Genome Sequence of a Food Spoilage Lactic Acid Bacterium, *Leuconostoc gasicomitatum* LMG 18811^T, in Association with Specific Spoilage Reactions^†

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Received 18 January 2011/Accepted 1 May 2011

*Leuconostoc gasicomitatum* is a psychrotrophic lactic acid bacterium causing spoilage of cold-stored, modified-atmosphere-packaged (MAP), nutrient-rich foods. Its role has been verified by challenge tests in gas and slime formation, development of pungent acidic and buttery off odors, and greening of beef. MAP meats have especially been prone to *L. gasicomitatum* spoilage. In addition, spoilage of vacuum-packaged vegetable sausages and marinated herring has been reported. The genomic sequencing project of *L. gasicomitatum* LMG 18811^T was prompted by a need to understand the growth and spoilage potentials of *L. gasicomitatum*, to study its phylogeny, and to be able to knock out and overexpress the genes. Comparative genomic analysis was done within *L. gasicomitatum* LMG 18811^T^T and the three fully assembled *Leuconostoc* genomes (those of *Leuconostoc mesenteroides*, *Leuconostoc citreum*, and *Leuconostoc kimchii*) available. The genome of *L. gasicomitatum* LMG 18811^T^T is plasmid-free and contains a 1,954,080-bp circular chromosome with an average GC content of 36.7%. It includes genes for the phosphoketolase pathway and alternative pathways for pyruvate utilization. As interesting features associated with the growth and spoilage potential, LMG 18811^T^T possesses utilization strategies for ribose, external nucleotides, nucleosides, and nucleobases and it has a functional electron transport chain requiring only externally supplied heme for respiration. In respect of the documented specific spoilage reactions, the pathways genes associated with a buttery off odor, meat greening, and slime formation were recognized. Unexpectedly, genes associated with platelet binding and collagen adhesion were detected, but their functionality and role in food spoilage and processing environment contamination need further study.

Industrially manufactured food must have a reasonably long shelf life due to the production chain, involving logistics and retail sale before the domestic storage and consumption of a product. CO2 in modified-atmosphere packaging (MAP) and refrigerated temperatures are two main extrinsic hurdles used by the food industry. They create negative selective pressure to aerobic Gram-negative spoilage bacteria. Under these circumstances, psychrotrophic, i.e., cold-tolerant, lactic acid bacteria (LAB) prevail in nutrient-rich foods, such as meat (4, 18, 21). Compared to the aerobic Gram-negative spoilage bacteria, the stationary growth phase associated with the production of sensory changes is reached more slowly by spoilage LAB. In addition, the end products of LAB carbohydrate fermentation are not sensed as unpleasant, as in protein or amino acid degradation. Therefore, the shift from aerobic Gram-negative bacteria to LAB is preferred.

Despite the generally moderate role of psychrotrophic LAB in spoilage, they are still spoilage organisms. The growth rate of psychrotrophic LAB can usually be predicted and the shelf life can be estimated with adequate accuracy. However, some psychrotrophic LAB may cause considerable hardship to the food industry. *Leuconostoc gasicomitatum* (3) is a LAB that was first encountered causing a spoilage problem of MAP, tomato-marinated, raw broiler meat strips in 1997. The packages already showed clear bulging due to CO2 formation in 5 days, even though the manufacturer-defined shelf life was expected to be 14 days. Since the first spoilage problem, *L. gasicomitatum* has been shown to form slime and CO2 in acetic acid-preserved herring (24), cause greening and off odor to value-added MAP, raw beef steaks (42, 45), and cause slimy spoilage and bulging of cooked vegetable sausages packaged under vacuum (45). In addition, this species has been documented to prevail in MAP, marinated broiler meat strips (38) and minced meat (29). Table 1 shows reported *L. gasicomitatum* spoilage with descriptive spoilage characteristics verified in food challenge tests. However, none of these have yet been confirmed through expression or other types of molecular analyses in food *in situ*.

