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Full Paper

Maltose adaptive mutant of heterotrophic marine bacterium, *Alteromonas espejiana* Bal-31: Changes in the growth and the induction of extracellular α-amylase

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Growth of the heterotrophic marine bacterium, *Alteromonas espejiana* Bal-31 was inhibited in the presence of sucrose, maltose and even glucose, but not with starch. Extracellular α-amylase was induced with a lag phase of 2 h in the presence of starch. In contrast, cell growth of the S2a mutant was not affected by the addition of maltose, and starch was ineffective in the induction of extracellular α-amylase in this mutant. Activity of extracellular α-amylase was induced from the S2a mutant with a 4-h lag phase in the presence of maltose, and the high level of enzyme activity was maintained for at least 24 h. Activity of α-amylase induced by both wild type starch and S2a mutant maltose cultures were mainly observed in extracellular locations. This activity could be stopped by tetracycline treatment, indicating that enzyme induction was dependant on gene expression and not on enzyme protein secretory mechanisms. Our results showed that the mutation in S2a changed the growth and the modulation of the specific α-amylase in response to carbon nutrients.

Key Words——*Alteromonas espejiana* Bal-31; carbon nutrient; extracellular α-amylase; marine bacterium; mutation

Introduction

Plankton is the main primary producer of marine ecosystems. Heterotrophic bacteria exist in the base position of the food chain providing prey for phagotrophic algae and protozoans. Growth of heterotrophic bacteria depends highly on the organic nutrients present in the environment. Polymeric organic compounds such as polysaccharides constitute the dominant form of carbohydrates in the ocean above the minimum level of oxygen (Benner et al., 1992; Pakulski and Benner, 1994). However, in the nature of marine systems the concentration and composition of polymeric compounds are usually subject to fluctuations as a consequence of biological, chemical and physical conditions.

Heterotrophic bacteria are mainly responsible for polymer hydrolysis by means of extracellular enzymes (Hoppe, 1983). Extracellular enzymes are mostly hydrolytic in nature and are necessary for bacterial feeding because most utilizable organic compounds can only be used after being hydrolyzed into low molecular weight compounds (Amon and Benner, 1996). Bacterioplankton assemblages possess various uptake systems operating at different ranges of substrate concentration (Azam and Hodson, 1981). Vetter and Deming (1999) demonstrated that the growth rates of four different marine bacteria on different particulate organic

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substrates are usually lower than rates using dissolved organic substrates. In the response of the microbial community to the changing nutrient sources in the environment, extracellular enzyme activities may exhibit a more dominant short-term variability than uptake activities (Karner and Rassoulzadegan, 1995). More recently it has been demonstrated that if the high-affinity hydrolytic extracellular enzymes and the low-affinity uptake systems are operating simultaneously, the potential rates of hydrolytic activity and uptake are tightly coupled (Unanue et al., 1999). Heterotrophic bacteria responded sensitively to change in nutrient conditions by regulating the synthesis and activity of their enzymes (Gotschalk, 1985). Extracellular enzyme activities have been found to respond positively to the concentration changes of polymeric compounds, but it is largely unknown how these hydrolytic activities are regulated (Chróst, 1991).

Alteromonas espejiana Bal-31 was originally isolated from seawater in a terrestrially polluted bay off the coast of Chile. The bacterium has a psychrophilic and halophilic nature and is a monotrichous, gram-negative, rod cell. It has unusual characteristics for *Pseudomonas* species including the absence of catalyzing activity and the ability to hydrolyze starch (Espejo and Canelo, 1968). Except for a few reports concerning nuclease (Hauser and Gray, 1990; Zhou and Gray, 1990), DNA repair (Zerler and Wallace, 1984), thermolabile α-amylase (Feller et al., 1992) and the chitinase gene (Tsujibo et al., 1993), the genetic organization of this organism is still unknown. In this report we carried out the induction of bacterial mutation and allowed the mutants to accommodate themselves to an environment with various organic nutrients in the laboratory, in order to assess the adaptive capability of the bacteria to survive. Changes in cell growth and the activities of extracellular α-amylase in response to the nutrient conditions of the bacterium and its mutants were described. The aim of this study was to investigate the effect of carbohydrate nutrients on cell growth represented by the adaptability of the bacteria, and to ascertain that this regulation of the activity of extracellular α-amylase was dependant on gene expression rather than on the secretion mechanism of enzyme proteins.

