Characterization of *Lactococcus garvieae* isolated from radish and broccoli sprouts that exhibited a KG^+ phenotype, lack of virulence and absence of a capsule

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Running: *L. garvieae* isolated from sprouts.

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ABSTRACT

AIMS: To identify isolates of Lactococcus garvieae from radish sprouts and broccoli sprouts, and compare these isolates with virulent strains obtained from yellowtail and the less virulent mutant strain for capsulation and virulence.

METHODS AND RESULTS: Six isolates were isolated from the radish sprouts and broccoli sprouts, and comparative 16S rRNA gene sequence analysis of the six isolates indicated that all the strains were *L. garvieae* (the percentage similarity among all the strains was >99%) The characterizations of these six strains were compared with isolates, KG9502, Lg2 and ATCC49156, obtained from yellowtail. We obtained the mutant Lg2-S after subculturing the strain Lg2 on Todd-Hewitt Agar (THA) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) four times. The mutant Lg2-S showed less virulence than Lg2 (wild type). Biochemical characterization of the six strains is almost the same as those of strains KG9502, Lg2, ATCC49156 and Lg2S, except for acidification of saccharose and tagatose and the activity of the enzymes hippuricase（HIP）. All the isolates from sprouts had no susceptibility for phages and were non- virulent to yellowtail. Pulsed-field gel electrophoresis (PFGE) analysis indicated heterogeneity among isolates obtained from different species. By transmission electron microscopy (TEM), isolates from both sprouts, ATCC49156 and Lg2-S have no capsule, while KG9502 and Lg2 have capsule.

CONCLUSIONS: *L. garvieae* strains were successfully isolated from the radish sprouts and broccoli sprouts in Japan. These strains showed no pathogenicity for yellowtail, KG+ phenotype without capsule. The less virulent mutant Lg2-S derived from virulent strain Lg2 was KG+ phenotype without capsule and the fact is similar to the isolates from sprouts. **SIGNIFICANCE AND IMPACT OF THE STUDY:** This study is the first report of the isolation of *L. garvieae* from the terrestrial plant, and isolates from both sprouts showed

genetic diversity, but have no pathogenicity for yellowtail and no capsule.

Keywords: *Lactococcus garvieae*, Japan, radish sprouts, broccoli sprout, Seriola, phathogenicity, Phage and pulsed-field gel electrophoresis (PFGE).

INTRODUCTION

Lactococosis caused by *Lactococcus garvieae* has been one of the most important diseases affecting the genus *Seriola* which includes yellowtail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), and kingfish (*Seriola lalandi*), with respect to the amount of damage it causes (Kitao 1982; Kusda *et al.* 1991) in Japan. The first outbreak of lactococcosis in the genus *Seriola* was occurred in a limited area of Shikoku Island, in1974 and the following year, it spread across western Japan (Kitao 1982). Still now the source and route of infection have not been cleared.

L. garvieae has also been isolated from cow and buffalos with mastitis and clinical specimens of human blood, urine, and skin in Europe and America. (Aguirre and Collins 1993; Doménech *et al*. 1993; Eldar *et al*. 1996; Texeira *et al*. 1996; Fefer *et al.* 1998; James, Hardman, and Patterson 2000; Vela *et al*. 2000). Now, *L. garvieae* is considered as an emerging pathogen of increasing clinical significance in the fields of fishery and veterinary and human medicine. However, there has been no report about *L. garvieae* isolated from any terrestrial plant or the other environmental materials still now.

In this study we isolated putative *L. garvieae* strains from radish sprouts and broccoli sprout among the research of presence of lactic acid bacteria in the vegetables. It was very important to investigate characteristics, especially virulence, of isolates from unprecedented origin, because *L. garvieae* is a microorganism which certainly has pathogenicity for *Seriola* and has the possibility of pathogen for human and animal as above mentioned.

There is a little report about the factor of pathogenicity of *L. garvieae* for yellowtail. To confirm the factor of pathogenicity we obtained the less-virulent strain by subculture a virulent isolate on THA supplemented with TTC like as before reported (Ooyama *et al,* 1999) and compared the various characters among isolates from sprouts, virulent isolates and less virulent.