The genomic sequencing project of *L. gasicomitatum* LMG 18811^T^T was prompted by a need to understand the growth and spoilage potentials of *L. gasicomitatum*, to study its phylogeny, and to be able to knock out and overexpress genes. This is the first complete genome sequence of a psychrotrophic food spoilage LAB, and it is presented in this study with particular emphasis on the food spoilage capabilities. Comparative genomic analysis was also carried out with the three other
**Genome and general aspects related to growth in food.** Table 2 shows the main properties of the genome of *L. gasicomitatum* LMG 18811T (GenBank accession no. FN822744). The genome is plasmid free and contains a 1,954,080-bp circular chromosome with an average GC content of 36.7%. Two prophages which are not located within the operons of interest in respect to spoilage reactions were detected. Figure 1 shows the genome map of *L. gasicomitatum* LMG 18811T colored according to the Automated Resource Classifier (ARC) classification based on the gene annotation. The proteome is presented for *L. gasicomitatum* LMG 18811T colored according to the Automated Resource Classifier (ARC) classification based on the gene annotation. The proteome is presented.
according to clusters of orthologous groups categories in Table S2 in the supplemental material.

Figure 2 shows a Venn diagram comparing the genome of *L. gasicomitatum* LMG 18811T to the three other publicly available *Leuconostoc* genomes: those of *L. carnosum* ATCC KM20 (20), *L. mesenteroides* ATCC 8293T (25), and *L. kimchii* ATCC 8293T (30). The unique genes for each of the four species are listed in Table S3 in the supplemental material. Like the other *Leuconostoc* LMG 18811T has a wide set of genes involved in the uptake of sugars, citrate, and amino acids. The genome includes the genes for the phosphoketolase pathway and three alternative pathways for pyruvate utilization by lactate dehydrogenase, pyruvate dehydrogenase, and α-acetolactate synthase. Compared to *L. mesenteroides* ATCC 8293T, it has fewer pathways involved in the biosynthesis of amino acids, vitamins, and cofactors (see Table S4 in the supplemental material). Pyruvate utilization is a hub for many of the spoilage reactions, and Table 3 summarizes the enzymes and coding genes attributed to the utilization of pyruvate.

**Utilization of ribose.** The majority of LAB does not utilize ribose, which is a pentose of interest in plant- and meat-derived foods. Within the genus *Leuconostoc*, the species *L. carnosum*, *L. gasicomitatum*, *L. gelidum*, *L. inhae*, and *L. kimchii*, which belong to the same 16S rDNA gene-based phylogenetic branch, can utilize ribose (14). Most ribose-utilizing LAB transport it via an H+ symporter, e.g., RbsU of *L. sakei* (37). *L. gasicomitatum* LMG 18811T and *L. kimchii* ATCC 8293T have the ribose ABC transporter RbsDACB (46) (LEGAL 0026 to LEGAL 0092 in *L. gasicomitatum*) generally more common in the bacterial domain. In the genomes of *L. mesenteroides* ATCC 8293T and *L. carnosum* ATCC KM20, genes do not exist either for the ribose ABC transporter or for the RbsU symporter.

In the pentose phosphate pathway, the enzyme ribose 5-phosphate isomerase A (RpiA, EC 5.3.1.6) plays an important role in the branching between the pentose phosphate pathway and the nucleotide pathways. All four sequenced *Leuconostoc* genomes contain multiple rpiA genes (LEGAL 0278, LEGAL 0031, and LEGAL 1232 in *L. gasicomitatum*), but the function and expression of them are unknown.

