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L-Glutamic Acid 生成菌에 關한 硏究(I)

—*Brevibacterium ammoniagenes*의 分離 및 同定에 關하여—

*洪淳佑 · 河永七 · 車勝熙

(서울대 文理大 *微生物學科 · 植物學科)

Studies on L-Glutamic Acid-Producing Bacteria(I)

—On the Isolation and Identification of *Brevibacterium ammoniagenes*—

***HONG, Soon Woo, Yung Chil HAH, and Seung Hee CHA**

*(Dept. of Microbiology · Dept. of Botany, Seoul National University)

ABSTRACT

Three strains which accumulated large quantities of L-glutamic acid as the chief product of metabolism in the presence of glucose and inorganic ammonium salt under the aerobic condition were newly isolated.

These strains have the general characters as follows: Gram-positive, short rod-shaped, non-sporulating, non-motile, and facultative anaerobe. A change of cell shapes was not almost observed in their life cycles and neither a phenomenon of cell-elongation nor a pleomorphism was recognized in any cases. Together with rigid stability in Gram-stain, these characters as above indicated evidences that newly isolates would belong to genus *Brevibacterium* clearly differentiated from genus *Corynebacterium*.

At the same time the newly isolates, in addition to nutritional requirement of biotin and thiamine, showed the distinctive character of requirement of special amino acid such as histidine or cysteine for their considerable growth.

These physiological characters including nitrates reduction, reaction on milk, and slow formation of acid from sugar also were useful in confirming that these bacteria would be *Brevibacterium ammoniagenes*.

INTRODUCTION

Synthesis of an individual amino acid by microorganisms was virtually unknown until the polyglutamyl peptides of *Bacillus anthracis* (Ivanovies and Bruckner, 1937), consisting of D- and L-isomers of glutamic acid, was isolated.

The sincere and tangible efforts to produce large amounts of amino acid employing microorganisms have been made since Dagley(1950) detected a small quantity of amino acid in the culture of *E. coli*.

A series of studies specially on the production of L-glutamic acid with microorganisms have been extensively carried out mainly by Japanese investigators on the ground that L-glutamic acid has been consumed as a seasoning in the largest amount among all amino acids. Thus Kinoshita *et al.*(1958) could accomplish to isolate a new L-glutamic acid-producing bacterium, *Micrococcus glutamicus*, and Asai *et al.*(1957) also isolated *Micrococcus varians* which was capable of producing extraordinary amounts of L-glu-

tamic acid.

Later, the extensive and continuous surveys by many investigators made it possible to isolate hundreds of L-glutamic acid-producing bacteria which could be employed for industrial fermentation of L-glutamic acid. At present a number of species belonging to genus *Micrococcus*, *Arthrobacter*(Veldkamp, 1963), *Corynebacterium*(Lee *et al.*, 1963), *Microbacterium*(Doi and Kaneko, 1960), *Bacillus* (Chao and Foster, 1959), *Aerobacter* (Fowler *et al.*, 1955; Takamura *et al.*, 1961), *Cephalosporium*(Kita, 1957), *Brevibacterium*, etc. are known to be favourable organisms for industrial L-glutamic acid fermentation.

Among them, *Brevibacterium* is a new genus proposed by Breed(1953), that is, Breed separated 22 species of the Gram-positive bacteria from genus *Bacterium*, *Achromobacter*, *Flavobacterium* and *Bacillus*, and unified them as one genus, *Brevibacterium*.

Practical studies on production of L-glutamic acid with microorganism belonging to genus *Brevibacterium* began to progress vigorously due to the isolation of *Brev. aminogenes* by Ota and Tanaka (1959) and *Brev. alanicum* by Ogawa *et al.* (1959).

After then, *Brev. divaricatum*(Su *et al.*, 1960), *Brev. lactofermentum*, *Brev. immariophilium*, *Brev. flavum*, *Brev. roseum* (Okumura *et al.*, 1962), *Brev. pentoso-aminoacidicum* (Yamada and Hirose, 1960), etc. together with *Brev. thiogenitalis*(Kanzaki *et al.*, 1967) were continuously isolated and identified as new L-glutamic acid-producing bacteria, and now about twenty of new species except for some L-glutamic acid-producing bacteria described in Bergey's Manual, 7th ed. are known to be the superior L-glutamic acid producers.

Meanwhile, the accompanied isolation of new species or strains belonging to other genera has been also performed in addition to that of *Brevibacterium*, consequently these successive isolations of microorganisms have brought the recognition of differences among them to be more difficult. From taxonomical viewpoint, above all, several similarities particularly among genera such as *Micrococcus*, *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, and *Microbacterium* have driven the current taxonomical system of L-glutamic acid producers to a great number of confusions.

Resulting from this newlydeveloped confusion, at present many taxonomists are in progress to create a new taxonomical system in order to classify all the L-glutamic acid-producing bacteria systematically (Okumura *et al.*, 1962; Takayama *et al.*, 1965; Hattori *et al.*, 1965).

