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Utilization of lactose and presence of the phospho-β-galactosidase (*lacG*) gene in *Lactococcus garvieae* isolates from different sources

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Summary. This study evaluates the utilization of lactose (Lac) and the presence of the phospho-β-galactosidase (*lacG*) gene as markers for distinguishing between fish ($Lac^-/lacG^-$) and dairy isolates ($Lac^+/lacG^+$) of *Lactococcus garvieae*, using a panel of *L. garvieae* isolates from different sources. None of the fish isolates produced acid from lactose (Lac^-), however $Lac^-/lacG^-$ isolates were observed in pigs, cows, birds and humans. Most of the dairy isolates (77.8%) were $Lac^+/lacG^+$, but some dairy isolates did not produce acid from this sugar. Data in the present study show that the ability to metabolize lactose and the presence of the *lacG* gene are heterogeneously scattered among *L. garvieae* isolates of different sources. Therefore, the use of these criteria as markers to differentiate between *L. garvieae* isolates of dairy and fish origin should be considered with caution. [**Int Microbiol** 2010; 13(4):189-193]

Keywords: *Lactococcus garvieae* · lactose metabolism · *lacG* gene

Introduction

Lactococcus garvieae is one of the most important bacterial pathogens that affect different farmed fish species in many countries. Its major impact, though, is on the trout farm industry [23]. In addition to farmed fish, this microorganism has been isolated also from a wide range of wild fish species, found both in fresh and marine water, as well as from giant fresh water prawns [24] and wild marine mammals [7]. The host range of *L. garvieae* is not limited to aquatic species, but is also associated to subclinical mastitis in cows and water buffalos, and pneumonia in pigs [4,5,22; Tejedor, PhD Thesis 2008]. In humans, *L. garvieae* has been isolated from patients affected by different clinical cases like urinary tract infections, pneumonia, endocarditis or septicaemia [15,17,25]. Like other animal pathogens of clinical relevance [3], *L. garvieae* is considered a potentially zoonosic bacteria [26]. In addition to its relevance as a pathogen, *L. garvieae* is widespread in nature, having been isolated from wild birds, rivers and sewage waters [Tejedor, PhD Thesis].

In the last years, *L. garvieae* has gained interest in the food industry because it has been isolated from different types of food, including vegetables [12], meat and meat products [19,21]. In particular, *L. garvieae* is considered to be a component of the natural occurring microbiota in dairy

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products from raw milk of different ruminants [8, 10] and is also associated with the naturally fermentation of different artisan cheeses [1]. Recently, *L. garvieae* has been isolated also from faecal samples of healthy people, suggesting this microorganism could be part of the human commensal microbiota or transient bacteria ingested with food [14].

Lactococcus garvieae strains have a genetic diversity related to the specific host they colonize [11] and molecular genetic studies have revealed a low genetic relatedness between fish and dairy strains [8]. According to Fortina et al. [9], the *L. garvieae* isolates of both origins can be differentiated by the ability of dairy isolates to utilize lactose (Lac^+) and the presence of the phospho-β-galactosidase (*lacG*) gene, detectable in all isolates of dairy origin but lacking in fish isolates. However, *L. garvieae* from sources other than fish and dairy products were not analyzed by Fortina et al. Due to the widespread nature of *L. garvieae*, the aim of the present study is to evaluate the usefulness of the acidification of lactose and the presence of the *lacG* gen as indicators of the origin of *L. garvieae* in a set of isolates from different sources.

Materials and methods

Bacterial isolates. A total of 57 isolates of *Lactococcus garvieae* from food (n = 10), diseased trout (n = 10), ruminants (n = 7), pigs (n = 10), birds (n = 2), water (n = 8) and humans (n = 10) were studied. Isolates were considered clinical when isolated from clinical specimens (trout, ruminants, pigs and human). Otherwise they were considered as non-clinical (food, birds and water). Additional information regarding the studied isolates is given in Table 1. Bacteria were grown on Columbia blood agar plates (bioMérieux España) incubated at 30ºC for 24–48 h. Identification of *L. garvieae* was carried out by using the commercial identification system Rapid ID32 Strept (bioMérieux España) and by PCR [27].

Lactose utilization. Acid production from lactose was determined using the Rapid ID32 Strept strips and also confirmed by using phenol red broth base medium (Difco) supplemented with 1% (w/v) sugar (Sigma-Aldrich), after 48 h of incubation at 30°C.

β-Galactosidase activity. The production of the enzyme β–galactosidase was determined using the Rapid ID32 Strept strips and also using the commercial diagnostic ONPG assay (Difco).