Materials and Methods

Cell cultures. The bacterial strain, *Alteromonas espejiana* Bal-31, was a gift of Dr. H. M. Huang, Institute of Radiation Biology, Chin Hua University. Bal medium containing 8 g/L bacto nutrient broth (Difco), 26 g/L NaCl, 0.7 g/L KCl, 1.5 g/L $CaCl_2 \cdot 2H_2O$ and 7.8 g/L $MgSO_4 \cdot 7H_2O$, was prepared according to the described method of Franklin et al. (1969). To measure cell density, cells from overnight culture were transferred to fresh medium at an initial concentration of 0.1 absorbance units at a wavelength of 600 nm, and incubated on a gyratory shaker at 160 rpm at 26°C in the dark. Two milliliters of cell culture were taken every 2 h to measure the absorbance at 600 nm and to count the cell numbers by plating after series dilutions. Concentration of carbon nutrient added to the Bal medium, such as soluble starch, sucrose, maltose and glucose, was 1%.

Mutagenesis and mutant isolation. Cells of *A. espejiana* Bal-31 were harvested when a density of 3×10^8 cells/ml was reached and pelleted by centrifugation. After being washed once with Tris buffered salts (TBS) medium, the cell pellet was resuspended in half volume TBS medium and the mutagenesis using ethylmethane sulfonate was done as described by Zerler and Wallace (1984). Mutants were selected from bacterial cells after treatment with ethylmethane sulfonate for 1–2 h (Table 1). After a series of dilutions, mutated cells were cultured on Bal agar plates. Each colony was picked up and inoculated in duplicate on Bal and starch agar plates. After incubation at 26°C in the dark for 4 days, potassium iodide (KI) solution was added to the starch plates. Colonies with either a large transparent area or no transparent area were selected. To eliminate the possibility that the isolated mutants were derived from contaminants, mutants were assayed for sensitivity to bacteriophage PM2 infection following the method of Franklin et al. (1969).

Enzyme analysis. Extracellular activity of α-amylase was assayed directly from culture medium. For

Table 1. Isolation of *Alteromonas espejiana* mutants by ethylmethane sulfonate treatment.

Time (h)	Viable cells $\times 10^6$		% Survival	Mutants selected
	Before	After		
1	984	553	56.2	3 from 2,000
2	1,030	163	15.8	5 from 1,500
3	994	1.44	0.14	—

determination of intracellular enzyme activity, bacterial cells were collected after being cultured for an appropriate time and centrifuged at 8,000×*g* for 3 min. The cell pellet was resuspended and washed in half volume 50 mM Tris buffer, pH 8.0, containing 10% sucrose. The washed cells were resuspended in the same buffer, and kept at −70°C for 5 min. After the cells had thawed, 0.1 ml of lysozyme (Sigma Chemical Co.) (10 mg/ml), 16 µl of 5 M NaCl and 6 µl of 0.1 M dithiothreitol (Sigma Chemical Co.) were added to the cell suspension. The suspension was kept on ice for 45 min and then at −70°C and 26°C for 5 min and 15 min respectively. The process of freezing and thawing was repeated four times in order to lyse the cells completely. The cell lysate was centrifuged at 5,200×*g* for 10 min and the supernatant was denoted as the intracellular fraction. Aliquots of medium, washed and intracellular fraction were used for enzyme and protein assays.

The activity of α-amylase was determined using a modified method from Doehlert and Duke (1983); Enzyme solution (0.1 ml) was added to 0.2 ml reaction mixture containing 0.1 M potassium succinate buffer, pH 6.8, 3 mM calcium chloride and 2% starch azure (Sigma Chemical Co.). After incubation at 30°C for 30 min, 0.3 ml of 20% trichloroacetic acid was added to stop the reaction. The solution was centrifuged and the supernatant was assayed for absorbance at 595 nm. A unit of activity was represented by an absorbance change (A_{595}) of 0.298/min. Protease activity was determined as described by Rick (1963). A 0.1 ml sample solution was added to a 0.9 ml reaction mixture containing 1% casein in 0.1 M phosphate buffer (pH 7.6). After mixing thoroughly, the solution was incubated at 37°C for 20 min. The reaction was stopped by the addition of 3 ml 5% trichloroacetic acid. It was centrifuged at 3,000×*g* for 20 min. Absorbance of the supernatant at 280 nm was determined. A unit of activity was calculated as 0.1 absorbance increase per min. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