In this study, we identified isolates from sprouts as *L. garvieae* by DNA sequence analysis of the 16S rRNA gene and compared those with isolates from yellowtail and less virulent mutant in biochemical characterisation, their susceptibility for three types of bacteriophages, their PFGE pattern, their KG+ and KG- phenotype, pathogenicity for yellowtail and mouse and the presence of capsule by TEM.

MATERIALS AND METHOD

Bacterial strains

Ten isolates of *L. garvieae* were studied (Table). Four isolates were obtained from radish sprouts and two from broccoli sprout in Japan. Both sprouts were cultivated in hydroponics plants and sampled at different days in 2002. 1g of each sprout was homogenate with a drop of distilled water and diluted by 100 ml saline. Then the saline was plated on MRA (Oxoid) agar containing 10 p.p.m. sodium azide and 10 p.p.m. cyclohezimide and was then incubated. Colonies were picked on MRS agar and maintained on MRS agar containing 0.5% $CaCO_3$. The isolates were putatively identified as *L. garvieae* based on Gram staining; growth on Bile Esculin Azide Agar (BEA) (Becton, Dickinson and Company, France); α-hemolysis on a Todd Hewit Broth (THB) (Becton) plate, supplemented with 5% defibrinated sheep blood and 1.5% agar; and the polymerase chain reaction (PCR) with specific primer as previously described (Zlotkin *et al.* 1998). Also we used the KG9502:KG- phenotype (Ooyama *et al* 2002), Lg2:KG-phenotype (Kawanishi *et al* 2005) and ATCC49156:KG+ phenotype (American Type Culture Collection Manassas, USA) strain isolated from yellowtail suffering from lactococosis in Japan. Lg2-S (KG+ phenotype) which

is obtained after subculture Lg2 on Todd Hewit Agar (THA) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) as previously described by Ooyama et al (1999) was also used. These isolates were cultured in THB for 20 h at 25˚C, suspended in 10% skimmed milk (Becton) solution, and then stored at –80˚C until they were used for the tests.

Biochemical and enzymatic characterization Biochemical and enzymatic tests were performed with the Rapid ID 32 Strep and API ZIM systems (bioMérieux, Japan Tokyo) by following the manufacturer's instructions, except for the temperature of incubation, which was always 30°C.

16S rRNA sequencing. Isolates from sprouts were further characterized by 16S rRNA sequencing. DNA preparation was performed using Insta Gene Matrix (Bio-Rad, Hercules, CA, USA). A 1-µl volume of the lysates was used for PCR; 1.5-kb fragments comprising the entire 16S rRNA open reading frame were amplified using primers 16s-fl-3 (5'- AGA GTT TGA TCC TGG CTC AG-3') and 16s-rl-3 (5'-ACG GTT ACC TGT TAC GAC TT-3') and *Taq* polymerase (TaKaRa, Kyoto.). PCR conditions consisted of 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30sec, followed by a hold at 72°C for 10 min. PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, Calif.) and they were sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems,) and an automatic DNA sequencer (model 310; Applied Biosystems). Sequencing was performed using primers 16s-fl-3 and 16s-rl-3. BLASTN search analysis was

used to compare the obtained sequence data with 16S rRNA sequence data deposited in GenBank.

Susceptibility to Phages

L. garvieae bacteriophage strains PLgY-16, PLgY-30, and PLgW-1 were used in this study (Park *et al.* 1997, 1998). The phages were propagated in *L. garvieae* NSS9310 (indicator bacterium) by the double agar-overlay method (Paterson *et al.* 1969), and the susceptibilities of the *L. garvieae* strains were then assessed by plaque formation by using the same method.