Detection and sequencing of the *lacG* gene. The presence of the *lacG* gene was determined by PCR using the primer set lac-F 5´-AGCTACTCGACGACCAACAC-3´ [9] and lacG-R 5´-AGCTACTCGACGACCAACAC-3´ [present study] designed to obtain an expected PCR product size of 969 bp (76.7% of the gene). *In vitro* amplification was carried out in a reaction mixture of 100 µl containing DNA template (10–20 ng), 1 µM of each primer, 100 µM of each dNTP (Biotools), 5 U of Ultratools DNA polymerase (Biotools) and its 1X amplification buffer. The amplifications were carried out in a Mastercycler gradient thermal cycler (Eppendorf) with the following parameters: an initial denaturation step of 94ºC for 2 min, 30 serial cycles of a denaturation step of 94ºC for 1 min, annealing at 50ºC for 2 min and extension at 72ºC for 2 min, followed by a final extension step of 72ºC for 10 min. Negative controls (no DNA template) were included in each batch of PCR reactions. PCR-generated products were detected by electrophoresis of 5 µl of each amplification mixture in 1% agarose gels supplemented with 1X SYBER safe (Invitrogen, Eugene, OR, USA). The *lacG* amplicons were purified (Geneclean Turbo Kit, MP Biomedicals LLC) and sequenced at the SECUGEN facilities (Biological Research Center, CIB-CSIC, Madrid, Spain) using the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit in an automatic ABI Prism DNA sequencer (Applied Biosystems). Multiple-sequence alignments and similarities (%) of the *lacG* sequences in the *L. garvieae* isolates examined in this study, and the one available in GenBank (accession no. EU153556.1), were obtained with CLUSTALW2 software available from the EMBL-EBI web server [http://www.ebi.ac.uk/Tools/clustalw2/index.html].

Statistical analysis. The Fisher's exact test was used to determine the relationship between the utilization of lactose and presence of *lacG* gen and the clinical/non-clinical origin of the *L. garvieae* isolates, using SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL). Differences were considered significant when probabilities were lower than 0.05.

Nucleotide sequence accession numbers. The *lacG* sequences of isolates T2-17, 1042, 306/79, MAM-77, DP1 and 21331-2, representatives of the different *L. garvieae* sources examined in this study, have been deposited in GenBank under the accession numbers FR687333-FR687338, respectively.

Results and Discussion

All *L. garvieae* isolates were properly identified with the Rapid ID32 Strept system. Biochemical identification was further confirmed by specific PCR, giving the expected amplification product of 1100 bp belonging to the 16S rRNA gene [27]. A good concordance was observed between the ability to acidify lactose and the presence of the *lacG* gene. All *L. garvieae* isolates that were able to utilize lactose (Lac^+) also carried the *lacG* gene (Table 1 and Fig. 1). The multiple-sequence alignment analysis revealed that *lacG* sequence is highly conserved among the different *L. garvieae* isolates analysed in this work and the previously available *lacG* sequence, showing similarities of between 98–99%.

In many bacteria, lactose catabolism is initiated by permease-promoted uptake of lactose and the generation of glucose and galactose by β-galactosidase. However, a small number of gram-positive bacteria follow a quite distinct pathway. This pathway begins with the phosphorylation of lactose upon uptake by the phosphoenolpyruvate-dependent phosphotransferase system (*lac*-PTS) and the catabolism of lactose phosphate into glucose and galactose 6-phosphate by the enzyme phospho-β-galactosidase, encoded by the *lacG* gene [2,16]. The ONPG assay was negative for all the isolates. These results indicate that *L. garvieae* does not catabolize lactose through the β-galactosidase enzyme and agree with the idea that in *L. garvieae* Lac^+ isolates, the lactose is

Table 1. Results of the lactose utilization (Lac) and presence of *lacG* gene for the *Lactococcus garvieae* isolates

Strain	Source	Pathological process	Lac and *lacG*
Food			
T1-1, T2-17, CAS-2	Casín cheese	–	+
4AB5, 3AA7	Cabrales cheese	–	+
1204	Salers cheese	–	–
N201	Saint-Nectarine cheese	–	+
1042	Raw milk	–	+
DK2-25	Kajmak[a]	–	–
LG-80	Morcilla Burgos[b]	–	–
Fish			
4876/006	Eel	Lactococcosis	–
8831/04, 98/4284, 5664, 1684, 8053/02, 5457, 5424, 5323, 8494/03	Trout	Lactococcosis	–
Human			
21881, BM06/00349	Human	Blood (bacteremia)	–
306/79	Human	Urine	+
240-88	Human	Urine	–
673-80	Human	Skin	–
2182-81, 1108-86, 364-88, 6690, 2486-87	Human	Blood	–
Ruminants			
1183, 1205	Buffalo	Subclinical mastitis	–
CECT[c] 4531[T], G-34, MAM-75	Cow	Subclinical mastitis	–
G-14, MAM-77	Cow	Subclinical mastitis	+
Pig			
1108/02, 1481/03, 1364/02, 1139/02	Pig	Pleuritis	–
205/03, 2497/03, 164/03	Pig	Pericarditis	–
391/03	Pig	Not known	–
396, 2487	Pig	Pneumonia	–
Wild bird			
H-14	Blue tit	–	–
30/02	Magpie	–	–
Water			
DP1, 2260-1	Sewage treatment plant	–	+
2260-2	Sewage treatment plant	–	–
2131-2, 2132-2	River	–	+
2544-1, 2134-1, 2133-1	River	–	–

[a]Serbian traditional fermented milk. [b]Spanish traditional black pudding. [c]Spanish Type Culture Collection.