Effect of carbon nutrients on cell growth

The successful infection of S1c and S2a cells by bacteriophage PM2 indicated the host cell infection pathway was not affected by mutation. Correlation between absorbance (A_{600}) and cell counting was tested in a preliminary experiment in which cell growth was measured 6 h after inoculation. The result showed a slight reduction in absorbance to cells of mutants grown in starch-supplemented media (data not shown). The effect of carbon nutrients on the time course of cell growth is shown in Fig. 1. Wild type cells grew very well in either Bal or starch-supplemented medium with the cell density reaching an A_{600} of 3.7 after 15 h growth. The maximum cell density of the S1c mutant was less than 3.0 at A_{600}. When sucrose was added to the medium, cell growth of the wild type and mutants was inhibited after 6 h and the absorbance was less than 1.0. Cell growth of the wild

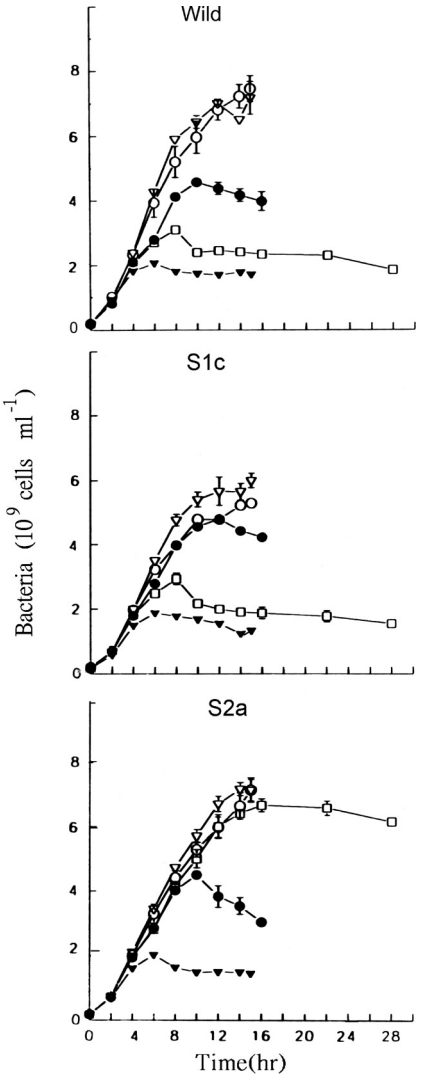

Fig. 1. Cell growth of *A. espejiana* Bal-31 and mutants cultured in Bal on various carbon nutrients supplemented media.

Figures were presented in the rank of wild type and S1c, S2a mutants. Supplemented media are Bal (○); starch (△); glucose (●); maltose (□) and sucrose (▼).

type and mutants was retarded in medium with added glucose after 10 h and then there was a further decrease in cell density. The pattern of cell growth of the wild type as well as mutants exhibited similar inhibition by the addition of sucrose or glucose to the medium. The same growth pattern of wild type and S1c mutant was observed in maltose-supplemented medium. Cell growth stopped at 8 h and an absorbance of 1.5 was reached. Within 28 h the absorbance was constantly maintained at 1.0. Surprisingly, cell growth of the S2a mutant was not significantly affected by the addition of maltose to the medium and the maximum cell density was maintained for at least 28 h.

Induction of extracellular α-amylase

When cells were grown in Bal medium, a very low α-amylase activity of the wild type culture was observed, and no enzyme activity could be detected for the S2a mutant. The culture medium of the S1c mutant showed a low level of enzyme activity. The highest activity was 1.0 unit measured after cell growth for 16 h (Fig. 2A). Starch was one of the carbon nutrients that did not affect cell growth, but was effective in the induction of α-amylase secretion, when it was added to the culture medium. After a 2 h lag phase, α-amylase activity of the wild type and S1c were similarly induced to a high level of 6.5 units with growth for 6 h and then declined after growth for 12 h (Fig. 2B). However, no enzyme activity was observed for the S2a mutant growth in starch-supplemented medium. Glucose-supplemented medium was not only unfavorable for cell growth, but also ineffective for α-amylase induction. Even for the S1c mutant only 0.2 units of enzyme activity were detected after cell growth for 6 h (Fig. 2C). A transient increase of α-amylase activity was observed both for wild type and S1c cells grown in the maltose-supplemented medium. Surprisingly, in the presence of maltose, a drastic increase of α-amylase activity in the culture of the S2a mutant was observed after a 4 h lag phase and a maximum activity of 8.5 units was reached at 14 h of cell growth (Fig. 2D). In contrast with starch induction, the high level of α-amylase activity was maintained at least for 28 h and no decrease of enzyme activity was observed.

