

# Plasmids of Raw Milk Cheese Isolate *Lactococcus lactis* subsp. *lactis* Biovar diacetylactis DPC3901 Suggest a Plant-Based Origin for the Strain<sup>∇†</sup>

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**The four-plasmid complement of the raw milk cheese isolate *Lactococcus lactis* subsp. *lactis* biovar diacetylactis DPC3901 was sequenced, and some genetic features were functionally analyzed. The complete sequences of pVF18 (18,977 bp), pVF21 (21,739 bp), pVF22 (22,166 bp), and pVF50 (53,876 bp) were obtained. Each plasmid contained genes not previously described for *Lactococcus*, in addition to genes associated with plant-derived lactococcal strains. Most of the novel genes were found on pVF18 and encoded functions typical of bacteria associated with plants, such as activities of plant cell wall modification (*orf11* and *orf25*). In addition, a predicted high-affinity regulated system for the uptake of cobalt was identified (*orf19* to *orf21* [*orf19-21*]), which has a single database homolog on a plant-derived *Leuconostoc* plasmid and whose functionality was demonstrated following curing of pVF18. pVF21 and pVF22 encode additional metal transporters, which, along with *orf19-21* of pVF18, could enhance host ability to uptake growth-limiting amounts of biologically essential ions within the soil. In addition, vast regions from pVF50 and pVF21 share significant homology with the plant-derived lactococcal plasmid pGdh442, which is indicative of extensive horizontal gene transfer and recombination between these plasmids and suggests a common plant niche for their hosts. Phenotypes associated with these regions include glutamate dehydrogenase activity and Na<sup>+</sup> and K<sup>+</sup> transport. The presence of numerous plant-associated markers in *L. lactis* DPC3901 suggests a plant origin for the raw milk cheese isolate and provides for the first time the genetic basis to support the concept of the plant-milk transition for *Lactococcus* strains.**

*Lactococcus lactis* strains are essential components of industrial starters for the manufacture of fermented dairy products. Their success in food fermentations is due largely to plasmid-encoded traits promoting efficient growth in milk (i.e., metabolism of lactose, milk proteins, citrate, and other complex nutrients), resistance to environmental stresses (e.g., bacteriophages, heavy metals, and temperature/osmotic stresses), and selective colonization advantages (i.e., production of bacteriocins and exopolysaccharides). Plasmids encoding these phenotypes are often mobilizable, which has greatly contributed to the genome plasticity of lactococci, enabling them to become adapted to and specialized for the dairy environment (25).

However, *L. lactis* is also naturally present on cows' skin and on plants, with the latter environment being the second most important ecosystem occupied by lactococci (21, 29). Plant strains are thought to be the natural source of dairy strains, since they can transfer from forage plants and meadow grasses to milk via cattle (37). Recent genotypic and phenotypic diversity analyses of large collections of dairy- and plant-derived *L. lactis* isolates have provided some evidence of such an origin (18) and showed that plant strains mostly belong to the subspecies *lactis* and exhibit a molecular diversity not observed in

dairy strains (33). A subsequent genome-scale analysis confirmed that plant strains exhibit an array of metabolic phenotypes exceeding that of dairy strains and including traits associated with the adaptation to grow on diverse plant cell wall substrates but also genes for defense and stress response (43). Natural isolates often demonstrate greater tolerance of stress as a result of specific protection mechanisms developed to resist the numerous cytotoxic compounds and organisms (i.e., antibiotics, heavy metals, bacteriocins, and bacteriophages) present in the plant and animal environments (34, 42, 49). They also exhibit phenotypes of industrial interest that can be utilized in dairy and other food fermenting applications, such as amino acid conversion to flavor compounds (i.e.,  $\alpha$ -keto acid decarboxylase and glutamate dehydrogenase) or the production of broad-spectrum bacteriocins (21, 44, 45). Again, many of these phenotypes are often plasmid encoded, which reaffirms the crucial contribution of these extrachromosomal entities to the performance, fitness, and ability of lactococci to colonize specific biotopes.

