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光陽灣에서 分離된 새로운 好鹽性 *Azotobacter insignis* 菌株에 關하여

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A new Salt requiring Strain of *Azotobacter insignis* isolated from Kwangyang Bay

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ABSTRACT

A strain of *Azotobacter* species was isolated from the surface sea water of Kwangyang Bay in Korea and was identified as *Azotobacter insignis*. In order to reveal the natural habitat of this microorganism, growth at various salt concentrations was tested with the result of 3% NaCl being optimum. Only slight growth was detected in the absence of NaCl.

This result was thought to prove (in part) that the natural habitat of the strain dealt with is sea water. Fairly good growth was obtained at 6% NaCl concentration.

The study of effects of salt on the growth of this strain to various temperatures and *p*Hs has shown that temperature 30°C and *p*H 7.0 are optimum.

INTRODUCTION

Many other workers have worked on the distribution of *Azotobacter* in soil (Rovira, 1956; Anderson, 1958 a and b; Strzelczyk and Katznelson, 1961; Ruinen, 1970; Hong and Choi, 1974b). Still others have tried to isolate this microorganism from aquatic environments including fresh water, estuarine water, and sea water (Pshenin, 1963; Wood, 1965; Norris and Chapman, 1968; Hong and Choi 1974b).

These works have revealed that *Azotobacter* is distributed widely in soil and fresh water, although this microorganism requires some special environmental conditions like *p*H and moisture (Anderson, 1958 b).

It was also reported that *Azotobacter* can be isolated in all depths of the sea and sediment in the Black Sea.

Hong and Choi (1974 b) also could isolate *Azotobacter* from surface sea water in Korea.

On the other hand there is other report that no *Azotobacter* could be isolated from marine scurces (Wood, 1965) including the estuarine environment and oceanic water at a number of depths to 10,000m.

This worker, moreover, expressed his doubt whether Pshenin's isolates were true *Azotobacter* or not indicating the peculiar environment from where Pshenin claimed to have isolated some *Azotobacter* species.

Hong and Choi (1974 b) themselves are

also doubtful about the origin of their *Azotobacters* because of the reason that samples were collected from the surface sea water at only 1 km away from land, where large influence of fresh water is expected.

Except these reports, it is quite difficult to find other reports dealing with the distribution of *Azotobacter* in the sea water.

Thus, some attempts were made to isolate and identify *Azotobacter* from the sea.

The salt requiring strain of isolated *Azotobacter* was tested in order to confirm, though partially, the natural habitat of this microorganism. It's ability of nitrogen fixation was also examined.

MATERIALS AND METHODS

1. Collection of sea water in Kwangyang Bay

Surface sea water was collected in Kwangyang Bay every other month from May, 1974 to March, 1975. Collected sea water was stored in sterilized cap tubes and transported to the laboratory in the ice box.

2. Isolation of *Azotobacter*.

N-free medium (Brown *et al.*, 1962) containing glucose 5g, $MgSO_4 \cdot 7H_2O$ 0.2g, $FeSO_4 \cdot 7H_2O$ 0.04g, Na_2MoO_4 0.005g, $CaCl_2$ 0.15g, K_2HPO_4 0.8g (separate autoclave), agar 15g and dist. water 1,000ml *p*H 7.0±0.5) was used for the isolation of *Azotobacter* with the addition of 30g NaCl. 0.1ml of diluted sea water sample was inoculated by spreading method on the agar plate containing above medium.

Each colony of which the diameter was more than 2mm after 5-day cultivation of 30°C was isolated and purified by repeated streaking on the same media. Pure culture was maintained on the slopes of Norris medium (Norris & Chapman, 1968), containing additional 30g NaCl per liter which was subcultured every month. Norris medium is composed of glucose 10g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.2g, $CaCO_3$ 1g, NaCl 0.2g, $Na_2MoO_4 \cdot 2H_2O$ 0.005g and agar 20g (*p*H 7.0±0.5) in 1 liter of dist. water. They were always incubated for 5 days at 30°C and then stored at 5°C.

3. Identification of isolated bacteria.

Isolated bacteria was identified after the methods of Hong and Choi (1974a). Morphological characteristics such as cell size, form, colonial appearance were observed. Motility was examined in the hanging drop preparations. Flagella were observed by Leifson's staining method (1958). Microcyst formation was induced with butanol treatment (0.3% butanol to the medium instead of glucose) by the method of Layne and Johnson (1964) and microcysts were observed by Vela and Wyss (1966) staining method. Pigmentation and Gram staining were also observed by routine methods.