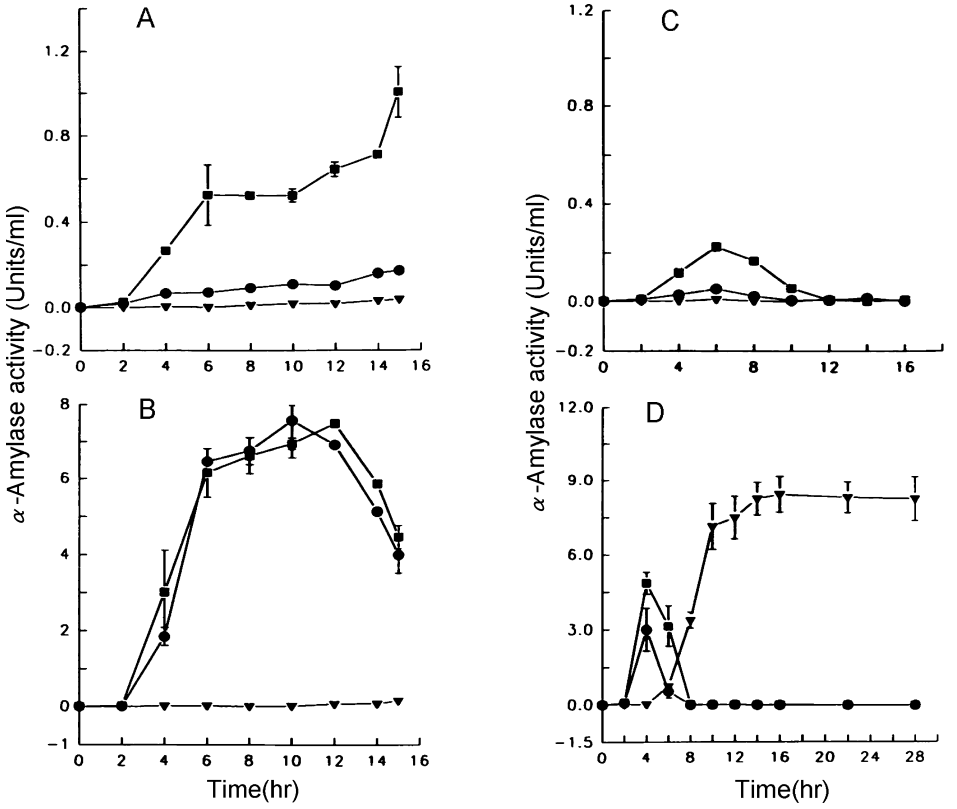

Fig. 2. Activity induction of extracellular α-amylase in bacteria cultured with various carbon nutrients.
Supplemented media are A. Bal; B. Starch; C. Glucose; and D. Maltose. Bacterial cells are wild type (●); S1c mutant (■); S2a mutant (▼).

Table 2. Distribution of α-amylase activity in the culture of *Alteromonas espejiana* Bal-31 during cell growth.

Cell growth:	6 h				16 h			
	α-Amylase activity (units/ml)	Intracellular fraction			α-Amylase activity (units/ml)	Intracellular fraction		
	Medium	α-Amylase (units/ml)	Protein (mg/ml)	Specific activity	Medium	α-Amylase (units/ml)	Protein (mg/ml)	Specific activity
Bal								
Wild	0.088	0.031	0.856	0.036	0.188	0.074	1.188	0.062
S2a	0	0.008	0.342	0.023	0.025	0.256	0.994	0.258
S1c	0.108	0.003	0.379	0.008	0.162	0.037	1.019	0.036
Starch								
Wild	10.92	0.091	0.841	0.108	0.647	0.008	1.521	0.005
S2a	0	0.011	0.356	0.031	0.026	0.351	0.379	0.926
S1c	11.85	0.053	0.57	0.093	1.336	0.026	1.992	0.013
Maltose								
Wild	0.455	0.003	0.688	0.004	0.004	0	0.379	—
S2a	0.893	0.043	2.009	0.021	11.54	0.454	0.4	1.135
S1c	0.273	0.002	0.573	0.003	0	0	0.275	—

Intracellular enzyme and protein were determined from the extract of washed cells. The data presented are the averages of duplicate experiments.