Analysis of chromosomal DNA restriction patterns by PFGE

In case of PFGE, DNA-embedded agarose blocks were prepared and endonuclease digestion was performed as described previously (Kawanishi *et al* 2005). The restriction endonuclease *Apa* I (TaKaRa, Kyoto, Japan) were used for digestion. The blocks containing the digested DNA were placed on 1% PFGE–certified agarose gel (Bio-Rad) and migration was performed for 21 h using CHEF-DRIII (Bio-Rad), with ramped pulse times of 0.1–25 s in order to obtain an optimal separation of the digested fragments.

The fingerprints generated with the standardized protocol were evaluated using Molecular Analyst Software Fingerprinting Plus, version 1.0 (Bio-Rad). A 0.5% band tolerance was selected for use during the comparisons of the DNA profiles. Cluster analysis was performed by the unweighted pair group method using arithmetic averages (UPGMA), and DNA relatedness was calculated based on the Dice coefficient.

Agglutination with antiserum against KG+ and KG－ phenotype

The phenotype was confirmed by the reaction with the immune sera, which were raised in rabbits as described by Yoshida *et al*. (Yoshida *et al.* 1996), against the KG9502, KG － phenotype (Ooyama *et al.* 1999) and NSS9310, KG+ phenotype (Ooyama *et al.* 2002). The agglutination was confirmed by a slide agglutination reaction.

Confirmation of cell capsule by TEM

KG9502, Lg2, Lg2-S, ATCC49156, NRIC0607, NRIC0612 and NRIC0611 were selected as representative isolates from *Seriola* and sprouts for TEM, respectively. TEM was performed as described by Yoshida et al. (1997) with a brief modification. Briefly, strains were grown overnight in 10 ml of THB. The bacteria were suspended in 0.3% formaldehyde solution and held overnight at 4℃. They were washed 3 times with PBS and resuspended in 10ml of homologous yellowtail antiserum (aggutlination titer above 1:512) diluted 200 times with PBS and incubated for an additional hour before staining with 0.15% ruthenium red in 0.1mol l^{-1} cacodylate buffer, pH 7.4 for 2h. Bacterial cells were embedded and cut into thin sections as described by Yoshida et al. (1997).

Challenge experiments

Pathogenicity in yellowtail

The bacteria were cultured in THB supplemented with 1.5% agar (THA) at 25C for 24 h. A bacterial suspension was prepared in phosphate-buffered saline (PBS) at a final concentration of about 10^9 cells/mL by optical density (620nm, 1cm path length), and viable counts were determined by plating on THA. The virulence of each strain was tested using 10 fishes (approximately 100 g body weight) that were inoculated intraperitoneally (i.p.) with 0.1 mL of each bacterial suspension; 1×10^9 CFU/mL. Fish were held for 14 d in a 200-L tank at

24–26C with continuous aeration. In all cases, the visceral organs (kidneys, livers, and brains) of both dead and surviving fish were analysed for the presence of *L. garvieae*. In addition, we calculated the 50% lethal dose (LD_{50}) by Probit method (Finney 1971) values of isolates KG9502, Lg2 and Lg2-S.

Virulence in mice

In order to assess the pathogenicity of *L. garvieae* in mammals, a mouse pathogenicity assay was performed using 10 ddY mice (4-week-old, female). These mice were i.p. inoculated with 0.1 mL of each strain (10^8 cells). The mice were maintained at 22C for 14 days in separate cages. Control animals received injections of 0.1 mL PBS.

RESULTS

All 10 isolates showed were Gram positive ovoid cells that formed short chains, grew on BEA agar, exhibited α-hemolysis, and gave the expected 1100-bp PCR amplification product that is species-specific to *L. garvieae*. In brief, all 10 isolates were putatively identified as *L. garvieae.*

Biochemical and enzymatic tests by Rapid ID 32 Strep and Api zym showed little biodiversity among the isolates except for acidification of saccharose, tagatose, and the presence of the enzymes hippuricase（HIP）(Table).

The sequence of 16S rRNA was aligned with *Lactococcus garvieae* sequences. 100% similarity of sequences was shown between the isolates from radish sprouts and *Lactococcus garvieae*, only one bace differences was shown between the isolates from broccoli sprouts and *Lactococcus garvieae*.