By virtue of continuous surveys by Jensen(1952), Alfold(1955), Takahashi *et al.* (1967), Kinoshita *et al.* (1960), Itagaki *et al.*(1959, 1961), and Takayama *et al.* (1965, 1967), current trends of taxonomy are under way to the direction that it is most reasonable that some of L-glutamic acid-producing bacteria belonging to genus *Microbacterium*, *Arthrobacter*, and *Micrococcus* should be combined in one genus *Corynebacterium* on account of their overlapping physiological and morphological similarities.

Of course, the distinguishable difference between *Brevibacterium* and *Corynebacterium* is not clear until now as described

in Bergey's Manual, 7th ed., p. 1011: "Criteria for separation of these two genera are inadequate." Furthermore, Veldkamp(1963) reported that even *Brev. linens*, type species of *Brevibacterium*, had changeable cell-shape during the process of its life cycle and so this bacteria had to be put in genus *Arthrobacter*.

Okumura(1962) and Oishi(1963), meanwhile, concentrated their eyes on the facts that changes of cell-shapes, branching or filamentation of cells, and pleomorphisms were never recognized in all *Brevibacterium* species isolated by themselves, and then they insisted that their strains might well belong to *Brevibacterium*. Based on their studies, many investigators nowadays tend to put their strains into genus *Brevibacterium*.

There are still sufficient overlaps to question the validity of each genus as a distinctive criterion for generic differentiation. Moreover, the newly-isolates are intended to regard as new species, providing that showing even slight differences from other organisms identified previously, which follows that the current taxonomical system has been prompted into more confusions. But these problems are expected to be solved according to pursuing the continuous and systematic studies.

The authors considered various characters of newly-isolates sufficiently comparable with organisms reported already in morphological and physiological respects and now is to report the results obtained.

MATERIALS AND METHODS

1. Organisms

Twenty-one strains of microorganisms were first isolated from Jungrang-Cheon sewages by using the medium containing benzoic acid as a sole carbon source according to the report of Yamamoto (1972). Yamamoto *et al.* stated in their reports that the benzoate assimilability is a particular property in the L-glutamic acid producers and it may become a useful point to characterizing "Glutamate producing-bacteria" taxonomically, like the requirement of biotin.

They were purified by three successive subcultures of a single colony. Among them, 14 strains were selected by first screening method using paper chromatography. Subsequently, three strains which produced L-glutamic acid more than 30% of initial sugar in the synthetic medium were chosen by quantitative determination of L-glutamic acid and named

Table 1. Composition of various media used.

Medium used for isolation

Chemicals	%(W/V)	Chemicals	%(W/V)
Benzoic acid	0.5	$MnSO_4 \cdot 7aq.$	0.0008
KH_2PO_4	0.2	Malt extract	0.02
K_2HPO_4	0.7	Biotin	10μg
$(NH_4)_2SO_4$	0.3	Thiamine	100μg
$MgSO_4 \cdot 7aq.$	0.05	Yeast extract	0.02
$FeSO_4 \cdot 7aq.$	0.001	Phenol red	0.0003

Slant medium

Chemicals	%(W/V)	Chemicals	%(W/V)
Peptone	1.0	NaCl	0.25
Beef extract	0.5	Agar	2.0
Yeast extract	0.5		

Synthetic medium used for L-glutamic acid fermentation

Chemicals	%(W/V)	Chemicals	%(W/V)
Glucose	5.0	$MgSO_4 \cdot 7aq.$	0.02
Urea	1.2	$FeSO_4 \cdot 7aq.$	0.001
K_2HPO_4	0.2	Yeast extract	0.2
		Beef extract	0.2

strain T—1, YR—2, and Y—2 respectively. Purified organisms were maintained on agar slant shown in Table 1.

2. Experimental test

Diagnostic tests were carried according to "Manual of Methods for Pure Culture Study of Bacteria" 6th ed. 1950, Society of American Bacteriologists, and "A Guide to the Identification of the Genera of Bacteria" by V.B.D. Skerman 2nd ed. 1967, Williams and Wilkins, and referred to "Handbook of Practical Bacteriology" by Mackie & MacCartney 9th ed. 1953, E. & S. Livingstone, Edinburgh and London.

3. Medium

Various media are shown in Table I. Urea was sterilized separately by filtration with Seitz filter and added to the medium. Glucose and mineral salts were also sterilized repectively by autoclaving at 121°C for 15mins in order to prevent the medium from caramelizing.

4. Culture

Temperature used for incubation of bacteria newly-isolated was generally 30°C in most cases tested except in the case of gelatin liquefaction test. For the fermentation test of L-glutamic acid the organisms was cultured on a reciprocal shaker (275rpm) at 30°C for 72hrs. These cultures were attained with 190mm by 21mm test tubes, where the medium was dispensed in 6m*l* quantity.

