TAXONOMIC STUDIES ON THE HIOCHI-BACTERIA, SPECIFIC SAPROPHYTES OF SAKE II. IDENTIFICATION AND CLASSIFICATION OF HIOCHI-BACTERIA

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In the previous paper⁽¹⁾ it was concluded that the hiochi-bacteria, both the so-called 'true hiochi-bacilli' and the 'hiochi-lactobacilli', belong to the category of lactic acid bacteria. The question arose then as to what systematical positions are occupied by these hiochi-bacteria. Important characteristics for determining the systematical position of lactic acid bacteria are the type of fermentation (such as homo or hetero), optical property of the lactic acid produced and the type of sugars which are fermented by the bacteria.

FERMENTABLE SUGARS AND OPTICAL PROPERTY OF LACTIC ACID.

Using culture medium IV (described in the preceding paper) in which glucose was substituted by various sugars, the fermentable sugars of 64 strains of hiochi-bacteria were examined. The results are summarized in Table 1, together with the optical properties of lactic acid which were investigated in our previous study.

Heterofermenters.

Among heterofermenters, three strains designated as H-50, H-34 and H-60, which were hiochic acid-independent, showed fermentation activity with glucose, fructose, galactose, sucrose, xylose, and α -methyl glucoside, producing inactive lactic acid. Dextran formation in sucrose-gelatin could not be observed, but mannitol was formed distinctly when fructose was used as substrate. In view of these characteristics, these may be regarded as being identical with *Lactobacillus fermentum* BEIJERINCK α -type KITAHARA⁽²⁾, although the optimum temperature for these strains is a little lower than that for typical strains of *L. fermentum* α . Strain H-67 is somewhat different from the above mentioned three strains, in being incapable of fermenting the sugars other than monosaccharides, although it can ferment xylose. Considering the slightrness of difference, this strain may be classed in the same species.

All 44 strains of heterofermentative 'true hiochi-bacilli' were found to be totally incapable of fermenting sugars tested in this experiment except glucose and fructose.

Table 1. Fermentable Sugars and Optical Forms of Lactic Acid.

	Heterofermenters				Homofermenters						Spherical strain	
	‘True hiochi-bacilli’		‘Hiochi-lactobacilli’		‘True hiochi-bacilli’		Trans- itional strains		‘Hiochi-lacto-bacilli’			
Representative strains	H 5	H 46	H 67	H 50	H 14	H 40	H 13	H 38	H 7	H 76	H 26	H 35-1
Number of analogous strains	42	2	1	3	4	3	3	1	2	1	1	1
Xylose	—	—	+	+	—	—	—	—	—	—	—	+
Arabinose	—	—	—	—	—	—	—	—	—	—	+	+
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	—	—	—	—	+	+	+	+	+	+	+	+
Galactose	—	—	—	+	—	—	—	—	+	+	+	+
Maltose	—	—	—	+	+	+	+	+	+	+	+	+
Sucrose	—	—	—	+	—	—	—	+	+	+	+	+
Lactose	—	—	—	—	—	—	—	—	—	+	+	—
Raffinose	—	—	—	+	—	—	—	+	—	—	—	+
Dextrin	—	—	—	—	—	—	—	—	—	—	—	—
Dextran	—	—	—	—	—	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	—	—	+	—	—	—
Starch	—	—	—	—	—	—	—	—	—	—	—	—
Glycogen	—	—	—	—	—	—	—	—	—	—	—	—
α—Methyl glucoside	—	—	—	+	—	—	—	—	(+)	+	—	+
Salicin	—	—	—	—	+	+	+	—	+	+	+	—
Glycerol	—	—	—	—	—	—	—	—	—	—	—	—
Optical form of lactic acid	DL	L	DL	DL	D	DL + D	DL + D	DL + L	DL + L	DL + L	DL	D

As they do not ferment sucrose, the dextran formation could not be examined. It is concluded that they belong to α -typed hetero-lactic acid bacteria, because they have the ability to change over 30 % of fructose to mannitol (Table 2). As a rule they produced DL-lactic acid. It is worth while to mention here that L-lactic acid was formed by two heterofermenting rods, H-45 and H-46. This phenomenon offers a serious problem pertaining to the classification of lactic acid bacteria, but we will reserve the discussion on this problem for some future occasion.