Physiological tests such as litmus milk test, gelatin liquefaction, catalase excretion, Methyl Red and Voges-Proskauer test, indole test, growth on starch medium and carbohydrate fermentation were made by routine methods.

4. Effects of temperature, *p*H and salt concentrations on the isolated bacterium.

Salt tolerance of *A. insignis* isolated was investigated. As control species, *A. insignis*, *A. agilis* and *A. vinelandii* isolated from soil were used. The basal medium was a N-free transparent Burk's type medium (Shuler and Tsuchiya, 1975);

KH_2PO_4 0.41g, Na_2SO_4 0.05g, $CaCl_2$ 0.2g, $MgSO_4 \cdot 7H_2O$ 0.1g, $FeSO_4 \cdot 7H_2O$ 0.005g, $Na_2MoO_4 \cdot 2H_2O$ 0.00025g, dextrose 10g and K_2HPO_4 0.52g (separate autoclave) in distilled water 1,000m*l* (*p*H 7.0±0.5). NaCl concentration levels were 0%, 3%, 6%, 9% and 12%. Each strain was cultured with shaking for 5 days at 30°C in 500m*l* flask containing 100m*l* of Burk's type broth with indicated amounts of NaCl.

At each NaCl concentration level, the effects of *p*H(5.5, 6.5, 7.5 and 8.5) and temperature (20°C and 30°C) on growth of *A. insignis* isolated were measured.

Inoculum was prepared as follows. Two loopfuls of bacteria grown on the maintenance agar slope were inoculated in 500m*l* flask containing 100m*l* Burk's type broth and cultured at 30°C for one day. 0.1m*l* of this was used as inoculum in each case.

Growth of the cells was determined by measuring transmittance at 660nm, using Turner Spectrophotometer (Model 350).

5. Nitrogen fixation of bacteria at various salt concentrations.

A. insignis isolated from Kwangyang Bay and *A. insignis* isolated from soil were inoculated in 500m*l* flask containing 100m*l* Burk's type broth and cultured for 5 days at 30°C. After cultivation, cells were harvested at 5,000 rpm with centrifuge. Protein contents were measured by the semi micro Kjeldahl method.

RESULTS AND DISCUSSION.

1. Isolation and identification of *A. insignis* from Kwangyang Bay.

Twenty eight *Azotobacter* like strains were isolated after various morphological and physiological examinations. As a result, one strain among them was identified as *A. insignis*. Table 1. shows the result of the identification of *A. insignis*.

Characteristics to distinguish the *Azotobacters* from other bacteria are their relatively large cell sizes, motility and morphology, gram-negativeness, nitrogen fixing ability, strict aerobe and catalase positiveness (Bergey's manual, 1974).

Among the Genus *Azotobacter*, the important characters to distinguish *A. insignis* from other species are non-microcyst formation, polar flagellation and light brown insoluble and green soluble pigmentation (Norris and Chapman, 1968), and other biochemical reactions. One strain showed these characteristics and so was identified as *A. insignis*.

2. Salt requirement of *A. insignis* isolated from Kwangyang Bay.

A. insignis isolated grew best in the medium containing 3% NaCl (Fig. 1). The period of lag phase was the shortest and the maximum yield amounted to 5% of transmittance after 5-day culture. In the medium containing 6% NaCl concentration, it grew to 20% transmittance and lag phase became longer than in the medium containing 3% NaCl. Growth of the cells was decreased and the length of lag phase was increased as NaCl concentration increased. In the absence of NaCl, *A. insignis* isolated grew very slow and the maximum yield amounted to 85% of transmittance. Thus, 3% was considered to be the optimum NaCl concentration for this strain, which suggests that this is a marine-borne microorganism. The control species, *A. insignis*, *A. agilis* and *A. vinelandii* from soil showed similar growth patterns at various salt concentrations (Fig. 2, 3 and 4).

Fustec-Mathon *et al.* (1970) reported that a salt sensitive upper beach strain, a salt

Table 1. Morphological and physiological characteristics of the bacterium isolated in Kwangyang Bay.