**Salvage and utilization of nucleotides, nucleosides, and nucleobases.** All four fully assembled *Leuconostoc* genomes show the ability for *de novo* synthesis of purines and pyrimidines. They do not have the genes encoding nucleotide phosphoribosyl transferase, which catalyzes the exchange reaction of the nucleobase of deoxynucleobase to salvage the deoxyribose moieties and convert between the different deoxynucleobases (8). *L. gasicomitatum* LMG 18811T has external nucleotides and nucleosides as both a carbon and an energy source; and the amino group of nucleotides and free nucleobases can be utilized as a nitrogen source (see Table S1 in the supplemental material), or they can be salvaged and rescued for nucleotide synthesis. Both guanosine and xanthine, but not adenine or cytidine, can be used as nitrogen sources, whereas inosine, uridine, and adenosine, but not thymidine, can be utilized as carbon sources (see Table S1 in the supplemental material). 2’,3’-Deoxyadenosine and 2-deoxyribose can also be utilized as carbon sources (see Table S1 in the supplemental material), although it is not clear how. The *deoQKPS* genes, for the uptake and utilization of 2-deoxyribose in other species (9), are not found in *Leuconostoc* genomes. Instead, genes for nucleotidases dephosphorylating the nucleotides to the corresponding nucleoside exist (*L. gasicomitatum* LEGAL 1431 and LEGAL 0848), as do genes for a nucleoside permease, *nupC* (LEGAL 0024), and *nrsACS*, encoding a nucleoside ABC transporter (LEGAL 1844 to LEGAL 1847) with two separate nucleoside-binding subunits, *nrsB1* and *nrsB2* (LEGAL 1460 and LEGAL 1179), enabling transportation of nucleosides into the cell. Genes for three transporters of nucleobases exist: a uracil transporter, *pyrP* (LEGAL 1771), and two guanine/hypoxanthine transporters, *pbuG1* and *pbuG2* (LEGAL 1320 and LEGAL 0450). Ribonucleotides can also be hydrolyzed by the ribonucleoside hydrolases (rhaA1, rhaA2, rhaB, and rhaC, corresponding to LEGAL 0022, LEGAL 1534, LEGAL 0456, and LEGAL 0023, respectively), to generate free nucleobases and ribose. The ribose formed can subsequently be fed into the pentose phosphate pathway.

**Citrate metabolism and butyrate of odor.** *L. gasicomitatum* LMG 18811T can utilize citrate. It has a citMCDEFGX system citrate locus (LEGAL 0211 to LEGAL 0219), which encodes the enzymes necessary for the uptake and conversion of citrate to pyruvate. The genes for the diacetyl/acetoin pathway are present. This pathway consumes pyruvate, forming α-acetolactate by the catalytic α-acetolactate synthase (LEGAL 0514). Under aerobic circumstances, α-acetolactate may be decarboxylated to acetoin, either via diacetyl, by a nonenzymatic decarboxylative oxidation followed by an NAD(P)H-dependent reduction to acetoin by diacetyl reductase (LEGAL 0209, LEGAL 1299), or directly to acetoin by acetaldehyde decarboxylase (LEGAL 1326) (Fig. 1). Acetoin and diacetyl can also be formed from the amino acid aspartate in the presence of α-ketoglutarate (22), but *Leuconostoc* do not have the glutamate dehydrogenase, which can convert glutamate to α-ketoglutarate as in a few other LAB species (41). Instead, *L. gasicomitatum* LMG 18811T has transporters for both aspartate (LEGAL 1791) and α-ketoglutarate (LEGAL 1138), and in the presence of α-ketoglutarate, the aspartate aminotransferase (LEGAL 1168) may convert aspartate to oxaloacetate and glutamate. The oxaloacetate can enter the diacetyl/acetoin pathway via the last
enzyme of the citrate pathway oxaloacetate decarboxylase (LEGAS_0212). Acetoin can in turn be converted to 2,3-butanediol by 2,3-butanediol dehydrogenase (LEGAS_1018).

Enhanced growth when heme and O₂ are available and the genes associated with the electron transport chain. All four Leuconostoc genomes possess genes encoding cytochrome bd terminal oxidase and for synthesizing menaquinone. However, unlike L. mesenteroides DSM 20343ᵀ, L. gasicomitatum LMG 18811ᵀ has a functional electron transport requiring only externally supplied heme for respiration. The presence of heme increased the produced biomass by ~50% under aerobic and high-oxygen MAP cultivation, while the addition of heme had no effect on anaerobically growing cells (Fig. 4). Cell size did not alter between the experiments. The nonfunctional cytochromes of L. mesenteroides ATCC 8293ᵀ were also reported by Brooijmans et al. (5). The L. gasicomitatum LMG 18811ᵀ genome encodes a cytochrome bd terminal oxidase (LEGAS_1333 and LEGAS_1334), an NADH dehydrogenase (LEGAS_1702), and the entity related to the ability to synthesize menaquinone in eight enzymatic steps.
Genes associated with H$_2$O$_2$ production and meat greening.