5. Determination of cell yield

Growth of cells was determined as turbidity. Cultured broth was diluted ten times and treated with Beckman model DU spectrophotometer at 620nm.

6. Assay of L-glutamic acid

As a first screening method L-glutamic acid identified by comparison with standard on paper chromatography using two solvent systems. The solvent were butanol, acetic acid and water(4 : 1 : 1 v/v) and other solvent was phenol water(75 : 25 w/w). L-Glutamic acid was also determined by manometric method using Warburg Manometer by treatment with L-glutamic acid decarboxylase of *E. coli* strain according to the improved method by Takawa *et al.*(1961) which pyridine-pyridine hydrochloride buffer (*p*H 4.0) was used instead of acetate buffer in order to enhance the sensitivity and swiftness of the activity of L-glutamic acid decarboxylase.

RESULTS AND DISCUSSIONS

1. Decision of family

The newly isolated bacteria have six properties which most of the glutamic acid-producing bacteria should possess, i.e., accumulation of L-glutamic acid in large quantity under the aerobic condition, Gram-positive, non-motile, non-sporulating, short rod-shaped usually and requirement of biotin(10r/*l*). Distinguishable changes of cell-shapes and pleomorphisms in all strains isolated were not observed in any cases tested. The newly-isolates never changed their cell-forms on agar or liquid media even supplemented with citrate and varied biotin.

The newly-isolates also showed no formation of branching or filaments and their reproductions were by snapping cell division. In addition, the newly isolated cells exhibited the rigid stability on Gram-staining in the young or even in the old cultures and always appeared in Gram-positive organisms.

Besides the morphological characters as above, these organisms had some ph-

ysiological properties that acid formation from glucose resulted from fermentative metabolism of cells and relation to free oxygen was facultatively anaerobic.

These all distinctive characters of newly-isolated bacteria indicated that among Order IV Eubacteriales three all newly-isolates would belong to Family IX Brevibacteriaceae described in Bergey's Manual, 7th ed. rather than Family XII Corynebacteriaceae and Family VII Micrococcaceae.

Though there is a possibility that they belong to Family XII Corynebacteriaceae because of mutual cellular similarities in morphological respects, the facts that members of this family are usually pathogenic cells showing severe pleomorphism, while the newly-isolates have the distinctive characters of cell-shapes, which could assure all species described in this family not to be possibly in accord with newly-isolates.

Breed(1953) stated the characteristics of *Brevibacterium* gen. nov. as follows: "Typically short, unbranching rods, generally non-motile; type of motility of motile, species peritrichous or uncertain; sometimes chromogenic, with non-water-soluble reddish, reddish orange, yellow, or brown pigments; may or may not reduce nitrates; glucose broth usually become acid, lactose not fermented; proteolytic action varies according to the species; aerobic or facultatively anaerobic, rarely microaerophilic; found in dairy products, soil, salt and fresh-water, and decomposing substances of a great variety of types." From this statement, typically short and unbranched rod shapes are considered to be the clearest characteristics of this genus distinguishable from other family.

On the other hand, Skerman (1958) stated his view concerning this genus as follows: "It is not possible to define this genus clearly. It was introduced primarily to provide a grouping (taxon) into which an assemblage of Gram-positive, non-sporing, non-acid-fast rods, having no clear-cut affinities with other genera of Gram-positive rods, could be placed. Only a general description of the genus can be given."

In conformance to such a statement by Skerman and Jenson's report(1952), Kinoshita *et al.* (1960) demonstrated it was reasonable that L-gutamic acid-producing bacteria belonging to *Brevibacterium* together with *Microbacterium*, *Micrococcus* and *Arthrobacter* should be put into Family Corynebacteriaceae on the basis of their morphological and physiological similarities among them. However, their thoughts might be permissible limitedly in *Micrococcus*, *Microbacterium*, and *Arthrobacter*, for example, *Micrococcus glutamicus* which was identified by themselves. This species shows the extremely pleomorphic character and branching or filament and now it has been called *Corynebacterium glutamicum*.

Regarding genus *Brevibacterium*, only two species of *Brev. linens* and *Brev. helvolum* were reported to have changeable cell-shapes, as it were, multicellular elongated form and club-shaped form. Most species of this genus except these two species have been regarded to maintain stable cell-shapes enough to allow to belong to *Bervibacterium* surely(Okumura *et al.*, 1962).

Although, during the cell growth, the newly-isolated bacteria changed their

cell-forms to short rods, rods and ellipsoidal spheres, now that these organisms had the distinctive characteristics of stable cell-form, it was right that these organisms belong to Brevibacteriaceae in all respects.

If newly-isolates were to be regarded as cocci, genus *Micrococcus* and *Staphylococcus* would be related with newly-isolates, but the former is different from newly-isolates in its action on sugar and distribution of DAP-component in cell wall and the latter is aberrant in reaction with litmus milk and acid formation from sugar. Among these two genera, *Micrococcus glutamicus* and *Micrococcus varians* were most similar to newly-isolates but the former was aberrant in sugar metabolism and M.R.&V.P. reaction and the latter was different in hydrolyzation of starch, production of ammonia, and action on sugar.

