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Growth and Polyamine Production of *Alteromonas spp* in Fish Meat Extracts Under Modified Atmosphere

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Abstract

Both marine and terrestrial types of *Alteromonas spp* were studied for their growth and polyamine production in fish meat extacts (FME). Growth of the marine type strain (Alt S5B) was inhibited in a carbon dioxide atmosphere, whereas the terrestrial strain (Alt S29) was not. Differing polyamine productivity was observed in each strain of *Alteromonas spp* in both pollock and squid FME's while under various gas conditions. It suggested that properties of polyamine production in both strains of *Alteromonas spp* are altered by environmental conditions such as free amino acid compositions and atmosphere.

Introduction

Alteromonas (formerly included into *Pseudomonas* III/IV) (Horie, et al., 1972, Okuzumi, et al., 1972) has been implicated in the spoilage of various marine fishes (Horie, et al., 1972, Okuzumi, et al., 1972, Shewan, et al., 1960). This is in spite of the fact that the microflora present in the process of putrefaction experiences various changes. From a food hygiene point of view, it is important to control the growth of putrefactive microorganisms.

Recently, there have been many reports on modified atmosphere (MA) as a useful approach in preventing putrefaction or for extending shelf life of foods (Brown, et al., 1980, Layrise and Matches, 1984, Parkin, et al., 1981, Straham and Bremmer, 1989, Wang and Ogrydziak, 1986). However, suitable conditions of MA for various marine foods have not yet been established. Straham and Bremmer (1989) have recommended an atmosphere of 100% carbon dioxide or mixed gas (carbon dioxide : nitrogen : oxygen = 40 : 30 : 30) as an MA for fish fillet. To develop suitable MA conditions for marine foods, it is necessary to understand the susceptivility of putrefactive bacteria to various gasses.

We have focused on polyamines of marine putrefactive bacteria because of their values as an index for evaluating marine food freshness (Mietz and Karmas, 1978, Yamanaka, et al., 1987, Yamanaka, et al., 1986) and their importance in regulation of life for most organisms. On this basis, we believe information on biochemical properties of bacterial polyamines and its control should be useful from both the view point of developing the control systems of microorganisms, and in studying fundamental biochemical bacterial functions.

It has already been found that halophilic and non-halophilic *Alteromonas spp* have different properties in polyamine production (Matsui, et al., 1989, Suzuki, et al.,

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1988). We report herein the inhibiting effect of nitrogen and carbon dioxide gasses against growth and polyamine production of *Alteromonas spp* in extracts of pollock and squid meat.

Materials and Methods

1. *Bacteria and Its Culture*

The non-halophilic *Alteromonas* strain S29 (Alt S29) and the halophilic *Alteromonas* strain S5B (Alt S5B) were graciously provided by Professor Masayo Okuzumi, Tokyo University of Fisheries. In this laboratory, both strains were examined for their GC mole percentages (%) to confirm their classification status. They displayed 46% (Alt S29) and 38% (Alt S5B) in their GC mole %, respectively. The result supports the decision that both strains should be classified as genus *Alteromonas*. Culture conditions for both strains were reported previously (Matsui, et al., 1989).

2. *Preparation of Fish Meat Extract (FME)*

Frozen walleye pollock (*Theragra chalcogramma*) and short-finned squid (*Illex argentinus*) were obtaine commercially and used as materials. Thawed flesh was homogenised in 3- or 4-fold volumes of 1% NaCl solution. This homogenate was centrifuged at 3,000 rpm for 10 min. Supernatant was then filtrated through membrane filter (0.22 μm pore size). This was used as FME for the culture. Salt concentration of each FME was adjusted to 1% and 4% with NaCl solution before use. Into each test tubes, 4.95 ml of FME was transferred. The test tubes were then completely sealed after bacterial inoculation. The pH of pollock-FME was 6.92 and that of squid-FME was 6.33.

3. *Culture in the FME*

The bacterial cells were washed with a phosphate buffered saline (PBS) and were appropriately suspended with the solution. Following this, 0.05 ml of these cells were inoculated into the test tubes with FME. The head space of the tube (21 ml) was then completely replaced with nitrogen or carbon dioxide. The gas was injected with a needle after passing through a membrane filter (0.22 μm). All cultures in this study were incubated at 25°C. Viable cell counts in the cultures were examined with time using the plate count method.

