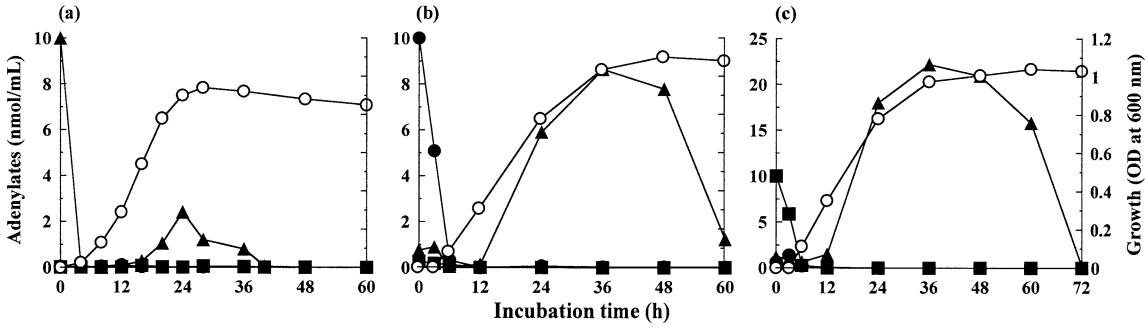

Fig. 9 The concentration of adenosine phosphates in the culture during growth in the media containing 10 nmol/mL of (a) AMP, (b) ADP, or (c) ATP in Type 2 strain (Strain PS344) having an optimum temperature of 30℃. (▲), AMP; (●), ADP; (■), ATP; (○), growth.

the absence of ATP as mentioned above. The adenylate levels, however, were almost the same between the cultures grown in the absence and presence of ADP.

In Type 2 strain (Fig. 9), when growing in the presence of individual adenosine phosphates, all of the added adenylates disappeared abruptly during the lag phase for growth and changes with growth time in adenylate concentrations after the disappearance of the added adenylates were similar to the results obtained from the culture grown in the absence of the adenylates as mentioned above. In addition, AMP concentration after the disappearance of the added adenylates was different among the added adenylates and the tested strains. AMP concentration in the Type 2 strain represented here increased remarkably in the cultures grown in the presence of ADP or ATP.

Adenylate concentrations in supernatant fluid of the culture during growth

Growth curves and adenylate concentrations in the culture and in the supernatant fluid of the culture in Type 1 and 2 strains are shown in Fig. 10. In Type 1 strain, adenosine phosphates were detected in the supernatant fluid but their concentrations were very low. In Type 2 strain, adenosine phosphates were not detected in the supernatant fluid, whereas AMP was detected in the culture.

Adenylate concentrations in the halophilic-psychrotrophic and the mesophilic isolates during growth

Growth curves, adenylate concentrations in the culture, and energy charge in the halophilic-psychrotrophic and the mesophilic isolates during growth are shown in Fig. 11. In both of the halophilic-psychrotrophic and the

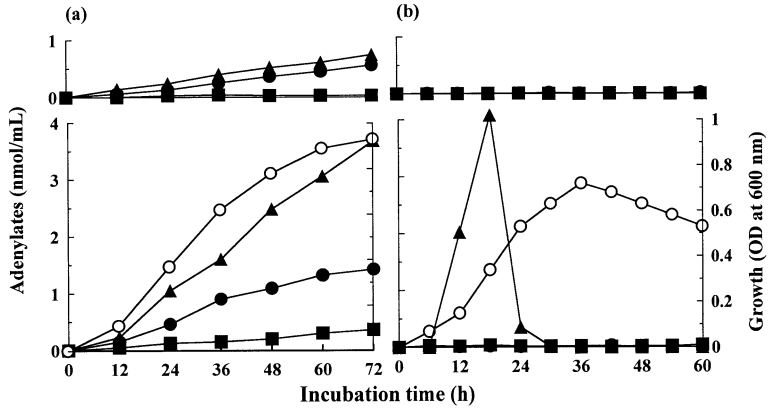

Fig. 10 The concentration of adenosine phosphates in the culture (lower) and the supernatant fluid of the culture (upper) during growth in (a) Type 1 strain (Strain PS1044) and (b) Type 2 strain (Strain PS845). (▲), AMP; (●), ADP; (■), ATP; (○), growth.