Characteristics	N-fixing bact.	*A. insignis**
Shape	ovoid	ovoid
Size	1.8×10	1.7×1.1
Gram stain	−	−
Pigment	soluble green and insoluble light brown	soluble green and insoluble light brown
Flagella	polar	polar
Microcyst	−	−
Colony	circular raised entire	circular raised entire
Growth on starch medium	−	−
Gelatin liquefaction	+	+
Catalase	+	+
Indole	+	+
Methyl-Red	+	+
Voges-Proskauer	+	−
Litmus milk		
Color	DE	DE
Coagulation	−	−
Rhamnose utilization	+	+
Carbohydrate fermentation		
Mannitol	Ac(−)	Ac(−)
Saccharose	Ac(−)	Ac(−)
Dextrose	Ac(−)	Ac(−)

abbreviation: DE=decolorization of milk, Ac(−)=alkaline product, no gas
*=isolated from soil of Korea by Y.K. Choi

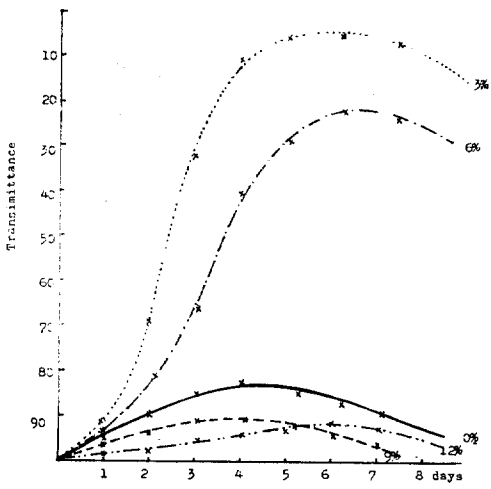

Fig. 1. Effect of salt at various concentrations on growth of *A. insignis* isolated at 30°C

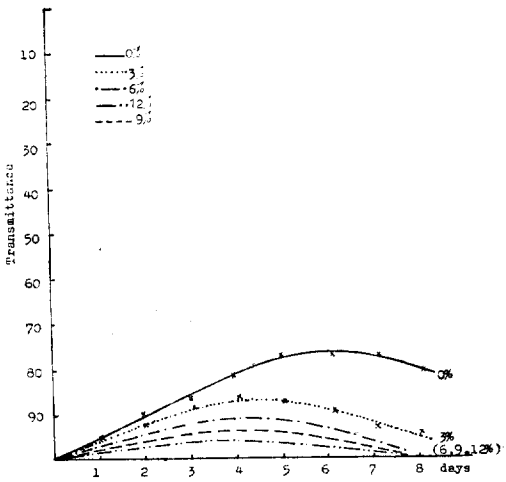

Fig. 2. Effect of salt at various concentrations on growth of *A. insignis* at 30°C

tolerant lower beach strain and halophilic strains were isolated from soils closely influenced by sea water. They also suggested the possibility of adaptation of these strains to salt.

In the present study, the results show that *A. insignis* from sea is rather a salt requiring microorganism than a salt-tolerant one regarding the fact that the growth of this bacterium was best when the salt concentration of the culture medium resembled that of the sea water. It showed fairly good growth at 6% NaCl concentration and only in this respect, it can be called a salt tolerant bacterium. Within the limit of present results, it is not clear whether the salt requirement and/or salt-

