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https://doi.org/10.5109/23943

出版情報：九州大学大学院農学研究院紀要. 35 (1/2), pp.1-7, 1990-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 35 (1・2), 1-7 (1990)

General Character and Taxonomic Study of *Lactococcus lactis* IO-l, JCM 7638

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(Received *February 1, 1990)*

A mesophilic L-lactate producing coccus, *Lactococcus lactis* IO-1 previously labeled *Streptococcus* sp. IO-1, was isolated and taxonomical study was carried out. This strain grew under anaerobic conditions, however, it was also capable of growing under microaerophilic conditions. In TGC medium, the strain grew with peculiar filamentous shooting from the top the yellow zone (anaerobic zone) to the bottom in the standing test tube and in double layer plate culture, the convex lens shape colony with dark green color was developed after a few weeks culture. Electron micrograph showed that this microbe was ovoid with the size of 0.8-0.9 μm width x 1.1-1.2 μm length. The strain fermented various carbohydrate to produce L-lactate with high conversion rate and no other volatile fatty acid was detected. The optimal temperture for growth as well as fermentation was 37°C and the strain tolerant in 6.5 % NaCl. It grew in bile esculin but it did not possessed Lancefield's D antigen. It possessed Lancefield's N antigen and puoduced nisin like peptide antibiotics which was sensitive to genus *Lactococcus*. The mol % G+C of the DNA was 38%. Glutamic acid was one of the essential amino acid for its growth. DNA-DNA hybridization between this strain and *Lactococcus lactis* NCFB 604^T showed very high homology value as 62-77 %. Thus, this strain was indentified as the variant strain of *Lactococcus lactis*. This strain has been deposited to the Japan Collection of Microorganisms as *Lactococcus lactis* IO-1 JCM 7638.

INTRODUCTION

Lactic acid bacteria are very common microbe and those are closely related to the almost all fields of the human life. There are two types of lactic acid bacteria, rods and coccus. Taxonomy for the rods, mainly belonging to genus *Lactobacillus,* has almost been completed while the coccus, they are so sensitive to be isolated from natural sources, are still one of the unestablished field of the taxonomy and morphology of bacteria. Lactic acid bacteria are particularly important in food industry including the traditional fermentation food. The role of lactic acid bacteria in Japanese traditional fermentation products such as sake brewery (Kitahara et *al.*, 1957), miso and shoyu (Nakano, 1967) are well known. Numbers of *Lactobacillus* acts on the process of traditional Japanese salted vegetable pickles fermentation (nukamisozuke) (Nakano, 1967) but novel *Lactococcus* was ever since isolated from such fermentation products as a screening source. We isolated an organism from the drain pit of the home kitchen in downtown Fukuoka and it is suspected that this microorganism came from common Japanese food. The organism was a homofermentative L-lactate producing coccus with the highest specific growth rate of 1.2 hr^{-1} at 37°C (Ishizaki and Ohta, 1989, Ishizaki *et al.*, 1989). As almost all lactate fermentation bacteria produce mixture of stereochemical isomers of lactic acid, L-lactate is one of the desired products for the current fermentation industry. However, the species used for such fermentation, *Lactococcus cremoris,* needs low temperature as 27°C (JΦrgensen and Nikolajsen, 1987, Bibal *et al., 1988) so* that requires high utility consumption. The isolated strain seemed to be valuable for industrial application of L-lactate production. Phenotypically, this organism did not resemble any established species of the genus *Streptococcus, Lactococcus,* and *Enterococcus* (Mundt, 1986, Schleifer and kilpper-Balz, 1984, Schleifer *et al., 1985).* Thus, we studied the character of this strain to specify the taxonomical position for this novel lactic acid producing coccus.