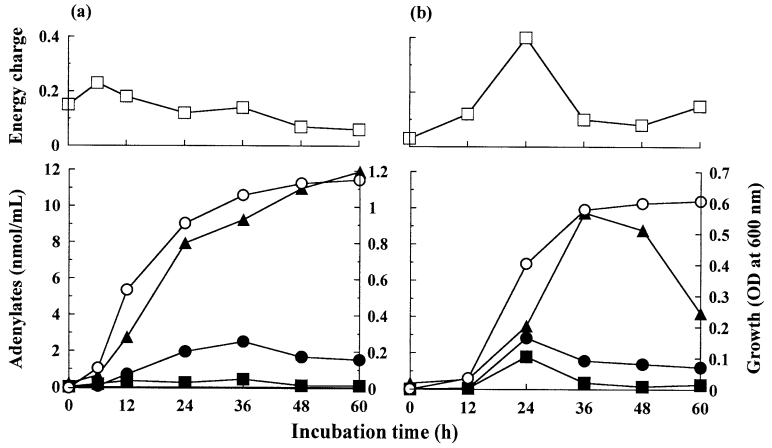

Fig. 11 The concentration of adenosine phosphates in the culture (lower) and adenylate energy charge (upper) during growth at optimum temperatures of 37 and 20℃ in (a) the mesophilic (Strain PM22) and (b) the halophilic-psychrotrophic Pseudomonas isolates (Strain PH614), respectively. (▲), AMP; (●), ADP; (■), ATP; (○), growth; (□), energy charge.

mesophilic isolates, AMP, ADP, and ATP were detected in the culture, the concentration levels were high for AMP, moderate for ADP, and low for ATP, and the charge values were very low (0.23-0.06 in the halophilic psychrotrophs and 0.4-0.08 in the mesophiles) in the same way as the results obtained from Type 1 strain. AMP concentration increased throughout growth for the most part of the mesophiles and decreased rapidly after the logarithmic growth phase for the large part of the halophilic psychrotrophs. ADP and ATP concentrations decreased slightly during the stationary growth phase for both of the mesophiles and the psychrotrophs.

Discussion

The psychrotrophic *Pseudomonas* isolates (non-halophiles) were divided into two groups (Type 1 and 2 strains) from growth curves. The growth curves showed an increased slope throughout growth in Type 1 strains but a decreased slope after the stationary growth phase in Type 2 strains, suggesting that autolysis progress quickly after the stationary growth phase in Type 2 strains but not in Type 1 strains. This may derive from the difference in chemical and physical characteristics of protein between the two Type strains to be published in a successive paper (Morii H, unpubl. data). When growing on excess glucose (0.5%), the final pH values in the cultures were below 4.74 in Type 1 strains but above 6.3 in Type 2 strains, therefore the growth was inhibited in Type 1 strains but preferably facilitated in Type 2 strains, consequently adenylate concentrations in the cultures also was very low in Type 1 strains but high in Type 2 strains, suggesting that the ability of acid production from glucose and cell viability attended on the acid production differ between the two Type strains. These facts suggest that strains of the same Type comprise the same ecological group. However, strains of each Type may be composed of different taxonomic species because various species are usually contained in the isolates and actually the utilization of various carbohydrates and amino acids varied from the isolates (Morii H, unpubl. data). On the other hand, the levels of adenylate concentrations and changes with growth time in adenylate concentrations were different between the two Type strains, regardless of the growth conditions (the growth temperatures and the growth media with altered concentrations of glucose), suggesting that adenylate metabolism is different between the two Type strains. In addition, adenosine phosphates in the culture came mostly from intracellular substances, since adenylatye concentrations were very low in the supernatant fluid of the culture compared to the culture. Additionally, the utilization of inulin was positive in Type 1 strains and negative in Type 2 strains; and the utilization of valerate, succinate, and pyruvate was positive in Type 2 strains and negative in Type 1 strains (Morii H., unpubl. data). These facts suggest that Type 1 and 2 strains are composed of different ecotypes.

