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Comparative genomics of *Lactobacillus curvatus* enables prediction of traits relating to adaptation and strategies of assertiveness in sausage fermentation

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ABSTRACT

Lactobacillus (L.) *curvatus* reaches high numbers in a variety of habitats, which suggests a high (genomic) diversity within this species. Empirically selected strains are used as starter cultures in sausage fermentation. Determinants for the assertiveness of a strain in this environment are assumed to be multifactorial. We used comparative genomics and *in silico* proteomics of 10 *L. curvatus* strains, which were representative of its genetic and physiological biodiversity, to possibly derive genetic determinants for strain or group specific assertiveness in sausage fermentation. Their genome sizes ranged from 1.7 Mb up to 2.0 Mb. The estimated pan- and core genomes were 3.0 Mb and 1.4 Mb, respectively. The accessory genome, GC-content and coding density revealed a significant genomic diversity within this species. Plasmids were found, which were either closely related or unique in several strains. Putative assertiveness determinants including CRISPR/Cas systems, prophages, bacteriocin production, or specific metabolic settings were detected. Such traits of the accessory genome could not be correlated with the source of isolation. Pathways, which previously have been predicted for a relation with adaptation to meat of *L. sakei*, are part of the core genome of *L curvatus*. Intraspecies differences in the accessory genome of *L. curvatus* comprise ribose metabolism, enzymes involved in nucleotide metabolism (nucleoside phosphorylases, phosphopentomutase, adenosine deaminase, ribose transporters), and tyrosine decarboxylases, ornithine decarboxylases. One group of the strains encoded a phosphotransferase system (PTS) as ribose transporter, whereas the second group encoded an ATP binding cassette (ABC) transporter. Analysis of the ribose uptake by HPLC analysis revealed different efficiencies of both transporter systems. Except for bacteriocin formation, no strain specific traits were identified predicting assertiveness of single strains. This fits our previous observation that single strains of *L. curvatus* could not override others in a competitive setting. Rather, pairs or sets of strains, comprising metabolically synergistic or non-assertive partner strains were able to dominate the fermentation. Indeed, this work suggests that assertive partnerships can be predicted along their complementary accessory genomes.

1. Introduction

Lactobacillus curvatus can be isolated from a variety of habitats, and some strains of these species can reach high numbers in spontaneously fermented sauerkraut, sourdough or raw sausages. Strains of *L. curvatus* are therefore frequently used as starter culture for meat fermentation (Hammes et al., 1990; Hammes and Hertel, 1996), namely when fast acidification is envisaged (Tichaczek et al., 1992, 1994). Furthermore, some strains produce bacteriocins, which can inhibit closely related strains of the autochthonous microbiota, as well as *Listeria monocytogenes* (Dicks et al., 2004; Straub et al., 1994; Vogel et al., 1993). Generally, meat is a rich environment (Lauret et al., 1996), and a lot of substrates are available, which can be used for growth and energy generation by many members of the autochthonous microbiota competing with the starter strain. As carbohydrates as glycogen and glucose in meat are generally limited and may vary with meat quality, glucose is added frequently to raw sausage batters enabling fast growth and acidification by lactobacilli. Again, simple carbohydrates like glucose are mostly considered as non-selective with respect to enabling assertiveness of a specific *Lactobacillus* strain and rapidly depleted. Therefore differences in the assertiveness of *L. curvatus* strains in the fermenting sausage environment should be attributed to their abilities to use alternative or additional substrates and pathways for energy generation during the onset of fermentation and persistence during the ripening of sausages. These can be assumed to comprise lipids and proteins, amino acids, nucleotides as well as ribose from RNA. While analysis of