MATERIALS AND METHODS

Bacterial strain

The strain *Lactococcus Zactis* IO-1 is isolated from the water collected at the drain pit of the sink of home kitchen in Higashi-ku, Fukuoka-shi, Japan in the middle May 1986. A half ml of the sample water was spread directly on the surface of a TGC agar plate and the plates were incubated in a gas-pak at 30°C for two days. Selected colonies were diluted with sterilized saline and TGC liquid medium to prepare double layer solid plate for single colony isolation. A typical convex shape with dark green color was isolated and the strain was purified by repeating the single colony isolation. The purified strain was stored in the TGC liquid medium and the stock culture was transplanted at every two weeks.

Type strains were purchased directly from NCFB (National Collection of Food Bacteria, U. K.), ATCC (American Type Culture Collection, U. S. A.), and NCTC (National Collection of Type Cultures U. K.). Strains used were *Lactococcus lactis* NCFB 604^T, *Lactococcus garvieae* NCFB 2155^T, *Lactococcus raffinolactis* NCFB 617^T, *Streptococcus dysgalactiae* ATCC 27957^T, *Streptococcus bovis* NCFB 579^T, *Streptococccus salivarius* ATCC 7073^T, *Enterococcus faecalis* NCTC 775^T, *Enterococcus faecium* NCTC 7171^T, and *Escherichia coli* ATCC 12435. *Lactococcus lactis* subspecies *cremoris* TUA 13446L (=ATCC 19257) is a kind gift from Department of Agricultural Chemistry, Tokyo University of Agriculture, Tokyo, Japan.

Medium and culture methods

TGC medium (Bacto thioglycolate w/o dextrose dehydrated, Difco Laboratories U. S. A.) was generally used and 10 g/Z of dextrose and 20 g/l of agar-agar were supplemented to TGC basal medium. Fermentation medium was glucose broth consisted of yeast extract 10 g, polypeptone 10 g, sodium chloride 5 g and glucose 10 g in 1 l of deionized water. Anaerobic solid culture were carried out by double layer agar plate in the gas-pak (Oxoid Limited, England) unless otherwise described. Liquid culture without shaking was also applied. Microaerophilic condition were given by shaking culture and surface culture in a ordinary incubator.

Analytical methods

Lactate was analyzed by enzymatic methods using L-lactate dehydrogenese and n-lactate dehydrogenese. The enzyme were purchased from Boehringer Mannheim Yamanouchi Co. Ltd. Optical density of the reacted solution was observed by spectrophotometer with the wave length of 340 nm (Okada et al., 1967). L-lactate determination was also carried out by the YSI lactate analyzer (model 23L, YSI, U. S. A.). Identification and determination of other organic acids were carried out by the HPLC using the Hitachi Model 655A analyzer with the column Hitachi # 2618 (cation exchange resin) 8 mmϕ x 500 mm. Elution was carried out with 0.1% phosphoric acid at 60°C. Optical absorbance was monitored at a wave length of 210 nm.

Electron microscopy

Cells grown on the TGC plate were harvested at the late logarithmic phase and suspended in deionized water. The cell suspension was negatively stained with potassium phosphotungustate (pH 6.0), and placed on grids coated with collodioncarbon. Electron micrograph was taken with a JEM-100B electron microscope (Japan Elecron Optics Laboratory Ltd. Japan) (Ogata et al., 1980).

Procedure for biochemical characterization

Biochemcal characterization of the IO-1 strain was carried out according to the description of Facklam and Carey (1985). Amino acids requirement was determined according to the method described by Tsunoda (1954).

D and N antigen were detected by the Slidex Strepto-kit (BioMerieux, France) and antiserum of Wellcome Laboratory, respectively.

The mol % of G+C of DNA was determined by the HPLC using Hitachi Model 638-30 with the column packed 10C18. DNA extracted by the Marmur's method (Marmur, 1961) was hydrolyzed by P1 nuclease and then alkaline phosphatase according to the method described by Tamaoka and Komagata (1984). DNA base compositon was determined by the HPLC using the Hitachi Model 638-30 with column Nakarai 4.6 mmϕ x 150mm packed 10C18. Elution was carried out with 0.1 M ammonium phoshpate at room temperature at a flow rate of 1.5 ml/min. Optical absorbance was monitored at a wave length of 260 nm using the Shimadzu Model SPD -6A UV detector. The equimoler four mixtures of nucleotides (Yamasa, Japan) was used as the standard (Kaneko et al., 1986).