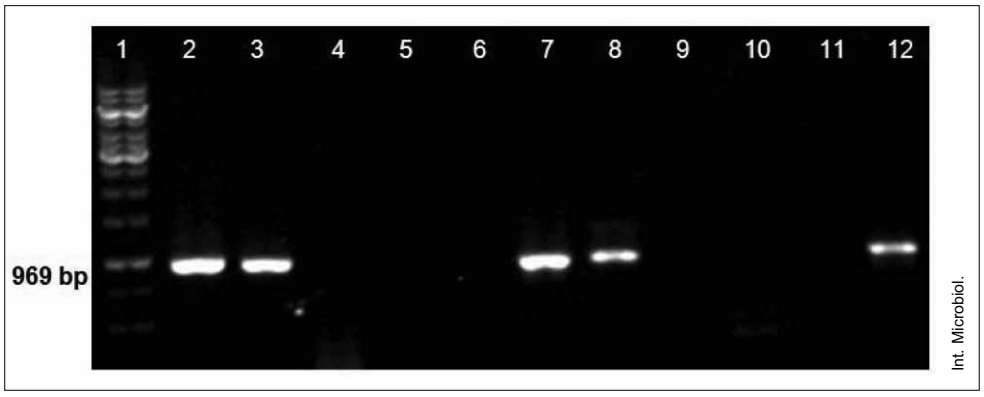

Fig. 1. Gel electrophoresis of *lacG* PCR amplicons from representative isolates of *L. garvieae*. Lane 1, molecular weight marker 1kb ladder (Biotools); lane 2 to 12, isolates T2-17, MAM-77, DK2-25, 8831/04, H14, 2132-2, DP1, 2133-1, 1108/02, 21881 and 306/79, respectively.

transported by the *lac*-PTS and hydrolyzed by the phospho-β-galactosidase enzyme. Unlike other lactic acid bacteria, in *L. garvieae* the *lacG* gene is chromosomally located [9].

None of the fish and pigs isolates of *L. garvieae* produced acid from lactose (Lac$^-$). Likewise, most of the *L. garvieae* isolates recovered from raw milk from cows with subclinical mastitis and most of the human isolates were Lac$^-$ (Table 1). In contrast, most of the dairy isolates (seven out of the nine isolates analyzed; 77.8%) were Lac$^+$; only two dairy isolates (DK2-25 and 1204) did not produce acid from this sugar. The isolate from black pudding, as well as both isolates from birds were Lac$^-$. The occurrence of Lac$^+$ and Lac$^-$ isolates of *L. garvieae* from the water samples of rivers and sewage treatment plants was comparable. In agreement with these results, Lac$^+$ isolates of *L. garvieae* have been detected also in soil and grass environmental samples [13].

The majority of the *L. garvieae* isolates from clinical specimens (91.9%) were Lac$^-$/*lacG*$^-$, a percentage that statistically was significantly higher ($p<0.05$) than that observed in non-clinical isolates (Table 1). This difference suggests that the inability to metabolize lactose and the absence of the *lacG* gene in *L. garvieae* could be related to the clinical origin of the isolate. Nevertheless, the high proportion of Lac$^-$/*lacG*$^-$ isolates observed also among the non-clinical isolates makes it difficult to use this phenotypic characteristic to anticipate the clinical origin of a *L. garvieae* isolate.

The ability to metabolize lactose and/or the presence of the *lacG* gene have been proposed as markers for distinguishing between dairy (Lac$^+$/*lacG*$^+$) and fish (Lac$^-$/*lacG*$^-$) *L. garvieae* isolates [8,9]. In the present study, in addition to fish, Lac$^-$/*lacG*$^-$ isolates of *L. garvieae* were also observed in pigs, cows, birds and humans (Table 1). Nevertheless, Lac$^+$ isolates of this microorganism had been isolated previously from fish [18,20].

Regarding the isolates of *L. garviae* of dairy origin, although most of them were Lac$^+$/*lacG*$^+$, some isolates (22.2%) were Lac$^-$/*lacG*$^-$. This percentage could be even higher considering that most of the *L. garvieae* isolates from raw milk from cows with subclinical mastitis were Lac$^-$/*lacG*$^-$ (71.4%; Table 1). *Lactococcus garvieae* is associated with a considerable number of subclinical mastitis in ruminants [5,6,22,23; Tejedor, PhD Thesis] and milk of these animals is commercialized to elaborate different dairy products. Consequently, it is likely to be expected that *L. garvieae* Lac$^-$/*lacG*$^-$ isolates present in the milk of animals with subclinical mastitis were further isolated in dairy products.

According to the results of the present study, as well as previous studies, the ability/inability to metabolize lactose and/or the presence/absence of the *lacG* gene are not exclusively related to dairy and fish isolates of *L. garvieae.* Therefore, the use of these criteria as markers to differentiate between *L. garvieae* isolates of dairy and fish origin should be considered with caution.

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