Table 2. Fermentation of Fructose by Heterofermentative Hiochi-Bacteria.
Culture: 2.5 g fructose/300 ml., 30°C, 7 days.

Strain	Sugar consumed g	Products				Remarks
		Mannitol		Lactic acid g	Acetic acid g	
		Quantity g	Yield %			
H 34	2.40	0.64	25.8	0.64	0.26	In the presence of CaCO ₃
H 60	2.40	0.68	28.3	0.56	0.22	
H 1	2.34	0.76	32.4	0.46	0.31	In the absence of CaCO ₃ Initial pH: 5.0
H 11	2.40	0.72	30.0	0.49	0.32	

There may be some reason to assume that these strains might be mutants of *L. fermentum* which had been simplified in their fermentative ability by adapting to limited nutritional environments⁽³⁾. This assumption seems, however, to be inadequate when we consider that these strains appear most frequently with vigorous growth activity in saké which can hardly be regarded as a poor nutritive medium. Thus the present authors want to propose a name *Lactobacillus heterohiochii* nov. sp. from the meaning of heterofermentative hiochi-bacteria.

Strains belonging to Type AF 1 in YAMASAKI's⁽⁴⁾ collection are probably identical with this species. This species has appropriately been used for biological determination of hiochic acid⁽⁵⁾.

Homofermenters.

Among homofermenters, strain H-26 ferments maltose, sucrose, lactose, arabinose, and mannitol besides hexoses and forms DL-lactic acid. So it may be identified as a typical strain of *Lactobacillus plantarum* (ORLA-JENSEN) HOLLAND.

Two strains, H-7 and H-68, have no ability to ferment mannitol. There may be some reason to consider these strains as *Lactobacillus japonicus* KITAHARA et ISHIDA⁽⁶⁾, but it will be more probable to identify them as *Lactobacillus acidophilus* (MORO) HOLLAND which lost the fermentability of lactose, as they produce DL+L-lactic acid instead of DL- or DL+D-acid which characterizes *L. japonicus*⁽⁷⁾. This conclusion is reinforced by the fact that

there exists another strain, H-76, which is identified as a typical strain belonging to *L. acidophilus*. This strain retains the abilities of lactose fermentation and of milk-coagulation, but in other properties it cannot be differentiated from above mentioned two strains. *Lactobacillus hyochi* OTANI⁽⁸⁾ may probably be a synonym of this species. It is noteworthy that H-7 had a strong fermentability of inulin. Though this property is not useful for classifying lactic acid bacteria, except in the case of *Streptococcus inulinaceus*, we are occasionally surprised by the phenomenon on the way of examining hundreds of newly isolated strains.

Seven strains, which require hiochic acid as an essential nutrient, ferment glucose, fructose, mannose, maltose, salicin and mannitol, and produce laevorotatory lactic acid, i.e. pure or partly racemized D-lactic acid.

Species belong to so-called Thermobacterium (ORLA-JENSEN)⁽⁹⁾, such as *L. lactis* and *L. delbrückii* (Syn. *Thermob. cereale*), which can grow and ferment actively at 48-50°, are well-known as D-lactic acid forming homofermenters. Hiochi-bacteria, however, can never be included in *Thermobacterium* from their growth temperature range. So these strains should be considered very curious. Only one species, *Lactobacillus leichmannii* HENNEBERG⁽¹⁰⁾, has been known as D-forming mesophilic homofermenter.

We acquired *L. leichmannii* ATCC 4797, and ATCC 7380, as strains for determining vitamin B₁₂. Here, we used these strains and an authentical strain of *L. delbrückii* for comparing with the D-forming hiochi-bacilli. Table 3 shows the summary of the experiments.