Resulting from these facts, family Corynebacteriaceae and Micrococcaceae were eliminated. Hence, all three strains isolated are confirmed to probably belong to Family Brevibacteriaceae.

2. Decision of genus

Brevibacteriaceae has only two genus, i.e., *Brevibacterium* and *Kurthia*. Species of *Kurthia* do not utilize sugar and its usual form is long and unbranched rod-shaped. So newly-isolates were assured to belong to genus *Brevibacterium*. Following description of *Brevibacterium* can be seen in Bergey's Manual, 7ed., 1957: "Short, almost coccoid, unbranched rods which do not form filament. Acid usually produced from simple carbohydrates."

3. Bacteriological characters of newly isolated bacteria

1. Morphological characters

1) Short rods with rounded and 0.4 to 0.6μ by 1.0 to 1.2μ in size, non-motile, no pleomorphism and spores not formed.

2) Cell division: snapping type.

3) Gram-positive by Hucker's modification and not acid-fast by Ziehl-Neelsen's method.

4) Elongated forms of cell-shape not observed in citrate-added medium.

2. Cultural characteristics

1) Nutrient agar colony: 1—5mm in diameter, greyish yellow, circular, slightly raised, smooth and entire.

2) Nutrient agar slant: growth moderate, and greyish yellow filiform, flat or slightly raised, opaque and butyrous, no odor.

3) Nutrient agar stab: filiform growth, more abundant near the surface.

4) Nutrient broth: slightly turbid without pellicles and rings, yellowish flocculent sediment.

3. Physiological properties

1) Temperature: no growth at 45°C, optimum growth at 28°C—35°C, killed at 52°C in 10 mins. non-heat-resistant in skim milk.

2) *p*H: growth in the range of *p*H 5.0—9.0 and optimum *p*H 6.5—8.0.

3) Relation to free oxygen: facultative anaerobe.

4) Carbohydrate metabolism: fermentative acid production.

5) Methylene blue is reduced.

6) Gelatin: no liquefaction.

7) Blood serum: no liquefaction.

8) M.R.&V.P. test: negative.

9) Nitrite produced from nitrate.

10) Casein dissimilation: negative.

11) Citrate: no utilization in Koser's medium.

12) Starch: not hydrolyzed.

13) Litmus milk: slightly alkaline and not coagulated.

14) Catalase: extremely positive.

15) Urease: strongly positive.

16) Phosphatase: negative.

17) Mono-ammonium-phosphate is poorly utilized but good growth occurred in the presence of biotin and thiamine in Hucker's medium.

18) Indole: not produced.

19) H_2S is not produced in ferric ammonium citrate agar.

20) Production of organic acid: mainly α-ketoglutaric acid and lactic acid.

21) L-Glutamic acid is accumulated in large quantity in the presence of glucose and ammonium ion.

22) Yellow chromogenesis is produced.

23) Acid from some carbohydrates, as shown in Table 2, but no gas formation in all carbohydrates tested.

24) Assimilation of organic acid: succinic acid is best assimilated and next acetic acid and gluconic acid are assimilated. Assimilations of fumaric acid and α-ketoglutaric acid are poor. Citric acid

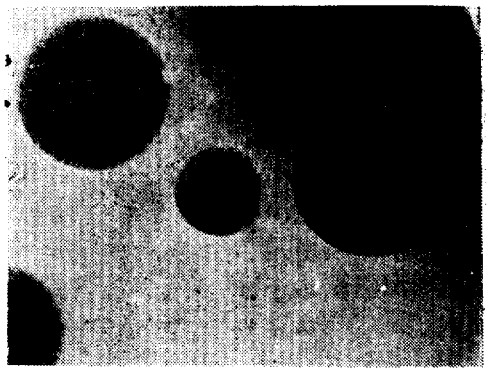

Fig. 1. Plated colony of strain T-1 newly-isolated, after 72-hours incubation at 30°C. Magnification ×7

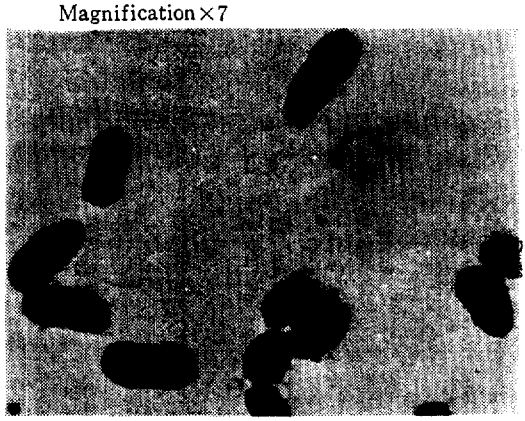

Fig. 2. Electron micrograph of strain T-1 after 12-hours incubation on nutrient agar. It shows snapping division. Magnification ×10,000

Table 2. Acid production from carbohydrates by bacteria newly-isolated.