Genetic implication in the mutation

In order to ascertain whether the mechanism of de novo synthesis or the secretion of enzyme protein had a more important role in this induction, α-amylase induction in starch- and maltose-supplemented cultures was further analyzed. Cells grown for 6 and 16 h, representing early and late phases of enzyme induction, were selected to determine enzyme activity in the medium as well as enzyme activity and protein content in the cells (Table 2). Both wild type and S1c cells cultured in Bal medium showed very low enzyme activity in the cells. Almost no enzyme activity of either extra- or intracellular compartments was detected from the S2a mutant cultured in Bal or starch-supplemented medium for 6 h, but there was a significant amount of intracellular enzyme activity present in the cell at 16 h. The wild type and S1c mutant showed high extracellular enzyme activity of 10.92 and 11.85 units/ml at 6 h, but intracellular enzyme activity remained less than 0.1 units/ml, when grown in starch-supplemented cultures. In the maltose-supplemented culture of the S2a mutant, α-amylase activity was highly induced. The ratio of extra- and intracellular enzyme activities was 20.7- and 25.4-fold at the growth for 6 and 16 h respectively. However, enzyme activity was almost undetectable in the cells of wild type and S1c cultures grown for 16 h in maltose-supplemented medium. A significant amount of intracellular enzyme activity was only observed from the S2a mutant grown for 16 h in all three kinds of media. There is a distinct difference in enzyme activity between extra- and intracellular locations in the cultures surveyed, indicating that the enzyme secretion mechanism did not play an important role in the induction of extracellular α-amylase and was not affected by mutation.

It is generally known that cyclic AMP is an effective chemical involved in catabolic repression in the gram-negative bacteria, *Escherichia coli* (Epstein et al., 1975). To determine whether the secretion of α-amylase was involved in the catabolic system of the heterotrophic bacteria, *A. espejiana* Bal-31, cells grown in starch-supplemented medium were transferred to glucose-supplemented medium containing 3 mM cyclic AMP. Despite pre-treatment, the same inductive pattern with a 2 h lag phase was observed in fresh starch-supplemented medium. Addition of cyclic AMP was ineffective for enzyme induction of the cells cultured in glucose-supplemented medium (Fig. 3A). A similar result was observed for the S2a mutant grown in maltose-supplemented medium. Cyclic AMP was ineffec-

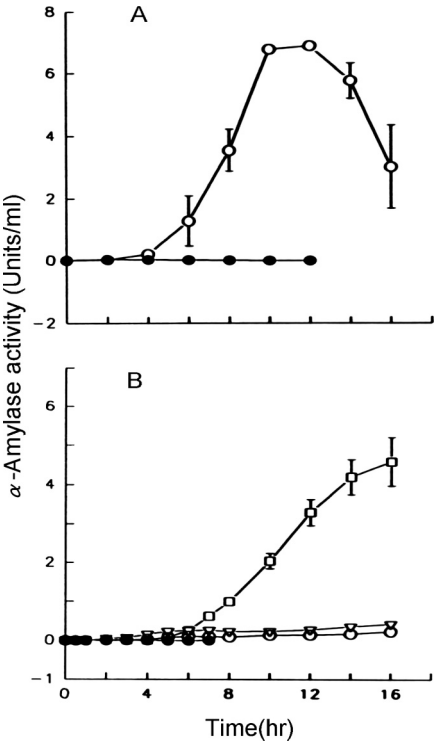

Fig. 3. Effect of cyclic AMP and tetracycline on the induction of extracellular α-amylase.