4. *Quantitation of Nitrogen and Amino Acids in FME*

Prior to culturing, the presence of nitrogen and amino acids in each FME were determined. Nitrogen content was measured by the micro-Kjeldahl method. FME protein was precipitated with 5% (w/v) trichloroacetic acid (TCA). Both supernatant and precipitation were subjected to quantitation of nitrogen content. Amino acids in the supernatant of the TCA extract were analyzed with an amino acid analyzer (Hitachi 835, Hitachi Manufacturing Co., Tokyo, Japan) located in the Centre for Instrument Analysis, Hokkaido University.

5. *Quantitation of Polyamine*

Cultures were harvested with time series' and added to the TCA solution. The TCA soluble material was subjected to high performance liquid chromatography (HPLC) after passing through a membrane filter (0.22 μm). The separation was carried out by reverse-phase chromatography on a Shim-Pack CLC-ODS column (Shimadzu Co., Kyoto, Japan). Post-column labeling reaction of amines with o-phtalaldehyde reagent was done through a teflon coil. Details of these analyses conditions were reported elsewhere (Suzuki, et al., 1990).

Table 1. Content of total nitrogen (T-N), non-protein nitrogen (NP-N) and protein nitrogen (P-N) in extracts of pollock and squid.

	T-N (μg/ml)	NP-N (μg/ml)	P-N (μg/ml)
Pollock	251	60	191
Squid	540	323	217

Table 2. Free amino acid composition in extracts of pollock and squid.

	Content (nmol/ml)	
Amino acid	Pollock (pH 6.92)	Squid (pH 6.33)
Asp	4.20	26.88
Thr	20.38	106.63
Ser	19.61	75.48
Glu	16.51	64.90
Gly	74.72	118.49
Ala	91.61	297.98
Cys/2	17.14	9.84
Val	16.22	57.84
Met	2.74	33.22
Ile	5.88	32.40
Leu	10.51	65.18
Tyr	nd*	15.29
Phe	nd	22.15
Lys	24.91	56.35
His	70.66	44.30
Arg	4.92	469.63
Pro	12.79	2084.59
Tau	155.04	1642.25
Orn	1.06	14.23
NH_3	178.15	211.66

* nd = not detected

Results and Discussion

1. *Nitrogen and Free Amino Acids in FME*

Nitrogen contents are shown in Table 1. Pollock-FME contained much more protein-nitrogen compared with non-protein-nitrogen. Opposite results were obtained from squid-FME. The total amount of nitrogen in the squid-FME was 540 μg/ml. This was 2-fold higher than that of pollock-FME. Data analysis of amino acids in each FME are summarized in Table 2. Ornithine and arginine, precursors of putrescine (Put) in prokaryote, were found to occur in much higher amounts in squid-FME. For example, the presense of arginine in squid-FME was approximately 100-fold higher than that observed in pollock-FME. Pollock-FME contained significant levels of histidine which is precursor of histamine (Htm). In contrast to pollock-FME, a noteworthy property of squid-FME was its high proline content.

2. *Growth and Polyamine Production of Alt S5B*

Growth and polyamine production of Alt S5B in pollock-FME are shown in Fig. 1. Growth of this strain was observed in the media containing 4% NaCl under atmosphere conditions using carbon dioxide (Fig. 1B) and nitrogen (Fig. 1D). However, its growth was not observed under conditions using carbon dioxide (Fig. 1E, F). The inhibition of growth under these conditions for Alt S5B results from the presence of carbon dioxide. Carbon dioxide is known to inhibit a growth of putrefactive bacteria (Brown, et al., 1980, Parkin, et al., 1981, Straham, et al., 1989, Wang and Ogrydziak, 1986). In addition, no growth of Alt S5B was observed in the media containing 1% NaCl. This confirms Alt S5B is halophilic. When the

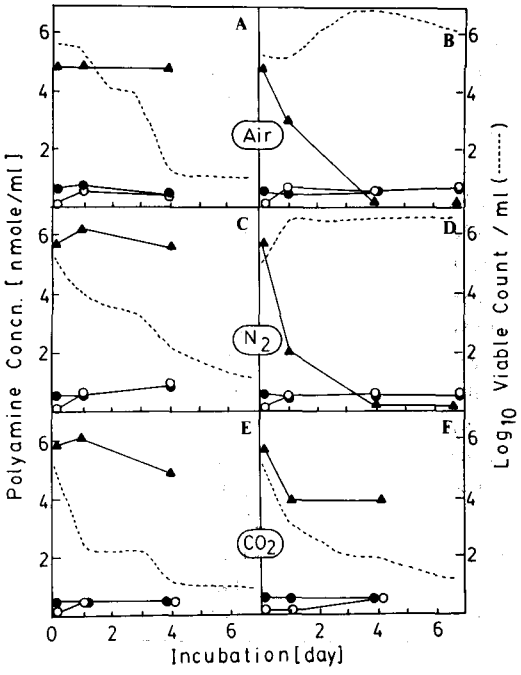

Fig. 1. Growth and polyamine production of Alt S5B in pollock-FME. A: With 1% NaCl under air, B: with 4% NaCl under air, C: with 1% NaCl under nitrogen, D: with 4% NaCl under nitrogen, E: with 1% NaCl under carbon dioxide and F: with 4% NaCl under carbon dioxide. Symbols, ●—●: putrescine, ▲—▲: cadaverine and ○—○: spermidine. Histamine and agmatine were not detected throughout incubation period.