Carbohydrates	Strains			Carbohydrates	S rains		
	T-1	YR-2	Y-2		T—1	YR-2	Y-2
Arabinose	—	—	—	Melibiose	—	—	—
Inositol	+	+—	+—	Cellobiose	—	—	—
Xylose	+—	—	—	Starch	—	—	—
Rhamnose	—	—	—	Inulin	—	—	—
Glucose	++	++	++	Dextrin	—	—	—
Fructose	+	+	+	Glycerol	—	—	—
Mannose	+	+	+	Adonitol	—	—	—
Galactose	—	+	—	Mannitol	—	—	—
Sucrose	+	+	+	Dulcitol	—	—	—
Maltose	+	+	+	Sorbitol	—	—	—
Lactose	—	—	—	Salicin	—	—	—
Raffinose	—	—	—	α-Methyl-glucoside	—	—	—

++: Acid is produced rapidly. +: Acid is produced slowly.
—: Acid is not produced. +—: Acid production is doubtful.

Table 3. Physiolgoical characters of *Brevibacterium* species descirbed in Bergey's Manual, 7th ed., 1957.

Species \ Characters	Motility	Pigment	Gelatin liquefaction	NO_2	H_2S	Litmus milk	Voges-Proskauer	Methyl red	Urease	Relation to free oxygen	Indole test	Acid from glucose	Hydrolysis of starch
Brev. linens	−	R, O	+	+	+	sAL	−	−		A	−	−	+
Brev. erythrogenes	−	R	+	+	+	Ac-Al				A, AF	−, +	+	
*Brev. fulvum**	−	rY	+	−		Ac, Ac-Al				〃	+		
*Brev. insectiphilium**	−	gY	+	−	−	Al				A	−	−	+−
Brev. brunneun	−	B	+	−		NC					−	−	
*Brev. vitarumen**	−	pY	−	+		Ac					−	+	−
Brev. maris	−	Y	−	+	−	sAL-sAc			+−	A, FA	−	+	
Brev. fuscum	−	B, Y	+	+		sAc-sAl				〃	−		
Brev. minutiferula	−		−	−	−	NC-sAC				A	−	+	−
Brev. socivivum	−		+	−	−	Dc			+	A, FA	−	+	+
Brev. immotum	−		+	−	−	〃				〃	−	+	+
Brev. marinopiscosum	−		+	−	−	〃			−	〃	−	+	+
Brev. tegumenticola	−	W	+−	−	−	NC				〃	−	+−	−
*Brev. stationis**	−		+	+	−	Al			−	A, FA	−	+	−
Brev. quale	−	W	+	+	−	NC					−	+	−
Brev. healii	−	W	+			sAc				A, FA	−	+	
Brev. incertum	+	gW	−	−	−	NC	−				−	+	−
*Brev. imperiale**	+	O-Y	−	−	−	NC-sAc				A	−	+	−
Brev. lipolyticum	+	G-Y	+	+−	−	Al				A, FA	+−	+	
*Brev. acetylicum**	+	Y	+	−		Ac	+			〃	−	+	+
*Brev. sulfureum**	+	Y	+	−		Al				〃	−	−	
*Brev. helvolum**	+	sY	+	−	+	Ac-Al				A	−	−	
*Brev. persicum***	+	R	+	−		Al						+	
*Brev. ammoniagenes**	−	G-pY	−	+	−	sAl			+	A, FA	−	−	

Abbreviations in this table can be seen in Table 4.
* These species are known to be L-glutamic acid-producing bacteria presently.
** This species was isolated and identified by Sasaki *et al.* (1960)

Table 4. Physiological characters of *Brevibacterium* species newly-isolated and not described in Bergey's Manual, 7th ed.