Susceptibility of each strain to bacteriophages PLgW-1, PlgY-16, and PLgY-30 is show in Table. None of the isolates obtained from radish sprouts and broccoli sprout were susceptible to any of the three bacteriophages. While, all isolates obtained from yellowtail and Lg2-S were found to be susceptible to bacteriophage PLgW-1, PLgY-16 and PLgY-30.

The results of the genomic analysis by PFGE after digestion with *Apa*I are shown in Fig. 1. The isolates from radish sprouts showed homogenecity, while isolates from broccoli sprouts were heterogenicity. And the similarity level between isolates from different species obtained by dendrogram based on cluster analysis was less than 30%.

All isolates from radish sprouts, broccoli sprout, ATCC49156 and Lg2-S strains were agglutinated by anti-KG+ phenotype cell serum, but Lg2 and KG9502 isolates were not agglutinated. All isolates were agglutinated by the anti-KG－ phenotype cell serum. Based on these results, it was determined that isolates from both sprouts, Lg2-S and ATCC49146 were KG+ phenotype strains; and the other 2 isolates were KG－.

None of the isolates obtained from radish sprouts and broccoli sprouts and ATCC49156 showed a distinct lethality for yellowtail. The fish that survived the challenge did not harbor the pathogen (strain) in their visceral organs (brain, kidney, and liver) 2 weeks after inoculation. KG9502, Lg2 showed strong virulence in yellowtail; the mortality was ~~more than~~ 100% after i.p. inoculation with 10^8 CFU/fish, and the LD_{50} values of Lg2 and KG9502 strains were less than 10^2 CFU/fish. Lg2-S showed no mortality at 10^2 CFU/fish, 10^4 CFU/fish and 10^6 CFU/fish i.p. inoculation but showed 40% mortality at 10^8 CFU/fish.

The results obtained in the mouse pathogenicity assay indicated that no strain

showed an obvious pathogenicity in mice.

We confirmed that KG9502 and Lg2 had capsule under the TEM, whilst Lg2-S, ATCC49156, NRIC0607, NRIC0612 and NRIC0611 had no capsule (Table and Fig.2)

DISCUSSION

Result from biochemical characterisation and determination of the 16SrRNA sequence isolates from radish sprouts and broccoli sprouts identified as *L. garvieae*. This is first report of *L. garvieae* isolate obtained from terrestrial plant.

There are a little difference in biochemical characterization among isolates from radish sprouts, broccoli sprouts and *Seriola*. Isolates from broccoli sprouts have the enzyme HIP and acidifiability of SAC, and those from radish sprouts have acidifiability of TAG. This biodiversity show the adaptation of the different environment and this ability might be needed to inhabit in these terrestrial plant.

Bacteriophages PLgW-1, PlgY-16, and PlgY-30 have been isolated and used in the typing of *L. garvieae* strains isolated from yellowtail（Park K. *et al.* 1997, 1998）None of the isolates from sprouts were susceptible to the bacteriophages, while isolates from *Seriola* show susceptibility to all Phages. These differences may be hypothesized that there might be difference in surface components of *L. garvieae* among the isolates obtained from the genus *Seriola* and those obtained from the other animals.

According to PFGE patterns, isolates obtained from sprouts were genetically not related to those from yellowtail. Previous study including Lg2 strain (Kawanishi *et al.* 2005) demonstrated that *L. garvieae* isolates from the genus *Seriola* from wide area in Japan have

homogeneity in the PFGE pattern. So, these isolate from radish sprouts and broccoli sprouts might be no epidemiological relation with isolates from *Seriola* prevalent in Japan. Vela *et al.* (2000) reported the analysis of PFGE pattern obtained by *Apa* Ⅰ digestion of *L. garvieae* isolates obtained from humans, cows, buffalos, trout and showed genetic diversity of isolates from terrestrial animal (human, cows buffalos) and genetic homogenesity of those from aquatic animal (trout). There is difference between radish sprouts and broccoli sprouts and also there is difference in each isolates from broccoli sprouts though the broccoli sprouts were sampled in same farm. These facts demonstrated the presence of genetic diversity of terrestrial plants as well as terrestrial animal but different from the homogenesity of isolates from aquatic animal (*Seriola* and trout).