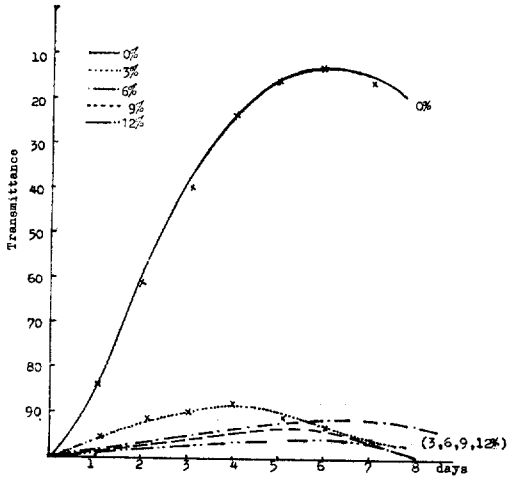

Fig. 3. Effect of salt at various concentrations of *A. agilis* at 30°C

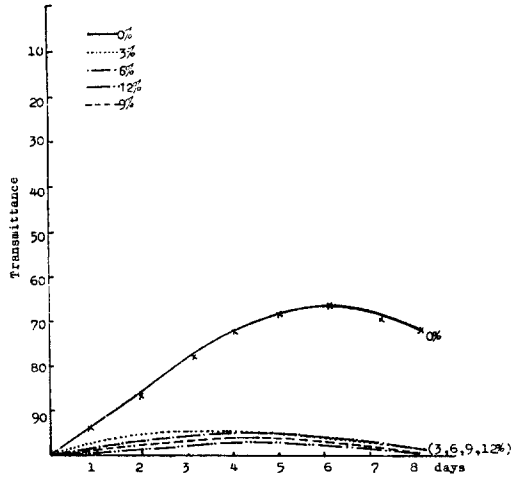

Fig. 4. Effect of salt at various concentrations on growth of *A. vinelandii* at 30°C

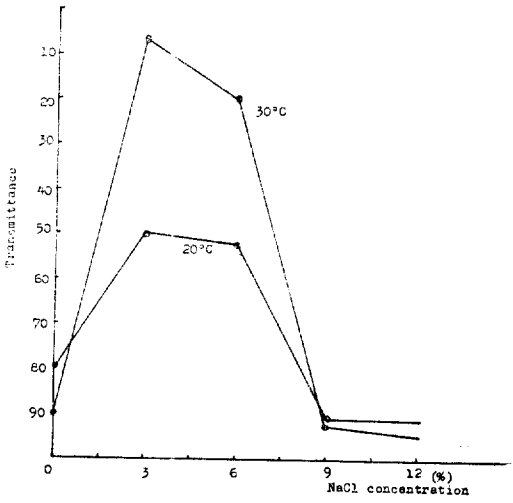

Fig. 5. Effect of temperature on growth of *A. insignis* isolated

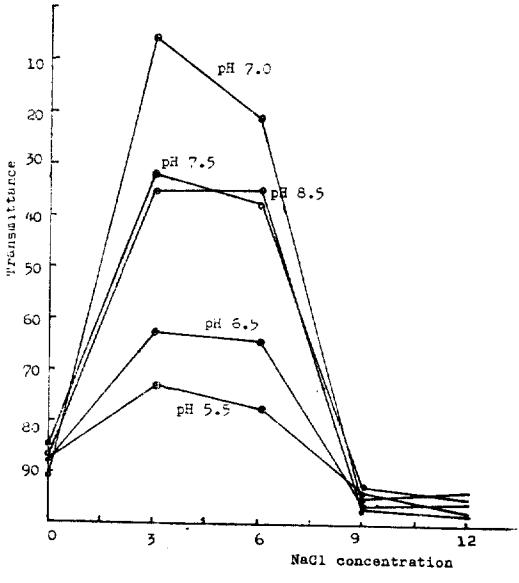

Fig. 6. Effect of *p*H on growth of *A. insignis* isolated

tolerance of this strain is because of adaptation or not. But the only thing certain is that the natural habitat of this strain is the sea water.

Such a fact is strongly supported by its poor (almost negligible) growth at NaCl-free culture condition.

At 30°C, *A. insignis* strain grew better than at 20°C but its growth pattern at both temperatures was similar at various salt concentrations. In medium containing 3% NaCl, the growth was best and in 9% and 12% NaCl concentration, the growth was slight (Fig.5). Considering the fact that optimum temperature of *Azotobacter* is 30°C in general, above results are the ones well expected.

A. insignis grew better at neutral or slightly alkaline(*p*H 7.5 and *p*H 8.5) than at *p*H 5.5 or 6.5 (Fig.6). Best growth occurred in *p*H 7.0. This coincides well with the observation that members of the genus *Azotobacter* usually prefer neutral or slightly alkaline conditions (Anderson, 1958b).