Species \ Characters	Motility	Pigment[1]	Gelatin liquefaction	NO_2	H_2S	Litmus milk[2]	Voges-Proskauer	Methyl red	Urease	Relation to free oxygen[3]	Requirement of biotin[4]	Authors
Brev. aminogenes	−	mY-gY	−	+	−	sAc	+	+−	+	FA	b	Ota *et al.*, 1959
Brev. alanicum	−	gW	−	+	−	sAc	−	−	−			Ogawa *et al.*, 1959
Brev. type A,B,C	Taxonomical characters are not published.											Kobayashi, 1959
Brev. divaricatum	−	pY-G	−	−	−	sAc	−	−	+	A	b	Su *et al.*, 1960
Brev. ureautilis												Murada *et al.*, 1960
Brev. pentoso-aminoacidicum	+	Y		−	+	NC	−	−	+	A		Yamada *et al.*, 1960
Brev. flavum	−	pY	−	+	−	sAl	+−	−	+	FA	b,bt	Okumura *et al.*, 1962
Brev. lactofermentum	−	Y-pY		+	−	Ac	+	+	+	FA	b	〃
Brev. saccharolyticum	−	mW		+	−	sAl	+, −	+, −	+	FA		〃
Brev. immariophilium	−	Y-mW		−	−	sAl	−	−	+, −	FA	b	〃
Brev. roseum	−	P		+	−	NC	−	−	+	FA	bt	〃
Brev. glutarium												Nasu *et al.*, 1963
Brev. sp. Teramoto	−	pY		+					+		b	Kita *et al.*, 1961
Brev. ketoglutamicum												Chen, Chou, 1964
Brev. sp. No.13	−	cY		−	−	NC	−	−	−			Hattori *et al.*, 1965
Brev. kerinus K-129	−	yB	+	+	+	NC	+			A,FA		Iizuka *et al.*, 1965
Brev. albus S-270	−	yB	+	−	+	NC	−			A		〃
Brev. thiogenitalis			-									Kanzaki *et al.*, 1967
Brev. sp. etc.												Oki *et al.*, 1998
Brev. sp. soya	Taxonomical characters are not published.											Hashida *et al.*, 1959
Brev. ammoniagenes T-103	−	pY		+					+		b	Su *et al.*, 1964
Brev. ammoniagenes Oishi	−	pY		+					+		b	Oishi *et al.*, 1963
strain T-1	−	gY	−	+	−	sAl	−	−	+	FA	bt	
strain YR-2	−	gY	−	+	−	sAl	−	−	+	FA	bt	
strain Y-2	−	gY	−	+	−	sAl	−	−	+	FA	bt	

1. mY: Milky yellow gY: Greyish yellow gW: Greyish white pY: Pale yellow G: Grey Y: Yellow mW: Milky white
   P: Pink cY: Creamy yellow yB: Yellowish brown R: Red O: Orange B: Brown W: White
2. sAc: Slightly acid NC: Not changed sAl: Slightly alkaline Ac: Acid Dc: Decolorized
3. FA: Facultatively anaerobic A: Aerobic
4. b: Biotin bt: Biotin and thiamine

not assimilated.

25) Habitat: may be soil and sewage.

4. Decision of species

Newly-isolated bacteria are all non-motile and yellow chromogenesis was produced. Nitrite was produced from nitrate and the liquefaction of gelatin was not recognized.

Among 23 species described in Bergey's Manual, only four species including *Brev. vitarumen*, *Brev. maris*, *Brev. fuscum* and *Brev. ammoniagenes* have similar characters described above, as can be seen in Table 3, and so all of other species were eliminated from discussion on morphological and physiological basis.

Brev. vitarumen, among them, turns litmus milk acid, *Brev. fuscum* liquefies gelatin and produces brownish to pale greenish yellow chromomogenes. And so these two species were regarded not to be in accord with the newley-isolates and also excluded. *Brev. maris* is different from newly-isolated bacteria in colonial colour and moreover its cells are encapsulated. Consequently, it could be suggested that newly-isolates possibly resembled *Brev. ammoniagenes* in all respects except for action on sugar.

Among the microorganisms isolated newly by mainly Japanese investigators since 1957 and identified to belong to Genus *Brevibacterium*, four species including *Brevibacterium flavum* have similar characteristics to newly-isolates in their morphological and physiological properties. All of these L-glutamic acid producing-bacteria newly identified are exhibited in Table 4 and compared with one another.

Brev. alanicum (Ogawa *et al.*, 1959) has distinguishable properties from newly-isolated strains in reaction with litmus milk and urease production. *Brev. aminogenes* (Ota *et al.*, 1959) is different in M.R.&V.P. reaction and carbohydrate metabolism, i.e., either by fermentative or by oxidative.

Brev. flavum (Okumura *et al.*, 1962) is a new species which was isolated and identified in Japan. Okumura *et al.* had classified all of species of L-glutamic acid-producing *Brevibacterium* isolated by them into 10 types, as presented in Fig.3. According to his works, *Brev. flavum*

Fig. 3. Classification of L-glutamic acid-producing *Brevibacterium* isolated from nature (Okumura *et al.*, 1962).

I. Yellow, pale yellow or milky white pigment.
- (A) Aerobic (carbohydrate metabolism; oxidative).
  - 1) Nitrites not produced from nitrates.
    - a. Acid produced in milk. ..... I *Brevibacterium* n.sp. (1)
    - aa. Alkaline reaction in millk. ..... II *Brevibacterium* n.sp. (4)
- (B) Facultatively anaerobic (carbohydrate metabolism; fermentative).
  - 1) Nitrites produced from nitrates.
    - a. Acid produced in milk. ..... III *Brevibacterium lactofermentum* (8)
    - aa. Alkaline reaction or not changed in milk.
    - b. Milky white pigment. ..... IV *Brevibacterium saccharolyticum* (6)
    - bb. Yellow or pale yellow pigment. ..... V *Brevibacterium flavum* (107)
  - 2) Nitrites not produced from nitrates.
    - a. Acid produced in milk. ..... VI *Brevibacterium* n.sp. (6)
    - aa. Alkaline reaction or not changed in milk. ..... VII *Brevibacterium immariophilium* (38)

II. Pink, brown pigment and facultatively anaerobic (Carbohydrate metabolism: fermen-

tative).