Concerning about virulence, it was interesting to note that *L. garvieae* strains isolates from sprouts and ATCC49156 were non-pathogenic to yellowtail, while Lg2 and KG9502 have strong virulence and Lg2-S have less virulence.

L. garvieae isolated from yellowtail was divided into KG+ (noncapsulated) and KG－(capsulated) strains. Moreover, it has been reported that the capsule contributes to virulence of *L. garvieae* isolated form *Seriola* (Yoshida et al. 1997) and the capsule plays roles invirulence with resistace to opsonophagocytosis by the head kidney phagocytes of yellowtail (Yoshida et al. 1997).

In this study Lg2-S was obtained by subculturing Lg2 on THA with TTC and Lg2-S showed less virulence than Lg2. Lg2 and KG9502 were KG- phenotype with capsule but Lg2-S and ATCC49156 were KG+ phenotype without capsule. ATCC49156 might lose capsule and virulence due to repeated culturing on the media with TTC like as Lg2-S. As for isolates from radish sprouts, broccoli sprouts, they showed KG+ phenotype without capsule and the fact is same to the less virulent strain, Lg2-S and no virulent strain ATCC49156.

With regard to avirulent ~~of~~ isolates obtained from sprouts, it was not confirmed whether these isolates have no pathogenicity for yellowtail from the moment of isolation or they previously had pathogenicity, but lost it due to repeated culturing. However, the facts that TTC was not used for isolation from radish sprouts and broccoli sprouts, the number of cultivation is limited and there are the differences in PFGE pattern and phage susceptibility between isolates from sprouts and *Seriola* might support the former idea.

Recently, clinical cases associated with *L. garvieae* infection have been reported in both humans and animals (Aguirre and Collins 1993; Doménch *et al*. 1993; Eldar *et al.* 1996; Texeira *et al*. 1996; Fefer *et al.* 1998; James, Hardman, and Patterson 2000; Vela *et al*. 2000). However, these were sporadic cases and it is not clear whether *L. garvieae* is pathogen or not. In this study, all strains isolated showed no obvious pathogenicity in mouse. Additionally, in Japan, there is no report of *L. garvieae isolate* being isolated from human clinical cases, although fish of the genus *Seriola*, radish sprouts and broccoli sprouts are consumed raw. These facts might suggest that *L. garvieae* from *Seriola* and radish sprouts and broccoli sprouts have not pathogenicity for terrestrial animals. Further studies that include isolates from a human clinical case are required to determine the pathogenicity of *L. garvieae* in terrestrial animals.

Result from the sequence of 16S rRNA, biochemical characterization and isolates from radish sprouts and broccoli sprouts were certainly identified as *L. garvieae,* however the results of susceptibility to the phages, PFGE pattern and virulence in yellowtail demonstrate that *L. garvieae* isolated from sprouts have different characteristics from the isolates obtained from the genus *Seriola* in Japan. Therefore there is no epidemiological relation between isolates from sprouts and *Seriola* in Japan. But this report demonstrates

that *L. garvieae* is present not only in animal but also in terrestrial plant and have genetic diversity than isolates from *Seriola*. And also, *L. garvieae* from sprouts have no virulence for yellowtail and showed KG+ phenotype without capsule as well as less virulent mutant.

In order to clarify the epidemiological relationship among aquatic and terrestrial animals, and terrestrial plant and factor of pathogenicity for yellowtail larger epidemiological study, biochemical and gene analysis are required.

REFERENCES

Aguirre, M. and Collins, M.D. (1993) Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology* **75**, 95-107.

Cai Y., Ng LK. And Farber JM. (1997) Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean-sprouts. J Appl Microbiol 83, 499-507.