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respective features of adaptation is lacking for *L. curvatus*, an overview on putative meat related traits is provided for its close relative *L. sakei* (Nyquist et al., 2011). Their comparative genomic approach, which did not yet use fully sequenced genomes, predicted strain specific differences, *e.g.* in ribose uptake and metabolism, citrate, gluconate, arabinose, malate and glycerol metabolism as well as putative utilization of lactate and *N*-acetyl-glucosamine. While it is clear that the direction of these pathways cannot be predicted from genomic settings, and these predictions need experimental biochemical proof, they may help to identify possible differential settings also in *L. curvatus*. Subsequently, traits can be differentiated, which putatively contribute to assertiveness in sausage fermentation. Indeed, strain specific differences in assertiveness can be observed in fermenting sausage models employing groups of *L. curvatus* strains in a competitive setting (Janßen et al., 2018). These authors deliberately use the term “assertiveness” to address the fact that out-competition of contaminants and persistence of a strain in a complex microbiome includes cooperation, decisiveness and even harshness towards competitors to achieve control of meat microbiota. Their data on the strain specific monitoring of *L. curvatus* and *L. sakei* revealed mechanisms of co-dominance of strains with putatively complementary metabolic settings. On the other hand bacteriocin formation proved as an important factor in single strain assertiveness against autochthonous microbiota. This suggests a complex, strain specific metabolic adaptation to the meat environment, which is supposed to be reflected in the genome.

With the increasing development of genome sequencing methods different whole genome sequences of lactobacilli from the meat environment have become available (Chaillou et al., 2005; Hebert et al., 2012; McLeod et al., 2013), The first complete genome sequence of *L. sakei* 23K (1.8 Mb), published by Chaillou et al. (2005), revealed an adaptation to meat at the metabolic level, including the ability of purine nucleoside metabolism. Moreover different genes for stress response are encoded as well as genes, which are involved in biofilm formation and cellular aggregation (Chaillou et al., 2005). The draft genome sequence of *L. sakei* LS25 (2.0 Mb) was published by McLeod et al. (2013). Above the study of Nyquist (Nyquist et al., 2011) the comparison of whole genomes of both *L. sakei* strains suggests that this species is genetically diverse. *L. sakei* LS25 encoded a citrate cluster, which is not orthologous to the one encoded in 23K. Furthermore, 250 protein-encoding genes were unique to LS25 (McLeod et al., 2013). For *L. curvatus* six genome sequences are currently publicly available. The first sequenced strain *L. curvatus* CRL705, which is used in starter cultures for sausage fermentation, was sequenced by Hebert et al. (2012). This strain is able to produce antilisterial bacteriocin AL705 and a two-component lactocin 705 (Castellano et al., 2003; Vignolo et al., 1993). Furthermore, the genome of *L. curvatus* NRIC 0822, which was isolated from kabura-zushi a traditional fermented sushi, was sequenced by Cousin et al. (2015). This study focussed on the rare property of motiliy of *L. curvatus* NRIC0822. It was shown that the motility genes are organized in an operon (Cousin et al., 2015). Other genomes were published, like *L. curvatus* FBA2 (Nakano et al., 2016) (Accession number: CP016028), which was isolated from radish and carrot pickled with salty rice bran and *L. curvatus* MRS6 (Jans et al., 2017) (Accession number: CP022474), which was isolated from the fermented sausage salsiz. Among two *L. curvatus* strains isolated from Kimchi, *L. curvatus* Wikim38 (Lee et al., 2017) (Accession number: CP017124) and *L. curvatus* Wikim52 (Accession number: CP016602), Lee et al. showed an adhesive ability to intestinal epithelial cells for *L. curvatus* WiKim38 (Jo et al., 2016; Lee et al., 2017). Based on the availability of these full genomes from *L. curvatus* strains isolated as predominant members of different fermented foods, an extended set of genomes could elucidate the degree of variability within *L. curvatus* strains and enable prediction of metabolic and other traits, which may affect the lifestyle and assertiveness of *L. curvatus* in the meat.