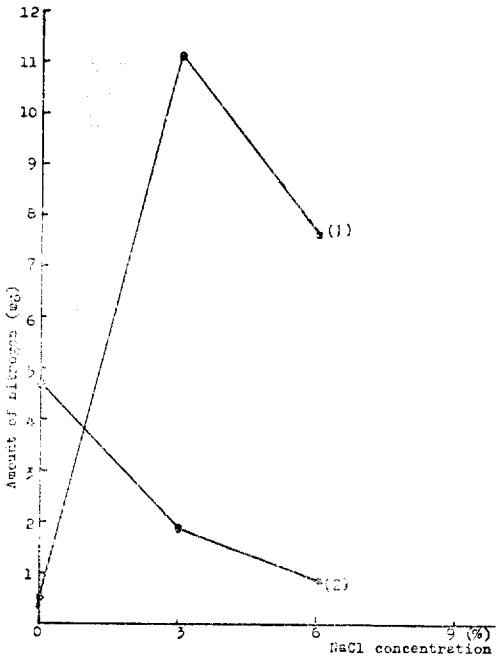

Fig. 7. Amount of nitrogen fixed by *A.insignis* isolated (1) and *A. insignis* in soil (2) at 0%, 3% and 6% NaCl concentrations.

3. Nitrogen fixation.

There is differential effect of increasing salinity on nitrogen fixation of two *A. insignis* strains (Fig. 7 and Table 2).

Table 2. Amounts of nitrogen fixed by *A. insignis* in K.Y. Bay and *A. insignis* in soil at 0%, 3% and 6% NaCl concentrations.

NaCl concentrations \ Strains.	*A. insignis* isolated in Kwangyang Bay	*A. insignis* isolated in soil by Y.K. Choi
0%	0.56mg	4.76mg
3%	11.06mg	1.96mg
6%	7.56mg	0.84mg

In case of *A. insignis* strain isolated from Kwangyang Bay, 11.6mg nitrogen was fixed, after 5-day culture at 30°C in the presence of 3% NaCl, indicating nearly 160 times as much increase as the initial nitrogen content. As was expected from the results of growth test, the amount of nitrogen fixation became less at higher concentrations. At 6% NaCl concentration, 7.56mg nitrogen was fixed. When no NaCl was added, only slight increase (0.56mg) in fixed nitrogen could be recognized.

In case of *A. insignis* from soil, nitrogen fixation was best at NaCl free condition (4.76mg). When 3% and 6% NaCl were added, only slight increase could be detected. Comparing two strains at their best nitrogen fixing conditions, the marine-originated one showed much higher nitrogen fixing ability (more than twice).

Regarding all the results stated above, it can be concluded that *A. insignis* isolated from Kwangyang Bay is a marine-

borne microorganism rather than a fresh water-originated one.

Although there is still the possibility that this salt-requiring and/or salt-tolerant character is due to its adaptation, the evidences available thus far indicate that the natural habitat of this strain is the sea water.

摘 要

光陽灣의 表面水에서 *Azotobacter* 屬에 屬하는 한 菌株를 分離해서 여러가지 形態的 性質과 生理的 性質에 대해서 調査한 結果, 이 菌株가 특히 polar flagella를 가졌고 soluble green and insoluble light brown pigmentation을 하고 microcyst를 形成하지 않았기 때문에 *A. insignis*로 同定하였으나 다소간의 好鹽性이 있음을 확인하였다.

일반적으로 土壤에서 채취한 같은 種 *A. insignis*에 比하여 이 菌株는 特異하게 NaCl 濃度 3%와 6%에서 成長이 좋았으며 NaCl이 없는 배지에서는 잘 자라지 않는다는 것으로 미루어 보아 好鹽性인 새로운 菌株라고 판단되었다.

이 菌株의 窒素固定量 측정에 있어서는 이는 NaCl이 없는 배지에서 보다 NaCl 濃度 3%와 6%에서 훨씬 높았으며 0%에서는 0.56mg이었음에 比하여 3% NaCl 濃度에서는 20배에 해당하는 11.06mg에 이르렀다.

溫度와 *p*H에 따라 NaCl이 이 菌株의 成長에 미치는 影響으로서는 最適 溫度는 30°C였고 最適 *p*H는 7.0이었음을 알았다.

그러므로 이 菌株의 自然 棲息地가 바다로 생각되며 土壤에서 分離한 *A. insignis*는 이러한 好鹽性 性質이 없는데 比하여 이 菌株가 이러한 性質을 가지는 것이 adaptation에 의한 것인지, mutation에 의한 것인지는 앞으로 밝혀져야 할 問題라고 생각된다.

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