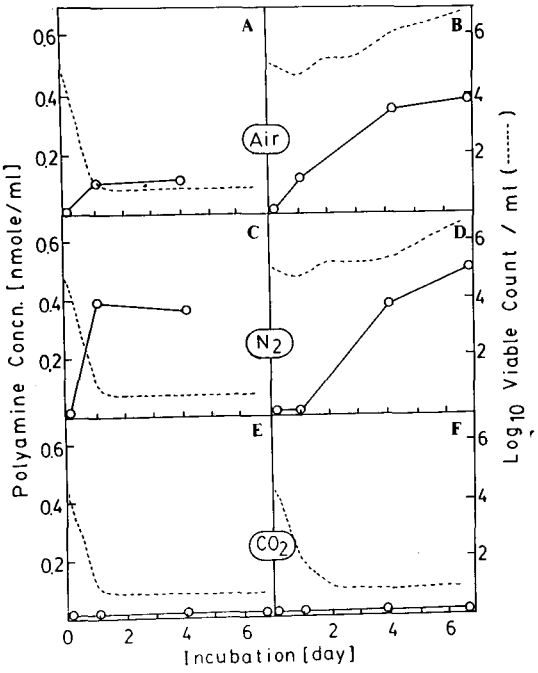

Fig. 2. Growth and polyamine production of Alt S5B in squid-FME. Culture conditions of A to F are the same as for Fig. 1. Symbols, ○—○ : spermidine. Other polyamines were not detected throughout incubation period.

bacteria grew, spermidine (Spd) was produced at the concentration of 0.5-0.7 nmol/ml but cadaverine (Cad) decreased (Fig. 1B, D). However, in the cases where bacteria did not grow, changes of Cad content were negligible (Fig. 2A, C, E, F). This suggests that Alt S5B consumed or decomposed Cad that was contained in pollock-FME. Results in squid-FME are shown in Fig. 2. Alt S5B could grow in squid-FME containing 4% NaCl under air and nitrogen atmosphere conditions. This is also the case for pollock-FME although growth was much slower (Fig. 2B, D). A0.4-0.5 nmol/ml of Spd was produced in media paralleling the bacterial growth. However, rapid productuion of Spd was observed when bacteria were inoculated into the squid-FME containing 1% NaCl but did not grow. This might be explained by the fact that salt depletion caused quick synthesis of Spd and the Spd could compensate for the lack of salt. Increases of Put was not observed in either pollock- or squid-FMEs.

3. *Growth and Polyamine Production of Alt S29*

Growth and polyamine production of Alt S29 in pollock-FME are shown in Fig. 3, and those for squid-FME are shown in Fig. 4. Alt S29, a non-holophile, grew in all culture conditions tested even under carbon dioxide. However, the growth in both FMEs in the presence of 4% NaCl in an atmosphere of carbon dioxide (Fig. 3F, 4F) were delayed compared to other conditions. This growth delay might be caused by the carbon dioxide effect as mentioned with respect to Alt S5B. Concerning the polyamine production of Alt S29 in pollock-FME, Put was produced in all experiments in concentrations between 2-5 nmol/ml. This strain gradually produced Htm after reaching a stationary phase. However, this was not found in the FME

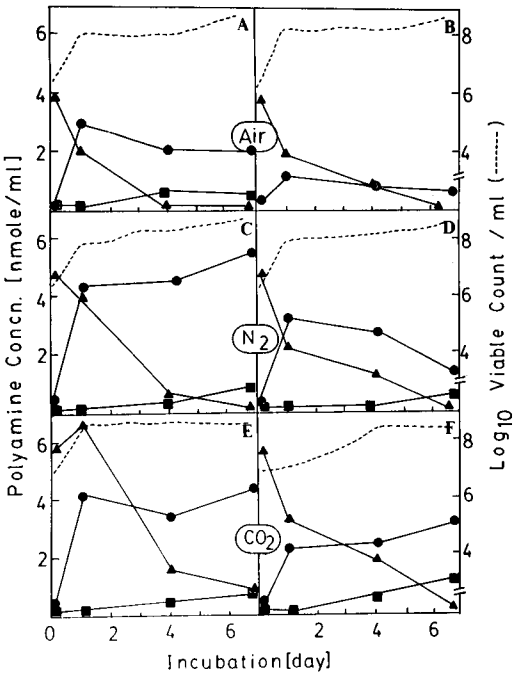

Fig. 3. Growth and polyamine production of Alt S29 in pollock-FME. Culture conditions of A to F are the same as for Fig. 1. Symbols, ●—●: putrescine, ▲—▲: cadaverine and ■—■: histamine. Spermidine and agmatine were not detected throughout incubation period.