Table 3. Comparison of the Characteristics of Homofermentative 'True Hiochi-Bacilli' with Those of *L. leichmannii* and *L. delbrückii*.

	Acid-formation from:											Optical form of lactic acid Long and twisted cells	Optimum temp.	Maximum temp.	Optimum pH	
	Glucose	Fructose	Mannose	Galactose	Maltose	Sucrose	Lactose	Pentoses	Salicin	Mannitol	Dextrin					
H 14 and analogous 6 strains	+	+	+	-	+	-	-	-	+	+	-	D	-	30 °C	40 °C	5.0
<i>L. leichmannii</i> ATCC 4797	+	+	+	+	+	+	+	-	-	-	-	D	+	37	48	6.5
<i>L. leichmannii</i> ATCC 7380	+	+	+	-	+	+	+	-	-	(+)	(+)	D	+	37	50	6.5
<i>L. delbrückii</i>	+	+	+	+	+	+	-	-	-	-	-	D	+	40	52	6.5

All strains coincide with one another in the character of forming D-lactic acid. This group of hiochi-bacteria cannot ferment sucrose which *L. leichmannii*

very readily can. On the other hand, the former can readily ferment mannitol and salicin, upon which the action of the latter is very limited or completely lacking. *L. leichmannii* is rather akin to *L. delbrückii*, that is Thermobacterium, not only in fermentable sugars but also in the temperature relationship and morphological nature.

In BERGRY's Manual, *L. leichmannii* is described as to be very similar to *L. delbrückii* except the lower optimum and maximum growth temperatures. It is a matter of regret that optical property of lactic acid is not recorded in the article of *L. leichmannii*.

We have met a typical strains of *L. leichmannii*, but at least from our results of comparative experiments, this species is more likely included in the Thermobacterium. Our hiochi-bacteria are, on the contrary, obviously mesophilic.

Furthermore, cells of *L. leichmannii* and *L. delbrückii* are, as described by HENNEBERG⁽¹¹⁾, occasionally markedly elongated and twisted, but in the hiochi-bacteria, such morphological changes could not be observed. It can be added as a difference that, because hiochi-bacteria of this type cannot grow in neutral media, fermentation cannot be carried out, unlike ordinary lactic acid bacteria, in the presence of CaCO_3 .

The present authors want to propose a name *Lactobacillus homohiochii* nov. sp. to this type of hiochi-bacteria, from the meaning of homofermentative hiochi-bacteria. YAMASAKI's strains of the Type AF-100 may be included in this species. This species can also be utilized for determination of hiochic acid as well as *L. heterohiochii*.

Among the four transitional strains, three strains, H-13, H-15 and H-16, are very close to the above mentioned 'true hiochi-bacilli', except in a slight difference in nutritive requirements. So that these strains are preferably included in *L. homohiochii*.

Another strain H-38 is further different from *L. homohiochii* not only in the nutritive requirement, but also in fermentable sugars, that is, this strain can ferment sucrose and raffinose on one hand, but cannot ferment mannitol and salicin on the other hand. There is no species into which this strain is suitably admitted. The authors, however, prefer to think it as a variety of *L. homohiochii*. *L. filamentosus* and *L. alcoholiphilus* described by OTANI are also to be considered as transitional type of *L. homohiochii*.

Spherical strain.

One strain of spherical shape which was exceptionally isolated from spoiled sake, carried out heterofermentation and produces D-lactic acid.

It is then easily concluded, even without examining its morphological characters of forming beaded chains, to be a member of *Leuconostoc*.

This strain produces dextran from sucrose and readily ferments pentoses, so that it is identified as *Leuconostoc mesenteroides* (CLENKOWSKI) VAN

Table 4. Brief Classification of Lactic Acid Bacteria.