Domenech, A., Prieta, J., Fernandez-Garayzabal, J.F., Collins, M.D., Jones, D., Dominguez, L. (1993) Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. *Microbiologia* **9**, 63-68.

Eldar, A., Ghittino, C., Asanta, L., Bozzetta, E., Goria, M., Prearo, M. and Bercovier, H. (1996) *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. *Current Microbiology* **32**, 85-88.

Elliott, J.A. and Facklam, R.R. (1996) Antimicrobial susceptibilities of Lactococcus lactis and Lactococcus garvieae and a proposed method to discriminate between them. *J Clin Microbiol*. **34**:1296-8.

Fefer, J.J., Ratzan, K.R., Sharp, S.E. and Saiz, E. (1998) *Lactococcus garvieae* endocarditis: report of a case and review of the literature. *Diagnostic Microbiology and Infectious Disease* **32**, 127-130.

James, P.R., Hardman, S.M. and Patterson, D.L. (2000) Osteomyelitis and possible endocarditis secondary to *Lactococcus garvieae*: a first case report. *Postgraduate Medicine* **76**, 301-303.

Kawanishi M., Kojima, A., Ishihara, K., Esaki, H., Kijima, M., Takahashi, T., Suzuki, S., and

Tamura (2005) Drug resistance and pulsed-field gel electrophoresis patterns of *Lactococcus garvieae* isolates from cultured Seriola (yellowtail, amberjack and kingfish) in Japan. *Letters in Applied Microbiology*. **40:** 322-328.

Kitao, T. (1982) The methods for detection of *Streptococcus* sp. Causative bacteria of streptococcal disease of cultured yellowtail (*Seriola quinqueradiata*). *Fish Pathology* **17**, 17-26 (in Japanese).

Kusuda, R., Kawai, K., Salati, F., Banner, C.R. and Fryer, J.L. (1991) *Enterococcus seriolicida* sp. nov., a Fish Pathogen. *International Journal of Systematic Bacteriology* **41**, 406-409.

Ooyama, T., Kera, A., Okada, T., Inglis, V., and Yoshida, T. (1999) The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae. Dis. Aquat. Organ*. **37,** 121-126

Ooyama, T., Hirokawa, Y., Minami, T., Yasuda, H., Nakai, T., Endo, M., Ruangpan, L. and Yoshida, T. (2002) Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Organ.* **51,** 169-177.

Park, K., H., Matsuoka, S., Nakai, T, Muroga, K. (1997) A virulent bacteriophage of *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) isolated from yellowtail *Seriola quinqueradiata*. *Dis Aquat Org* **29**,145-149.

Park K., H., Kato, H., Nakai, T., Muroga, K. (1998) Phge typing of *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) a pathogen of cultured yellowtail. *Fish Sci* (Tokyo) **64**, 62-64.

Paterson, W., D., Douglas, R., J., Grinyer, I. and McDermott, L., A. (1969) Isolation and preliminary characterization of some Aeromonas salmonicida bacteriophage. *J Fish Res Board Can* **26**, 629-632.

Teixeira, L.M., Merquior, V.L., Vianni, M.C., Carvalho, M.G., Fracalanzza, S.E., Steigerwalt, A.G., Brenner, D.J. and Facklam, R.R. (1996) Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. *International Journal of Systematic Bacteriology* **46**, 664-668.

Vela, A.I., Vazquez, J., Gibello, A., Blanco, M.M., Moreno, M.A., Liebana, P., Albendea, C., Alcala, B., Mendez, A., Dominguez, L. and Fernandez-Garayzabal, J.F. (2000) Phenotypic and genetic characterization of *Lactococcus garvieae* isolated in Spain from lactococcosis outbreaks and comparison with isolates of other countries and sources. *Journal of Clinical Microbiology* **38**, 3791-3795.

Yosida, T., Eshima, T., Wada, Y., Yamada, Y., Kakizaki, E., Sakai, M., Kitao, T., Inglis, V. (1996) Phenotypic variation associated with an anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*. *Disease of Aquatic Organism* **25**, 81-86.