1) Nitrites produced from nitrates.
   a. Pink pigment. .... Ⅷ *Brevibacterium roseum*(1)
   aa. Pale brown pigment. .... Ⅸ *Brevibacterium* n.sp.(4)
2) Nitrites not produced from nitrates.
   a. Brown pigment. ....Ⅹ *Brevibacterium* n.sp.(2)

* Figures in parenthesis indicate the number of strains concerned.

corresponds to type V and the newly isolated bacteria also came under the same type. *Brev. flavum* and the newly-isolates were clarified to have the very similar characteristics each other in morphological and physiological characters with only one clear difference between two species, like in the case of *Brev. ammoniagenes*. The former is capable of forming acid from salicin but the latters not. Although *Brev. flavum* shows slow positive in Voges-Proskauer test and the newly-isolates were certainly Voges-Proskauer-negative organisms in any stages of incubation, this difference was not regarded to be an apparent key taxonomically. Now that all of *Brev. ammoniagenes*, *Brev. flavum* and the newly-isolates were comparatively possessed of very clear similarities one another, it would be that the expressible differences among them and the decision of species of the newly-isolates could be represented merely in view of action on sugar.

In Bergey's Manual it is described that *Brev. ammoinagenes* has no action on carbohydrate.

According to the reports of Kinoshita *et al.* (1960) and Takayama *et al.*(1965, 1967), however, it was already elucidated that some strains of *Brevibacterium ammoniagenes* could produce acid from some carbohydrates except for salicin and the description of Bergey's Manual concerning this species turned out to be inadequate. Kinoshita elucidated that some strains of this species, for example, *Brev. ammoniagenes* ATCC 6871 and *Brev. ammoniagenes* ATCC 6872 exhibited the definite actions on carbohydrates such as glucose, fructose, mannitol and sucrose, even though weak and slow reaction, and consequently he could ascertain these strains not to conform to the description of Bergey's Manual, 7th ed.

Later, Takayama *et al.* also found that acid formation from glucose, fructose, sucrose, mannose, etc. could appear in *Brev. ammoniagenes* T-103 and *Brev. ammoniagenes* Oishi contrary to the description of Bregey's Manual.

All three strains newly isolated were characterized by the distinctive action on sugar as follows.

All of newlyisolates showed the undoubtful acidproduction from glucose, mannose and maltose in the differentiated manner. Acid production from glucose appeared within 48 hrs in all strains but from others acid production delayed. Among these three strains newlyisolated, strain T-1 could produce acid from inositol and galactose more excellently and rapidly than other two strains, but acid production by strain YR-2 was obvious only in galactose but doubtful in inositol. Acid production from galactose was not at all recognized in strain Y-2 and from inositol it was also doubtful, like in strain YR-2. Acid production from salicin could never be observed in all three strains. Resulting

Table 5. Acid productions from various carbohydrates by *Brevibacterium* species newly identified.

Species \ Carbohydrates	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
Brev. aminogenes	+−		+ (−)		+	++−	+− (+)	+	+−													− (+)
Brev. alanicum					+				+−	+		+−				+−						
Brev. type A,B,C	Taxonomical characters are not published.																					
Brev. divaricatum					+	+	+		+	+							+ (−)					− (+)
Brev. ureautilis																						
Brev. pentoso-aminoacidicum	+		+		+	+	+ −	+ −														+
Brev. flavum			+		+	+	+		+	+										+(−)		−(+)
Brev. lactofermentum			+ (−)		+	+	+	+ (−)	+	+	+ (−)	+ (−)	+ (−)	+ (−)		+			+ (−)	+ (−)	+	− (+)
Brev. saccharolyticum					+	+	+	+ (−)	+							+						− (+)
Brev. immariophilium								+	+	+		+	+									−(+)
Brev. roseum			+ (−)		+	+	+		+	+				+ (−)								− (+)
Brev. glutarium																						
Brev. sp. Teramoto					+	+			+										+	+		
Brev. ketoglutamicum																						
Brev. sp. No.13					+	+	+		+											+		
Brev. kerinus K-129			−		−	−			−					−		−						
Brev. albus S-270			−		−	−			−					−		−						
Brev. thiogenitalis																						
Brev. sp. etc.																						
Brev. soya	Taxonomical characters are not published.																					
Brev. ammoniagenes T-103					+	+			+	+												
Brev. ammoniagenes Oishi					+−					+												
strain T-1		+	+−−		+	+	+	+	+	+												
strain YR-2		+−			+	+	+	+	+	+												
strain Y-1		+−			+	+	+			+												