Quantitative hybridization was carried out in microdilution plate described by Ezaki et *al.*, *(1988,* 1989). Briefly, hybridization experiment was carried out at 37°C in 2 XSSC (Saline-Trisodium-citrate) (0.3 M NaCl and 0.03 M sodium citrate), 5 × Denhardt solution, 50 % formamide for 2 h.

RESULTS AND DISCUSSION

General character and morphorogy

The isloated strain was Gram-positve coccus and ovoid in shape with the size of 0.8-0.9 μm width x 1.1-1.2 μm length according to the electron micrograph (Fig. 1) The convex lens colony with deep green color was formed in the double layer of TGC agar after a week incubation in the gas-pak, however, the size and color of the colony

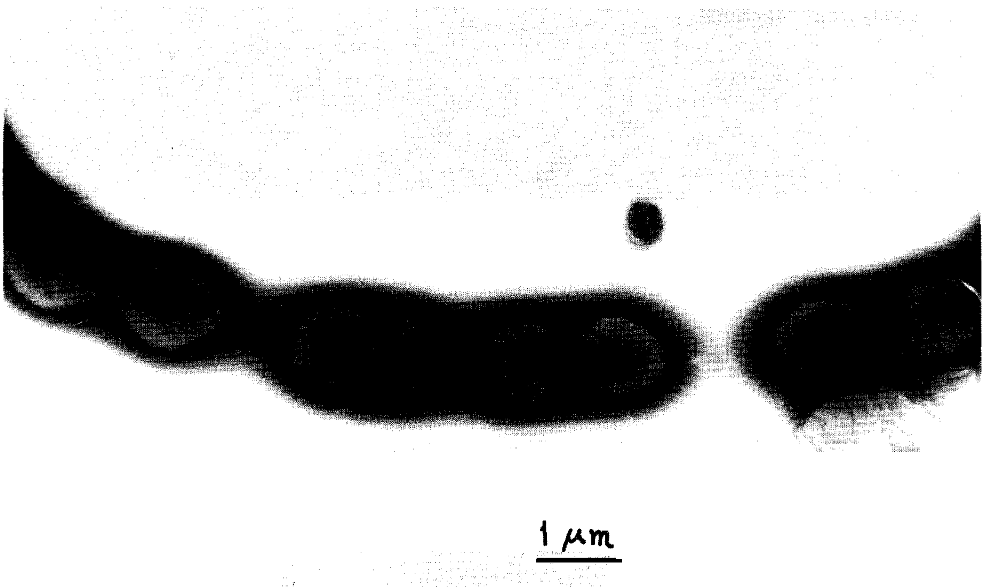

Fig. 1. Electron micrograph of *Lactococcus lactis* IO-1, JCM 7638

were slightly different from the type strains *L. lactis* NCFB 604^T, *L. lactis* subspecies *cremoris* TUA 1344L, *L.* ***garvieae*** NCFB 2155^T and *Enterococcus faecalis* NCTC 775^T. Growth of the standing culture using TGC liquid in the test tube were limited within the anaerobic zone and no growth was observed at the upper layer of the medium where the color was green due to oxydized methylene blue. Microorganism growth in TGC liquid developed a peculiar filamentous shooting from the upper layer to the bottom. However, organism is also capable of growing under microaerophilic condition by means of shaking culture using glucose broth.

The lowest temperature for growth was 10°C while the highest was 45°C, however optimal temperature was 37°C with the highest specific growth rate 1.2 h^{-1} (Ishizaki and Ohta, 1989).