In Type 1 strains, adenylate levels were usually high for AMP, moderate for ADP, and low for AMP, independent of the growth conditions. The concentrations of AMP, ADP, and ATP during growth generally increased almost parallel with an increment of growth (i.e. except for growth on excess glucose). It has been suggested from these results that the metabolic rate of adenosine phosphates is balanced among the three phosphate groups in microorganisms growing at a low temperature. Theoretically, ATP contains three phosphate groups attached to an adenosine molecule, and one or two of the terminal phosphate groups with high-energy bonds are broken off by hydrolysis[15]. The making of many biosynthetic bonds is coupled with the breakdown of a high-energy bond, so that the high-energy bonds (mainly ATP) in the cell generally have a very short life. Likewise, the activation of an amino acid is achieved by transfer of an AMP group from ATP. During protein syn thesis, the formation of each peptide bond is coupled with the breakdown of ATP to AMP. The ATP, not ADP, is the primary energy donor, and ADP cannot transfer a high-energy group. These theories support these observations and the suggestion noted above.

In Type 2 strains, adenosine phosphates consist of AMP alone during the growth phase. It is presumed that almost instantly the high-energy bonds (mainly ATP) are formed during a degradative reaction, it would be broken down to yield the energy needed to drive another reaction. Consequently, only AMP was accumulated because the formation of each peptide bond would not be coupled with the breakdown of ATP to AMP. The AMP concentrations during growth rose rapidly and then fell sharply from low to high growth temperatures (therefore, AMP concentration in the culture grown at an optimum temperature of 30℃ was very low compared to that grown at 20℃ and below), suggesting that Type 2 strains are controlled to speed the metabolic rate and consequently the cessation of growth. This suggestion may be confirmed by the rapid consumption of adenosine phosphates added to the medium.

The energy charge values in both of the Type 1 and 2 strains were different with strains, culture age, and the growth conditions. However, the values during growth were below 0.01 in Type 2 strains and below 0.49 in Type 1 strains (AMP is the predominant nucleotide). The low energy charge values ranging from 0.06 to 0.23 were also shown in the halophilic-psychrotrophic and the mesophilic

Pseudomonas isolates used for comparison in this experiment. The low energy charge values ranging from 0.16 to 0.60 (AMP is the predominant nucleotide), which have been previously reported for the *Pseudomonas aeruginosa* biofilm[6], support these results. Moreover, the psychrotrophic *Vibrio* isolates during growth at optimum temperatures or below have also been shown low energy charge values of 0.38 or below. These results differed from that energy charge values during growth of prokaryotic and eukaryotic microorganisms are above about 0.8 and therefore ATP is the predominant nucleotide[3-5,16], and that growth can occur only at energy charge values above about 0.8, viability is maintained at values between 0.8 and 0.5, and cells die at values below 0.54). It is hoped that this type of study will be continued in the future.

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異なる生育条件下で生育した低温性 *Pseudomonas* 属細菌の生育曲線とアデノシンリン酸

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低温貯蔵下のサバから分離した低温性 *Pseudomonas* 属細菌の生育曲線は，生育を通して増加（Type １型）または定常期以降に減少した（Type ２型)。Type １型菌の培養にはAMP，ADP，ATPが認められ，またこの順にその量を減じた。Type ２型菌ではほぼAMPしか検出されず，このことは，培地に加えたアデノシンリン酸の消費速度から，速い生育速度に起因すると考えられた。エネルギー充足度はType １および２型菌ではそれぞれ0.49および0.01以下であった。グルコースからの生酸能も両者で異なり，両者は生態系および生態型を異にすると考えられた。