A. Washed wild type cells were further grown in the starch-supplemented medium (○) or glucose-supplemented medium containing 3 mM cyclic AMP (●), after growth for 5 h in starch-supplemented medium. B. Washed S2a cells were further grown in the maltose-supplemented medium (□); maltose-supplemented medium containing 0.1 mg/ml tetracycline (○); Bal medium containing 3 mM cyclic AMP (▽); or glucose-supplemented medium containing 3 mM cyclic AMP (●), after growth for 10 h in maltose-supplemented medium.

tive both in Bal and glucose-supplemented media (Fig. 3B). However, α-amylase induction of wild type cells grown in starch-supplemented medium as well as of the S2a mutant grown in maltose-supplemented medium were abolished by treatment with tetracycline, indicating that protein synthesis was required for enzyme induction. Activity of extracellular α-amylase in wild type cells cultured in starch-supplemented medium decreased rapidly after growth for 12 h. To investigate the capacity of extracellular protein degradation, protease activity was determined in either starch or maltose media. Negligible activity was observed in starch media of both S1c and S2a mutants throughout the culture period. Significant activity was seen in the wild type culture after 12 h of growth (Fig. 4A). This activity correlated well with the decay of α-amylase activity. In the maltose medium only the S2a culture

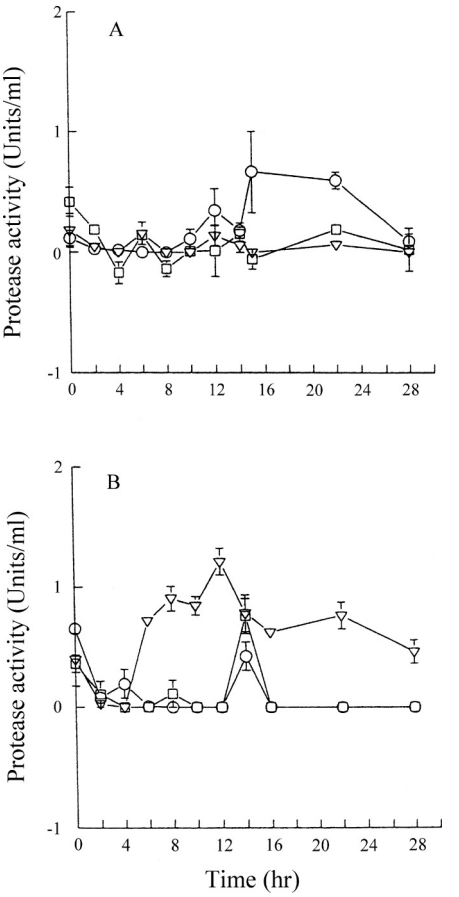

Fig. 4. Activity of extracellular protease in starch (A) or maltose (B) media of bacterial cultures.

Wild type (○); S2a (▽); or S1c (□).

showed increased protease activity after 6 h of growth. It also maintained a significant level of enzyme activity (Fig. 4B). Therefore, we suggest that molecular stability of α-amylase excreted from wild type and S2a cells was quite different.

Discussion

Heterotrophic bacteria are strictly dependent on nutrients from the environment to maintain their lives. In the natural habitat of *A. espejiana* Bal-31, polysaccharides are the dominant form of carbohydrate and an active component of the carbon cycle in the near ocean surface (Pakulski and Benner, 1994). Extracellular enzymes are necessary for bacterial feeding because most utilizable organic carbon has to be hydrolyzed into lower molecular compounds before uptake and metabolism (Amon and Benner, 1996). In general, bacteria respond to changing nutrient conditions by regulating the synthesis and activity of their

enzymes (Gotschalk, 1985). Vetter et al. (1998) proposed a model of bacteria foraging by attached or released extracellular enzymes to predict the contribution of these enzymes to particulate organic carbon hydrolysis. The cells of *A. espejiana* are monotrichous and mobile. In marine habitats, foraging bacteria actively search for particulate organic carbon. This organic carbon is then hydrolyzed by release of extracellular enzymes to fulfill the need for a carbon source. In the present study, we found that the halophilic nature of *A. espejiana* Bal-31 resulted in successful growth on the starch-supplemented medium. Extracellular α-amylase was induced after a 2 h lag period and enzyme activity decreased rapidly after 12 h, by which time the starch was mostly hydrolyzed. Therefore, *A. espejiana* Bal-31 adapts itself well to the circumstance in which it originally lived.