Biochemical characterization

Catalase was negative. It was tolerant to 6.5 % NaCl. It grew on bile esculin but none of hemolysis was observed. pH range for possible growth was the range between 4.5-10. The cells survived after heating to 60°C for 30 min. Those results suggested that the strain resembled to genus *Enterococcus.* However, the strain did not carry Lancefield's D antigen. The strain carried Lancefield's N antigen. The results of carbohydrate assimilation (Table 1) did not introduce the definite information to determine the genus of this strain. Glutamic acid, Leucine and Valine were found as essential amino acids for growth and this agreed well with the results for *L. lactis* reported by Marshall and Law (1984). In 1 % glucose broth, the strain dropped pH of the medium lower than 4 within a day and finally converted more than 90 % of

Table 1. Carbon source assimilation and acid formation of *Lactococcus lactis* IO-1

Carbon source	Growth	Acid formation	Final pH
Arabinose			
Cellobiose		+	4.3
CMC		−	
β-Cyclodextrin	+	+	4.6
Dulcitol	−		
Erythritol	n. t.		
Esculin	+	+	
Fructose	+	+	4.2
Fumaric acid	+		
Galactose	+	+	4.2
D-a-Galacturonic acid	+	+	6.0
Glucose	+	+	4.2
Inositol			
Inulin			
Lactic acid			
Lactose	+	+	4.9
Lignin			
Malic acid		−	
Maltose		+	4.3
Mannitol		+	4.5
Mannose		+	4.3
Melibiose	−		
Methy1-β-D-glucoside	+	+	4.5
Polygalacturonic acid	n. t.		
Raffinose			
Rhamnose			
Salicin	+	+	4.8
Soluble starch			
Sorbitol			
Succinic acid			
Sucrose	+	+	4.1
Treharose	+	+	
Xylitol	n. t.		
Xylose	+	+	4.6

Symbols (+) indicate clear growth and (−) indicate no growth and not very clear growth.
The abbreviation n. t.means “not tested”.

glucose into L-lactic acid. No other significant volatile fatty acids were detected in the cultured broth. The strain produced peptide antibiotics which is not nisin but similar to nisin to which other lactococci and some species of genus Bacillus and *Clostridium* were sensitive. Mol % of G+C of the DNA of the strain was 38 % and it resembled to the value for *L. lactis* ATCC 19435^T (Mundt, 1986). From those facts, the strain 10-l resembled to the genus *Lactococcus*, however, these phenotypic chracterization was not enough to assign the taxonomic position to the strain.

DNA-DNA hybridization

Quantitative hybridization among the selected strains of the genus *Enterococcus*, *Streptococcus* and *Lactococcus* clearly indicated that the 10-l strain is genetically more related to lactococci than to enterococci. The homology value between the type

Table 2. Determination of homology values for the strain IO-1 and *Lactococcus lactis* NCFB 604^T against the selected strains.

Unlabeled competitive DNA from	Homology value (%) Labeled DNA from	
	IO-1	*L. lactis* NCFB 604^T
IO-1	100	62
L. lactis NCFB 604'	77	100
L. garvieae NCFB 2155^T	15	15
L. raffinolactis NCFB 617^T	9	4
S. *dysgalactiae* ATCC 27957^T	0	8
S. bovis NCFB 597^T	1	6
S. *salivarius* ATCC 7073^T	1	6
E. faecalis NCTC 775^T	0	2
E. faecium NCTC 7171^T	0	1
Escherichia coli ATCC 12435	0	0

Lactococcus, *Streptococcus* and *Enterococcus* are abbreviated as *L.*, *S.* and *E.* respectively.

strain of *L. Zactis* NCFB 604^T and 10-l strain was in a range from 62-77% at optimal condition (Table 2). Thus, we identified the IO-1 as the variant strain of *Lactococcus lactis* NCFB 604^T. Since the optimal temperature of the strain *L. Zactis* subspecies *cremoris* for L-lactate production was between 27°C and 30°C (Rogers *et al.,* 1978), the strain IO-l is more mesophilic so that this strain is more favor to industrial application of L-lactete fermentation.

Acknowledgements

The authors express their sincere gratitude to Dr Takayuki Ezaki, associate professor for microbiology, school of medicine, Gifu university, for his instruction and guidance of antigen test and DNA-DNA hybridization.

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