Of the enzymes known to generate hydrogen peroxide in LAB, L. gasicomitatum LMG 18811T has only the genes for pyruvate oxidase (poxB, LEGAS_1053), NADH oxidase (nox, LEGAS_0926), and two unknown NADH:flavin oxidoreductase/NADH oxidases (LEGAS_0056 and LEGAS_1753). The NADH oxidases likely produce water and not hydrogen peroxide as an end product. For protection against peroxide, a capability for three different peroxidases, thioredoxin peroxidase (tpx, LEGAS_0306), glutathione peroxidase (bsaA, LEGAS_1017), and heme-containing Dyp-type peroxidase (LEGAS_1694), exists, whereas no potential for catalase production was detected.

Genes encoding EPSs, adhesion, and mucus binding. L. gasicomitatum LMG 18811T has genes for two dextransucrases: epsA (LEGAS_0699) is part of a large exopolysaccharide (EPS) cluster, while dsrA (LEGAS_1012) is located as a single gene in the chromosome. Three genes encoding proteins containing putative LPXTG anchors were detected. LEGAS_0414 encodes a putative mucus binding protein with unknown function. Orthologs were also detected in the genomes of L. mesenteroides and L. citreum (plasmid encoded). LEGAS_0537 encodes Srr-2, a serine-rich protein. The serine-rich domain shows homology to the platelet-binding protein GspB of streptococci (34), but since it is missing a large portion of the nonrepeat region, the function cannot be predicted. The genes secY2, asp1 to asp3, secA2, nss, and gftAB were located in the same locus with LEGAS_0537. They all are required for the secretion and glycosylation of Srr-2 (39). LEGAS_1063 is part of an intercellular adhesion locus (ica) ABC, where it encodes the putative collagen adhesion protein IcaC. The ica locus has been shown to be required for biofilm formation in Staphylococcus aureus (10) and among LAB is otherwise found in only a few Lactococcus species (35). No orthologs of LEGAS_0537 or LEGAS_1063 are found in other Leuconostoc genomes (see Table S3 in the supplemental material).

DISCUSSION

An unexpected story related to a novel food spoilage organism in Finland has been seen over the last 14 years. The first incident was considered to be related to the use of a specific tomato-based marinade (3), but the following years have shown that this species is persisting (44) and causing spoilage in many types of cold-stored MAP foods of several manufacturers in Finland.

An ability to grow well on MAP meat with no added carbohydrates (29, 44) is interesting, while this species is not able to obtain energy from proteinaceous substrates, lactate, or fatty acids. It has the genes required for energetic catabolism of nucleosides, and it also grows well on adenosine and inosine. Nucleosides, particularly inosine, are abundant in meat, and if glucose is exhausted, they provide an alternative source of energy. Differing from Lactobacillus sakei 23K, which is considered a meat ecosystem-adapted LAB (6, 7), L. gasicomitatum LMG 18811T cannot release amino acids from meat proteins or utilize arginine as an energy source. Bearing food safety aspects in mind, the genome analysis confirms the high-pressure liquid chromatography determinations (27) that the meat-derived amino acids are not decarboxylated as biogenic

![Venn diagram showing the distribution of orthologous relationships of genes between four Leuconostoc species.](image)