1. A: Arabinose B: Inositol C: Xylose D: Rhamnose E: Glucose F: Fructose G: Mannose H: Galactose
   I: Sucrose J: Maltose K: Lactose L: Raffinose M: Melibiose N: Cellobiose O: Starch P: Dextrin
   Q: Glycerol R: Adonitol S: Mannitol T: Salicin U: Esculin V: Trehalose
2. Marks in parenthesis exhibit the opposite results attained by Takayama *et al.* (1965).

from these facts, it could be explained that the differences of acid formation from salicin, galactose and inositol represented the most distinguishable characters enough to confirm the newly-isolates to be different from *Brev. flavum* although strain Y-2 showed no acid formation from galactose and doubtful in inositol.

Such an explanation was supported by the report of Takayama *et al.*(1965). As illustrated in Fig. 4, they divided all L-glutamic acid-producting bacteria into 12 types according to acid formation from glucose, sucrose, salicin, and mannitol, and stated that wheather acid formation from salicin by microorganisms occurs or not could hold the important key in classifying the L-glutamic acid-producing bacteria.

Fig. 4. Type of L-glutamic acid-producing strains(Takayama *et al.*, 1965).

I. Acid from glucose within 48 hrs
  A. Urease positive
    1. Nitrites produced from nitrates
      a. Acid from sucrose
      b. No acid from salicin strains
      c. No acid from mannitol·····Type 1···124
      cc. Acid from mannitol ·····Type 2···3
      bb. Acid from salicin
      c. No acid from mannitol ·····Type 3···8
      cc. Acid from mannitol
      d. Acid from maltose ·····Type 4···4
      dd. No acid from maltose ·····Type 5···9
      aa. No acid from sucrose
      b. No acid from salicin
      d. Acid from maltose ·····Type 6···2
      dd. No acid from maltose ·····Type 7···3
      bb. Acid from salicin ·····Type 8···3
    2. Nitrites not produced from nitrates·····
      ·····Type 9···6
  B. Urease positive
    1. Nitrites produced from nitrates·····
      ····· Type 10···5
    2. Nitrites not produced from nitrates ·····
      ····· Type 11···2
II. Acid production from glucose usually delayed
  Urease positive ·····Type 12···38

All of these expriences, as a result, provided logical explanations for identifying all three strains newly isolated as *Brev. ammoniagenes*. Besides of all facts described above, the authors could prefer to designate the newly isolated strains as *Brev. ammoniagenes* for the purpose that this investigation was not intended to be an exhaustive identification of microorganisms concerned.

However, it is now recognized that all of the L-glutamic acid producers do not always express the constant reaction on carbohydrates as like as demonstrated previously by other investigators, i.e,acid formation from some sugars by microorganisms are varied according to strains concerned. Moreover, as shown in Table 5, *Brev. flavum* ATCC 14067 do not produce acid from salicin contrary to strains of *Brev. flavum* isolated by Okumura *et al.*, (1962).

Meanwhile, Okumura *et al.* suggested that *Brev. flavum* would be a different species from *Brev. ammoniagenes* merely on the basis of the description, "No action on carbohydrate in *Brev. ammoniagenes*" in Bregey's Manual, 7th ed., 1957. Their thought therefore is supposed to be unreasonable from a currently logic point of view, and subsequently *Brev. flavum* is expected to be approximately one of the strains of *Brev. ammoniagenes*.

Additional works in detection of cell wall constituents and DNA component is necessary to fully define their comparative characters. Owing to further studies, the advent of the proper discriminating

of all L-glutamic acid producers in the near future.

According to the results from comparative tests among three strains newly isolated throughout the experiments, each of strains was named individually, *Brev. ammoniagenes* T-1, *Brev. ammoniagenes* YR-2, and *Brev. ammoniagenes* Y-2.

摘 要

今番 好氣的 狀態에서 糖과 암모니움鹽을 利用, 培地內에 對糖 30% 以上의 glutamic acid를 生産하는 菌 3株를 分離 考察하였다. 그 結果 本 菌들은 短桿狀, 無胞子, 通性嫌氣性, 非運動性으로서 glutamic acid 生成菌들이 가지는 一般的 特徵을 지님을 確認하였고 細胞의 生活環 內에서 營細胞의 形態的 變化 및 細胞의 長大化 現象이 觀察되지 않고 同時에 Gram 染色에 安定된 反應을 나타낸다는 點에서 *Corynebacterium*屬과는 分明히 相異한 *Brevibacterium*屬으로 判斷하였다. 아울러 炭酸鹽의 還元, litmus milk에서의 反應 및 糖에 對한 反應性의 諸特性과 biotin, thiamine 및 아미노酸에 對한 榮養要求性 等을 根據로 하여 本菌들을 *Brevibacterium ammoniagenes*로 同定할 수 있었다.

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