Cell growth was strongly inhibited by the addition of sucrose, indicating that cells might be defective in the utilization capacity of either transport or catabolic enzyme systems. Furthermore, the presentation of sucrose could increase osmolarity leading to unfavorable conditions for cell growth. Glucose or one of its metabolites acts as a repressor of respiration. In microorganisms, several enzymes are affected by glucose repression, although the degrees of repression are altered among organisms (Fiecher et al., 1981). Growth of *A. espejiana* Bal-31 was partially inhibited by the addition of 1% glucose in Bal medium. The effect may have led the cells to reach the stationary phase earlier. It is well established that the catabolite repression in *E. coli* is mediated by glucose via lowering the cyclic AMP level. When the level of cyclic AMP/CRP is increased, the expression of a series of genes is activated (De Crombrugghe et al., 1984; Pastan and Adhya, 1976). However, cyclic AMP did not relieve the repression of α-amylase excretion in the presence of glucose, indicating that cyclic AMP/CRP might not be involved in the α-amylase gene expression of *A. espejiana* Bal-31. In the presence of both starch and glucose for the wild type and of both maltose and glucose for the S2a mutant, the induction of extracellular α-amylase was completely abolished (Lee and Tong, unpublished data).

Maltose regulon in *E. coli* consists of four operons that encode the proteins required for the transport and metabolism of maltose and maltodextrin, and has been recently reviewed in detail by Boos and Shuman (1998). A similar regulation of gene expression on the utilization of carbon nutrients has also been reported in the gram-positive soil bacteria, *Streptomyces* species (Nguyen et al., 1997; Virolle and Gagnat, 1994). Unfortunately, there has been little information concerning the genetic and physiological mechanism of carbon nutrient utilization in the heterotrophic marine bacterium, *A. espejiana* Bal-31. The wild type strain of *E. coli* could not grow in medium with added starch, whilst the starch-utilizing mutants were well adapted and were induced to synthesize intracellular α-amylase in maltose as well as starch-supplemented media (Shibuya et al., 1986). On the other hand, *A. espejiana* Bal-31 grew well and extracellular α-amylase was induced in starch-supplemented medium, whilst the S2a mutant grew well both in maltose and starch added media; however, induction of extracellular α-amylase occurred in maltose rather than in starch-supplemented medium. These opposing characteristics of bacteria indicate that the utilization of carbon nutrients of *A. espejiana* Bal-31 is most likely to be an ecological adaptation to its marine habitat after having undergone a long period of evolutionary diversity from enteric bacteria.

A. espejiana Bal-31 grew well and was induced to secrete α-amylase in starch-supplemented medium, indicating that it has a utilization system for exogenous polysaccharides. However, the S2a mutant grew well in both maltose- and starch-supplemented media. Secretion of α-amylase was induced by the addition of maltose, but not by starch. The pattern of α-amylase induction in the wild type cell was obviously different from that in the S2a mutant. The lag phases of induction were 2 and 4 h for starch and maltose respectively, indicating different processes of gene expression. Induction of enzyme activity in both the wild type and the S2a mutant were abolished by tetracycline treatment. Furthermore, extracellular protease activity in wild types cultured in starch increased after 12 h of cell growth. This increase corresponded to a decrease in α-amylase activity. The protease activity increase with the activity of α-amylase, however, was shown in S2a cultured in maltose. This indicated differences in stability of enzyme molecules produced against proteolysis by wild type and S2a mutants. Therefore, we suggest that those two α-amylases might be encoded from different genes. A similar situation has been reported in *E. coli* (Raha et al., 1992; Schneider et al., 1992). An α-amylase gene, *amyA,* lies outside the known maltose regulon and lacks the *malT* box in its

upstream region with the result that both *amyA* and *malS* in the maltose regulon are regulated differentially.

We do not have direct evidence of the alternative regulation of those two α-amylase genes, including the effect of mutation on gene expression. However, shifting the inducers from starch to maltose and changing the stability of enzyme protein were unlikely to be triggered by a single point mutation, unless the mutation occurred in a regulatory sequence affecting more than one α-amylase gene. Comparing the gene sequences of α-amylases of *A. espejiana* Bal-31 with the S2a mutant might provide some information to understand the molecular mechanism of mutation. Thus, gene cloning is proceeding. Furthermore, S2a mutant cells grow well in the presence of maltose and are induced to secrete a large amount of stable α-amylase, which may be of industrial interest.

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