Zlotkin, A., Eldar, A., Ghittino, C. and Bercovier, H. (1998) Identification of *Lactococcus garvieae* by PCR. *Journal of Clinical Microbiology* **36**, 983-985.

Table

Title; Data on the *L.garvieae* strains analyzed in this study

Footnote;

*KG+; Isolate that was agglutinated with both anti KG+ phenotype and anti KG- phenotype serum. KG-; isolate that was agglutinated with only anti KG- phenotype serum.

Fig. 1

Title; Dendrogram of *L.garvieae* isolates based on UPGMA cluster analysis

Fig. 2

Title; Electron micrographs of *L. garvieae*

Arrow pointed the capsule. (a) Lg2 (capsule +) (b) Lg2-S (capsule -) (c) NRIC0607 (capsule -) (d) NRIC0611 (capsule -)

Fig.1

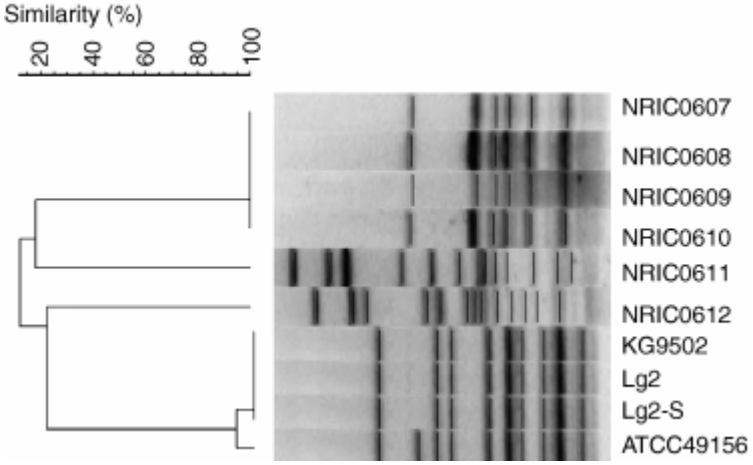
Similarity (%)
20
40
60
80
100
NRIC0607
NRIC0608
NRIC0609
NRIC0610
NRIC0611
NRIC0612
KG9502
Lg2
Lg2-S
ATCC49156