- A. GRAM-positive.
 - B. Spheres.
 - C. Catalase negative.
 - D. Homofermenters.
 - E. Racemiase negative (L-formers). Genus **Streptococcus**
Sc. cremoris, *Sc. thermophilus*,
Sc. lactis, *Sc. bovis*, *Sc. inulinaceus*, *Sc. faecalis*,
Sc. glycerinaceus, *Sc. liquefaciens*.
 - EE. Racemiase positive (L+DL~DL-formers).. Genus **Pediococcus**
Pc. hennebergi, *Pc. lindneri*.
 - DD. Heterofermenters (D-formers) Genus **Leuconostoc**
Leuc. dextranicum, *Leuc. mesenteroides* α ,
Leuc. mesenteroides β .
 - CC. Catalase positive (L-formers)..... Genus **Tetracoccus**
Tc. liquefaciens.
 - BB. Rods..... Genus **Lactobacillus**
 - C. Catalase negative
 - D. Homofermenters Subgenus True-Lactobacillus
 - E. D-Formers.
 - F. Racemiase negative or slightly positive.
(D-~D+DL-formers)..... *Delbrückii*-series
L. delbrückii, *L. lactis*, *L. leichmannii*,
L. homohiochii.
 - FF. Racemiase positive (DL-formers)..... *Plantarum*-series
L. japonicus, *L. plantarum* (Syn.
L. arabinosus)
 - EE. L-Formers.
 - F. Racemiase positive.
(DL-~DL+L-formers). *Acidophilus*-series
L. acidophilus, *L. saké*, *L. casei*.
 - FF. Racemiase negative (L-formers). *Bulgaricus*-series
L. bulgaricus, *L. xylosus*.
 - DD. Heterofermenters..... Subgenus Beta-Lactobacillus
 - E. Racemiase positive (DL-formers)..... *Brevis*-series
L. brevis α , *L. brevis* β , *L. fermentum* α ,
L. fermentum β , *L. heterohiochii*.
 - EE. Racemiase negative (D-formers)..... *Batatas*-series
L. batatas *L. fructosus*⁽¹²⁾.
 - CC. Catalase positive (L-formers)..... Subgenus Wild-Lactobacillus
L. thermophilus, *L. ciliatus*,
Microbacterium ORLA-JENSEN, So-called
spore-forming lactic acid bacteria.
- AA. GRAM-negative (D-formers). Genus **Escherichia**
E. acidi lactici.

TIEGHEM.*

Positions of Hiochi Bacteria in the Classification of Lactic Acid Bacteria.

The location of the hiochi-bacteria in the classification of lactic acid bacteria may be made clear, if they are inserted in the newly proposed system of lactic acid bacteria (Table 4) by one of the present authors (KITAHARA)⁽¹³⁾.

DESCRIPTION OF NEW SPECIES

Lactobacillus heterohiochii nov. sp.

Round-ended rods, ordinarily $0.7-0.8\mu$ by $2-6\mu$, but occasionally longer cells having $10-20\mu$ can be seen. Occurring singly, in pairs or in chains. Always GRAM-positive, non spore-forming, and non motile. (Fig. 1)

Fig. 1.
L. heterohiochii
 $\times 1,350$
Liver extract-saké medium
 30°C , 12 days.

Solid culture: Colonies in agar media are white glistering pinhead or fist-form. Cells composing the colony are strongly congealed with each other. Stab culture: Filiform or papillate growth, uniformly along the stab-canal, can be seen after 3-5 days, but no surface growth. Streak culture: As a rule no visible growth, but exceptionally slight growth can be observed.

Liquid culture: Liquid medium becomes somewhat turbid after 2-4 days, and reveals silky luster when being shaken. Afterwards a grayish white sediment is gradually formed. When old, the turbidity fades away and there remains a firm sediment at the bottom of the clear liquid.

It does not grow in ordinary culture media, such as glucose-yeast water, glucose bouillon, or milk, but does grow readily in saké. This is due to the essentiality of hiochic acid for the growth of the species. The hiochic acid is considered as a specific constituent of saké.

* A type of microorganism belonging to this species has been known as one of the important acidifiers in the cultivation of 'moto', yeast starter for saké brewing. But owing to the differences in alcohol-resistance and arabinose fermentation, there may be no use to have such a fear that the useful organism for preparation of saké occasionally take a bad turn and attack the newly produced beverage.