<table>
<thead>
<tr>
<th>Spoilage compound</th>
<th>Enzyme</th>
<th>Gene(s)</th>
<th>Locus tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>Acetate kinase</td>
<td>ackA1, ackA2</td>
<td>LEGAS_1085 to LEGAS_1559</td>
</tr>
<tr>
<td></td>
<td>Citrate lyase complex</td>
<td>citCDEF</td>
<td>LEGAS_0213 to LEGAS_0216</td>
</tr>
<tr>
<td></td>
<td>N-Acetylglucosamine-6-phosphate deacetylase</td>
<td>nagA</td>
<td>LEGAS_0472</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Acetolactate decarboxylase</td>
<td>alsD</td>
<td>LEGAS_1316</td>
</tr>
<tr>
<td></td>
<td>Oxaloacetate decarboxylase</td>
<td>ctaM</td>
<td>LEGAS_0212</td>
</tr>
<tr>
<td></td>
<td>6-Phosphogluconate dehydrogenase complex</td>
<td>gnd1, gnd2, poxABCD</td>
<td>LEGAS_1343 to LEGAS_1378</td>
</tr>
<tr>
<td></td>
<td>Pyruvate oxidase</td>
<td>poxB</td>
<td>LEGAS_1053</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>Acetolactate synthase</td>
<td>alsS</td>
<td>LEGAS_0526</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Pyruvate oxidase</td>
<td>poxB</td>
<td>LEGAS_1053</td>
</tr>
<tr>
<td>Slime</td>
<td>Dextransucrase</td>
<td>dsrA</td>
<td>LEGAS_1012</td>
</tr>
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<td></td>
<td>Protein cluster related to formation of an EPS with unknown structure</td>
<td>eps gene cluster</td>
<td>LEGAS_0699 to LEGAS_0710</td>
</tr>
</tbody>
</table>
amines. Few leuconostocs (17, 32) are capable of forming biogenic amine from tyrosine. Related to the metabolism of pentoses or citrate, \textit{L. gasicomitatum} LMG 18811\textsuperscript{T} has the central genes involved in the pyruvate-dissipating routes leading to the formation of acetate and diacetyl. Like the other leuconostocs (1, 47), \textit{L. gasicomitatum} is likely to metabolize pyruvate to acetate or diacetyl when intracellular pyruvate accumulates, for example, when oxygen, citrate, or fructose is available. Presence of oxygen has been reflected in the type of off odor (Table 1). The buttery off

![FIG. 3. Salvage and catabolic pathways for nucleosides in \textit{L. gasicomitatum}. External nucleotides are dephosphorylated by extracellular nucleotidases (step 1) (LEGAS_1431, LEGAS_0848) and are then transported into the cell by a nucleoside permease or nucleoside ABC transporter (step 2) (LEGAS_0024, LEGAS_1844–1846, LEGAS_1460, LEGAS_1179); there are also separate nucleobase transporters (step 3) (LEGAS_1771, LEGAS_1320, LEGAS_0450). Nucleosides entering the cell can be directly phosphorylated by the corresponding kinase (step 7) (LEGAS_0367, LEGAS_0687, LEGAS_0374, LEGAS_0393, LEGAS_0602, LEGAS_1125, LEGAS_1345, LEGAS_1390, LEGAS_1662, LEGAS_1710), or deoxyribonucleosides can first exchange nucleobases by a \(N\)-deoxyribosyltransferase (step 4) (LEGAS_0737). Ribonucleosides can be cleaved to free nucleobase and ribose by ribonucleoside hydrolases (step 5) (LEGAS_0022, LEGAS_0366, LEGAS_0456, LEGAS_0023), and the free nucleobase can be salvaged either by the \(N\)-deoxyribosyltransferase (step 4) or by one of the phosphoribosyltransferases (step 6) (LEGAS_0433, LEGAS_1197, LEGAS_1345, LEGAS_1261, LEGAS_1902). The ribose formed by the hydrolysis of ribonucleosides can be either salvaged by the phosphoribosyltransferase (step 6) or used as an energy source by feeding it to the pentose phosphate pathway. NMP, nucleoside monophosphate; \(N^1\)dR, a deoxyribonucleoside; \(N^2\)dR, another deoxyribonucleoside; \(N^3\), a nucleoside; PRPP, phosphoribosyl pyrophosphate.

\[\text{NMP} \rightarrow \text{N nucleoside} \rightarrow \text{N nucleobase} \rightarrow \text{PRPP}\]

![FIG. 4. Growth of \textit{Leuconostoc gasicomitatum} LMG 18811\textsuperscript{T} and \textit{Leuconostoc mesenteroides} DSM 20343\textsuperscript{T} in MRS broth with and without hemin supplementation (2 \(\mu\)g/ml) under aerobic and anaerobic conditions. Error bars show the differences obtained between three tests. Under a modified atmosphere containing 20\% \textit{CO}_2 and 80\% oxygen, growth was similar to growth under aerobic conditions.]
odor marking diacetyl has been associated with products packaged under an oxygen-containing modified atmosphere (MA) or containers with an aerobic atmosphere (24, 42), whereas the pungent acidic odor has occurred in foods packaged under oxygen-deprived atmospheres (3, 45). Notably, some Lactobacillus and Lactococcus strains can also form diacetyl via catabolism of aspartate (19, 22), an amino acid present in meat. The genes required for aspartate catabolism are present in L. gasicomitatum 18811T, but no gene exists for glutamate dehydrogenase, considered important for the formation of α-ketoglutarate, the amino group acceptor essential for the pathway (40). Instead, L. gasicomitatum LMG 18811T encodes an α-ketoglutarate transporter, suggesting that exogenous α-ketoglutarate may be exploited. However, whether L. gasicomitatum LMG 18811T produces diacetyl via aspartate catabolism and if this occurs in the meat ecosystem must be further studied.