Thus, it was the aim of this study to obtain more genome sequences of additional isolates of *L. curvatus* and enable the establishment of pan- and core genomes of this species. These strains were selected according their different genetic and proteomic fingerprints (RAPD and MALDI-TOF MS profiles), and physiological traits (Freiding et al., 2011). A more detailed genome and *in silico*-proteome analysis supported by physiological tests should enable prediction and (partial) proof of species-, group- or strain specific properties. In addition it could deliver insight in expectable biodiversity, lifestyle and adaptation to the meat environment, as well as competitive strategies. These may be exploited for the prediction of complementary or even synergistic partner strains for effective suppression of autochthonous strains in sausage fermentation, as observed in the study of Janßen et al. (2018).

2. Material and methods

2.1. Bacterial strains and cultivation

Bacterial strains of *L. curvatus* were obtained from strain collection of Technische Mikrobiologie Weihenstephan (TMW). The complex medium MRS (20 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ casein pepton, 10 $g \cdot l^{-1}$ meat extract, 5 $g \cdot l^{-1}$ yeast extract, 1 $g \cdot l^{-1}$ Tween 80, 2 $g \cdot l^{-1}$ K_2HPO_4, 5 $g \cdot l^{-1}$ Na-Acetate, 2 $g \cdot l^{-1}$ $(NH_4)_2$ citrate, 0.2 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.05 $g \cdot l^{-1}$ $MnSO_4 \cdot H_2O$) (de Man and Sharpe, 1960) was used for cultivation of all selected strains. The cultures were grown at 30 °C under anaerobic conditions. For DNA isolation a preculture was inoculated. After 14 h of incubation 2.5 ml were inoculated in a main culture of 45 ml MRS and incubated for 8 h at 30 °C.

2.2. Growth experiments

The bacteria were cultivated in precultures in MRS for 16 h and under anaerobic conditions. Afterwards 1 ml of the cell suspensions were washed twice with 0.9% NaCl solution to completely remove remaining MRS. In the next step cells were incubated for 24 h in PBS-Buffer (8 $g \cdot l^{-1}$ NaCl, 0.2 $g \cdot l^{-1}$ KCl, 1.42 $g \cdot l^{-1}$ Na_2HPO_4 or 1.78 $g \cdot l^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 0.27 $g \cdot l^{-1}$ KH_2PO_4). Afterwards, the cells were inoculated in chemically defined medium (CDM) like it is described by Hebert et al. (Hebert et al., 2004; Morishita et al., 1981) at an initial OD_{590nm} of 0.1. At five different time points (0 h, 24 h, 48 h, 72 h, 96 h) samples of 500 µl were taken for HPLC analysis.

2.3. HPLC analysis

Two biological replicates were analysed for each strain as well as a non-inoculated control. For precipitation of proteins 250 µl 10% (w:v) $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich, St. Louis, USA) were added to 500 µl cell suspension. In the next step 250 µl 0.5 M NaOH (Carl Roth, Karlsruhe, Germany) were added to the sample and the suspension was mixed well. After incubation at room temperature for 20 min, the sample was centrifuged 10 min at maximum speed (13,000*g*) to remove the precipitate. Analysis was carried out by using a REMEX™ RPM Pb^{2+} column (Phenomenex, Aschaffenburg, Germany) at 85 °C in combination with a Dionex UltiMate 3000 Pump (Dionex, Idstein, Germany) and a Shodex RI-71 detector (Showa Denko Shodex, Munich, Germany). Quantification was done by using external standards of ribose (Sigma Aldrich, St. Louis, USA) and the Chromeleon evaluation software version 6.80 (Dionex, Idstein, Germany).

2.4. DNA isolation and genome analysis

High molecular DNA was isolated by using Qiagen Genomic-tip 100/G (Qiagen, Germany) and Qiagen Genomic DNA kit (Qiagen, Germany). 9 ml of bacterial cells of late exponential growth phase were centrifuged at 5000*g* for 10 min. The harvested cells were resuspended in 1 ml TE-buffer to remove media and centrifuged at 5000*g* for 5 min. Afterwards, the bacterial cells were lysed according to the manufacturer's protocol with minor modifications. For better lysis 80 µl