Microaerophilic, catalase negative, nitrate not reduced to nitrite, and indole not formed. Readily ferments glucose and fructose, but cannot ferment other carbohydrates such as mannose, galactose, pentoses, oligosaccharides, polysaccharides, sugar alcohols, and glucosides.

Glucose fermentation: Considerable amounts of ethanol, acetic acid and CO_2 are formed besides lactic acid. The yield of lactic acid is about 50 % of sugar fermented. The lactic acid is generally of pure DL-form.

Fructose fermentation: Generally, mannitol is produced as a chief product. Lactic acid, acetic acid, CO_2 and more or less ethanol are also produced.

Growth temperature: Optimum temperature is about 30°C , maximum temperature for the growth is considered to be lower than 40°C .

pH for growth: Markedly acidophilic, optimum pH being 5.0; can grow slightly at pH 6.0, and can never grow at pH 7.0.

Strongly resistant against ethyl alcohol, viz. 13–16 vol. % or more. Willingly grows in saké and renders it undrinkable. This phenomenon is called 'hiochi'.

An overwhelming species which appears as hiochi-bacteria.

Habitat: Spoiled saké.

Lactobacillus homohiochii nov. sp.

Round-ended rods, with $0.7\text{--}0.8\mu$ in breadth and as a rule $2\text{--}4\mu$ and occasionally 6μ in length, the cells longer than 6μ can hardly be observed (Fig. 2).

Fig. 2.
L. homohiochii
 $\times 1,350$
Liver extract-saké medium
 30°C , 12 days.

GRAM-positive, non-spore-forming, and non-motile.

Characters in solid and liquid media, as well as the nutritive requirement toward hiochic acid, are almost identical with those of the foregoing species.

Microaerophilic, catalase negative, nitrate not reduced to nitrite, indole not formed. Ferment glucose, fructose, mannose, maltose, salicin and mannitol, whereas galactose, sucrose, lactose, raffinose, pentoses, polysaccharides, α -methyl glucoside, and glycerol are as a rule not fermented.

Almost quantitative amount of lactic acid is formed from hexoses. The

optical form of the lactic acid is more or less laevorotatory, viz. D- or D+DL-lactic acid.

Growth temperature: Mesophilic, grow and ferment most vigorously at 30°C, generally somewhat at 40°C, but never at 45°C.

pH for growth: Acidophilic, optimum pH lies about 5.0, slightly active at pH 5.5, no growth at 6.0 or higher pH values.

Strongly resistant against ethyl alcohol, viz. 13-16 vol. %. Willingly grows in saké and makes it to 'hiochi'.

One of the chief species of hiochi-bacteria.

Habitat: Spoiled saké.

SUMMARY

Further examinations have been carried out for identification and classification of hiochi-bacteria, from the stand point of lactic acid-bacteriology.

The characteristics of 'true hiochi-bacilli', both homo- and heterofermenters, do not coincide with those of any species which have hitherto been known. So the authors have proposed the names *Lactobacillus homohiochii* and *L. heterohiochii* nov. sp. respectively.

The 64 strains used throughout this research are classified as follows:

Spheres: Heterofermenting D-lactic acid former.

....*Leuconostoc mesenteroides* (1 strain).

Rods: Heterofermenting, as a rule DL-formers.

Hiochic acid independent, xylose is fermented.

....*Lactobacillus fermentum* (4 strains).

Hiochic acid essential, ferment only glucose and fructose.

....*Lactobacillus heterohiochii* n. sp. (44 strains)

Homofermenting

Hiochic acid independent, pentose-fermenting, DL-former

....*Lactobacillus plantarum* (1 strain).

pentoses not fermented, DL+L-former

....*Lactobacillus acidophilus* (3 strains).

Hiochic acid promotive, D+DL-formers.

....*Lactobacillus homohiochii* varieties (4 strains).

Hiochic acid essential, D- or D+DL-formers

....*Lactobacillus homohiochii* n. sp. (7 strains).

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