Vihavainen and Björkroth proposed (42) that H2O2 produced by an NADH oxidase in L. gasicomitatum strains caused green discoloration on beef steaks. Analysis of the genome of L. gasicomitatum LMG 18811T revealed that pyruvate oxidase is the only enzyme with a known ability to generate H2O2. Slime formation on vegetable sausages (45) and in a herring product (24) was proposed to be due to sucrose-derived homopolysaccharide dextran, since L. gasicomitatum produces slime from sucrose in vitro (3). Consistent with this, L. gasicomitatum LMG 18811T encodes a dextrantransaccharase, a cell wall-associated glycosyltransferase catalyzing the formation of dextran from sucrose. In addition, a gene cluster homologous to the heteropolysaccharide EPS gene cluster present in Streptococcus thermophilus (26) was detected. Heteropolysaccharide formation in leuconostocs has not been reported. Compared to homopolysaccharides, their biosynthesis is more complex (12, 26), ruling out prediction of the EPS structure, physical properties, and possible role in food spoilage.

Addition of heme to aerated MRS medium increased the biomass formation of L. gasicomitatum considerably, whereas addition of CO2 (20%) to the oxygen-containing atmosphere mimics the atmosphere used to create the high-oxygen MA for red meats did not limit the biomass increase. Since neither the present study nor that of Brooijmans et al. (5) showed functional respiration in L. mesenteroides ATCC 8293T, we did not anticipate this finding. Heme-induced respiration dramatically alters the phenotype of Lactococcus lactis, as it improves not only growth efficiency but also robustness as improved stress resistance (13, 16). No heme uptake transporters have been characterized in any LAB, despite numerous efforts (15, 31). Nevertheless, meat contains heme, and since the CO2 added in the atmosphere did not have any effect, L. gasicomitatum may respire while growing on high-oxygen MAP meats, leading to succession in the spoilage LAB population due to effective growth and improved stress resistance.

L. gasicomitatum has not been detected on skin or mucous membranes of broiler chickens (43) or pigs (23). The precise habitat of this species is not known, but on the basis of its growth temperatures and carbon source utilization (plant-derived pentoses), we have considered it an environmental LAB. Thus, it was interesting to detect unique genes (see Table S3 in the supplemental material) associated with adhesion and platelet binding. For genes encoding the putative mucus binding protein LEGAS_0414, orthologs were detected in the genomes of L. mesenteroides and L. citreum (plasmid encoded), but none of the other genomes contain orthologs for the putative colla- gen adhesion protein enabling biofilm formation in staphylo- cocci (10). The collagen binding capibilities might enable better survival in the meat environment. In preliminary analyses, L. gasicomitatum strain KG1-16, isolated in the spoiled vegetable sausages (47), did not harbor these genes. The role and expression of these genes in association with meat spoilage will be an interesting target of further studies.

With the help of the genome sequence, many of the spoilage reactions found their rationale. In addition, interesting new hypotheses arose, such as the potential increase of growth and stress resistance capabilities through respiratory capacity in high-oxygen meat products. L. gasicomitatum 18811T provides an interesting model of psychrotrophic spoilage LAB for future studies.

ACKNOWLEDGMENTS

The financial support of the Finnish Funding Agency for Technol- ogy and Innovation projects (TEKES 44074/20 and 4069/05) and the Academy of Finland support for the Centre of Excellence of Microbial Food Safety Research are gratefully acknowledged.

Markku Ala-Pantti, Janne Backman, Henna Niinivirta, Erja Merivirta, Riikka Räty, Eeva-Marja Turkkii, and Hannu Väinänen are thanked for their excellent technical assistance.

REFERENCES