Fig.2

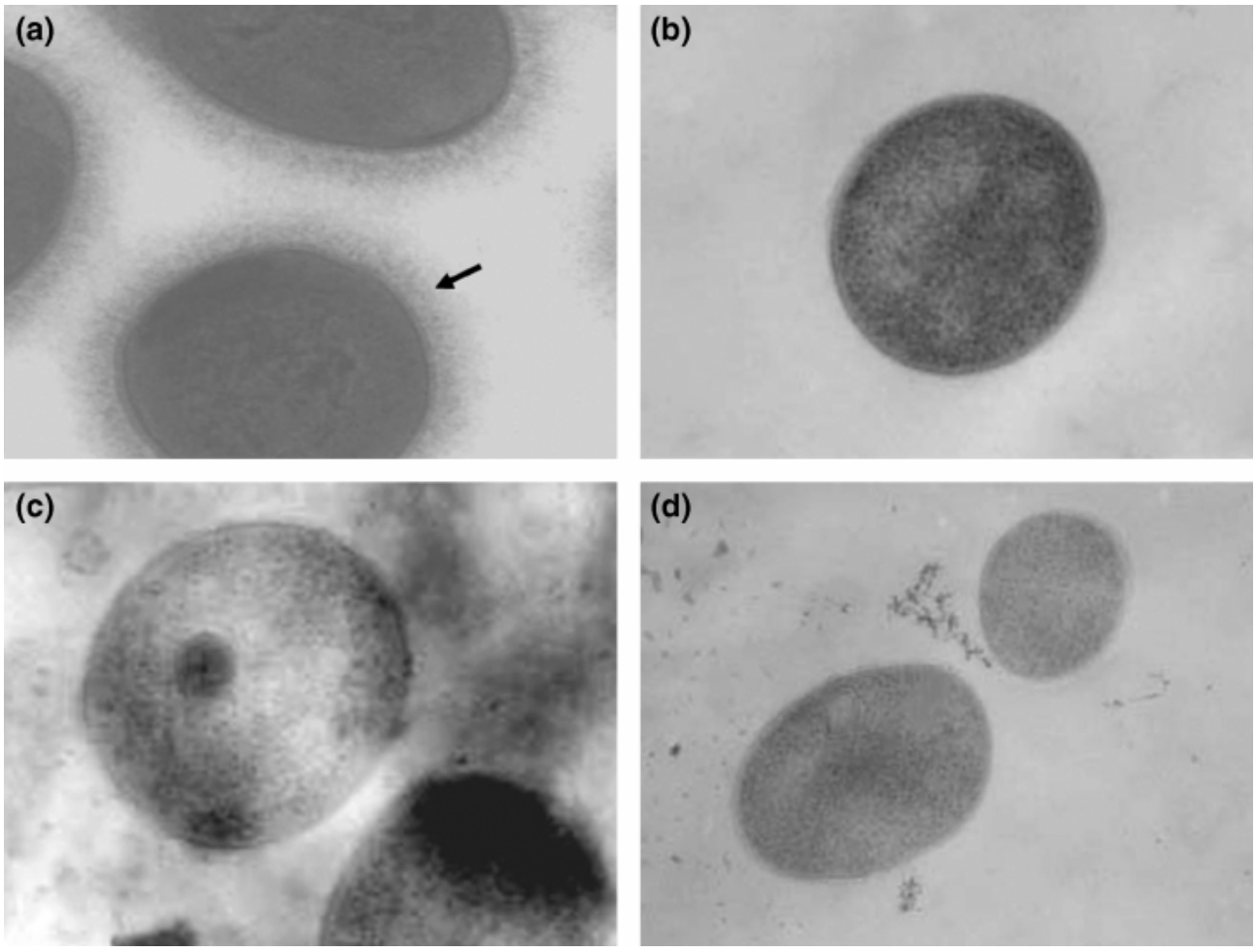
(a)
(b)
(c)
(d)

Table 1

Table 1 Data on the *L. garvieae* strains analysed in this study

Isolate(S)	Source	Year of isolation	Phenotypic characteristics			Sensitivity against phages			Mortality			Phenotype*
			Saccharose	Tagatose	Hippuricase	PLgW-1	PLgY-16	PLgY-30	Yellowtail	LD50	Mice	
NRIC0607	White radish sprout	2002	–	+	–	–	–	–	0/10		0/10	KG^+ ($capsule^-$)
NRIC0608	White radish sprout	2002	–	+	–	–	–	–	0/10		0/10	KG^+
NRIC0609	White radish sprout	2002	–	+	–	–	–	–	0/10		0/10	KG^+
NRIC0610	White radish sprout	2002	–	+	–	–	–	–	0/10		0/10	KG^+
NRIC0611	Broccoli sprout	2002	+	–	+	–	–	–	0/10		0/10	KG^+ ($capsule^-$)
NRIC0612	Broccoli sprout	2002	+	–	+	–	–	–	0/10		0/10	KG^+ ($capsule^-$)
KG9502	Yellowtail	1995	–	–	–	+	+	+	10/10	$<10^2$ CFU/Fish	0/10	KG^- ($capsule^+$)
Lg2	Yellowtail	2002	–	–	–	+	+	+	10/10	$<10^2$ CFU/Fish	0/10	KG^- ($capsule^+$)
Lg2-S	Yellowtail	–	–	–	–	+	+	+	4/10	$>10^8$ CFU/Fish	0/10	KG^+ ($capsule^-$)
ATCC49156^T	Yellowtail	1974	–	–	–	+	+	+	0/10		0/10	KG^+ ($capsule^-$)

*KG^+; isolate that was agglutinated with both anti KG^+ phenotype and anti KG^- phenotype sera. KG^-; isolate that was agglutinated with only anti KG^- phenotype serum.