containing 4% NaCl under air. A decrease of Cad was observed in pollock-FME (Fig. 3). This was similar to the case of Alt S5B in pollock-FME. Growth profiles of Alt S29 in squid-FME (Fig. 4) were similar to those in pollock-FME. With exception to the pollock-FME, agmatine (Agm) production, under all atmospheres, was observed in the amounts between 0.6–2.6 nmol/ml. This Agm should be derived from arginine contained in squid-FME. Significant amounts of Put (85 nmol/ml) were detected in samples under air condition (Fig. 4A). This phenomenon was found 1 day after inoculation and then decreased rappidly. Such a large amount of Put production was not observed under the nitrogen (Fig. 4C) or carbon dioxide atmosphere conditions (Fig. 4E). Understandably, this study has not completely explained the differing production profiles of Put under various gas atmospheres. However, it is postulated that Alt S29 could not synthesized Put or could not release it under nitrogen and carbon dioxide atmosphere. It has been known that Put is converted from ornithine by ornithine decarboxylase (Pegg, 1986, Pegg and Willium-Ashman, 1981, Tabor and Tabor, 1985). Another pathway is also known in bacteria (Pegg and Willium-Ashman, 1981, Tabor and Tabor, 1985), that is, Put can be converted from Agm by the combined action of arginine decarboxylase and agmatine ureohydlorase. In the Alt S29 strain, Put production in squid-FME was much higher than in pollock-FME. Arginine content in squid-FME was much more than pollock-FME. Therefore, it is reasonable to postulate that the source of Put biosynthesis in Alt S29 would be mainly from Agm, and the intermediate of Agm might have a short half life. However, for both strains tested in this study, the mechanism for maintaining the polyamine content is unknown. It was found that

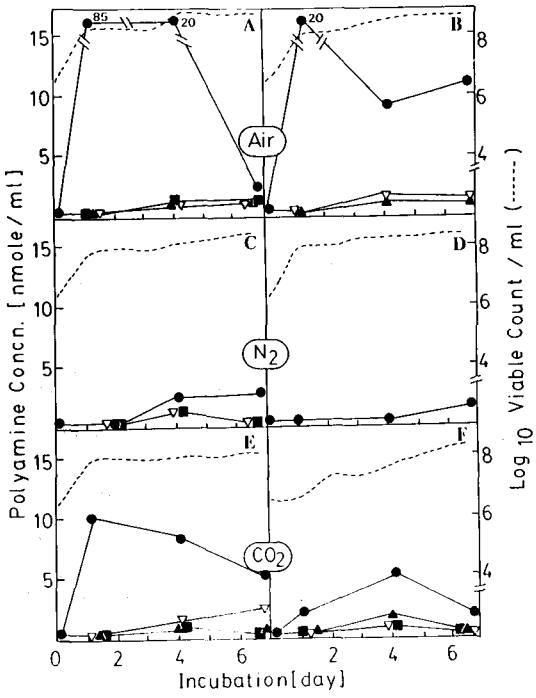

Fig. 4. Growth and polyamine production of Alt S29 in squid-FME. Culture conditions of A to F are the same as for Fig. 1. Symbols, ●—●: putrescine, ▲—▲: cadaverine, ▽—▽: agmatine and ■—■: histamine. Spermidine was not detected throughout incubation period.

both the media and atmosphere affected the growth and polyamine production in these two strains of *Alteromonas spp.* This study focused on the biosynthesis pathway(s) of polyamines in the bacteria tested. Physiological roles of polyamine in bacterial cells are reported to enhance DNA, RNA and protein synthesis (Pegg, 1986, Tabor and Tabor, 1985), and to stabilize cell membrane (Kamio and Nakamura, 1987, Kamio, et al., 1986, Souzu, 1986). The role of polyamines in membrane proteins of Alt S5B and S29 are of interest, because the various atmospheres could directly affect the membrane. To clarify these questions, further investigations are now underway.

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