The analysis of plasmid complement is therefore essential to evaluate the genetic potential and variability existing in dairy and natural lactococcal strains. Using this approach, novel genotypes useful to the dairy industry can be found, but also niche-specific traits determining the uniqueness and adaptive capabilities of the strain can be uncovered. Should the plant-milk transition theory be valid, strains recently transferred to milk and therefore potentially still carrying plant-specific traits could theoretically be retrieved in artisanal products such as raw milk cheeses, where the natural milk microflora, derived from the grass, remains intact (14, 37).

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristic(s)	Source <sup>a</sup> or reference
<i>L. lactis</i> subsp. <i>lactis</i> biovar diacetylactis DPC3901	Raw milk cheese isolate; contains four plasmids of 18, 21, 22, and 50 kb	TFRC culture collection
<i>L. lactis</i> subsp. <i>lactis</i> biovar diacetylactis DPC3901-c1	pVF18-cured derivative of <i>L. lactis</i> DPC3901	This study
<i>L. lactis</i> subsp. <i>lactis</i> biovar diacetylactis DPC3901-c2	pVF22-cured derivative of <i>L. lactis</i> DPC3901-c1	This study
<i>L. lactis</i> subsp. <i>cremoris</i> MG1363	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>cremoris</i> 712	16
<i>L. lactis</i> subsp. <i>lactis</i> biovar diacetylactis DRC3	DNA size marker strain; contains eight plasmids with sizes from 2 to 78 kb	24
<i>L. lactis</i> subsp. <i>lactis</i> SK1	Cheese starter	TFRC culture collection
<i>L. lactis</i> subsp. <i>cremoris</i> DPC4268	Cheese starter	TFRC culture collection

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In this study, we have sequenced and functionally analyzed the four-plasmid complement of *L. lactis* subsp. *lactis* biovar diacetylactis DPC3901, a raw milk cheese isolate isolated and phenotypically characterized during previous studies aimed at identifying novel strains or genetic traits to be used in programs of starter improvement (6, 11). *L. lactis* DPC3901 plasmids contain a number of gene functions not previously described for *Lactococcus* and which are typical of bacteria associated with plants, as well as several other genes displaying homology only to single genes found on the plant-derived lactococcal plasmid pGdh442 or pKF147A (41, 47). Additionally, two *L. lactis* DPC3901 plasmids exhibited high levels of homology over many loci with pGdh442, which is indicative of extensive horizontal gene transfer (HGT) as well as plasmid recombination and implies that *L. lactis* DPC3901 may have originated from a plant niche.

#### MATERIALS AND METHODS

**Lactococcal strains and media.** The natural strain *L. lactis* DPC3901 was isolated and partially characterized in previous studies (6, 11). Lactococcal strains (Table 1) were routinely propagated at 30°C in M17 medium (Oxoid, United Kingdom) supplemented with 0.5% (wt/vol) glucose (GM17) or lactose (LM17). Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, United Kingdom). Bacterial strains were stocked in M17 containing 40% glycerol at -80°C. Working cultures were stored at 4°C and transferred periodically.

**Classification of *L. lactis* DPC3901.** *L. lactis* DPC3901 was genotyped with the universal 16S rRNA primers CO1 (5'-AGTTTGATCTGGCTCAG-3') and CO2 (5'-TACCTTGTTACGACT-3') and using the PCR conditions described elsewhere (2). Aliquots (5 µl) of the amplified products were subjected to electrophoresis in 1% agarose (Sigma) gels and stained with ethidium bromide. PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Germany) and sequenced by single-run primer extension (Beckman Coulter Genomics). Sequencing data were searched for homology against sequences available in public databases (GenBank, EMBL, DDBJ, and PDB) by using the nucleotide MegaBLAST algorithm of the Basic Local Alignment Search Tool (BLAST) (56). Confirmation of the genotypically predicted citrate-fermenting phenotype was obtained by streaking a freshly grown culture of *L. lactis* DPC3901 on KMK medium and checking for the appearance of blue colonies after 48 h of incubation at 30°C (9).

**Plasmid DNA sequencing, assembly, and annotation.** Plasmid DNA was prepared as described elsewhere (32). Any residual genomic DNA was removed by treatment with Plasmid-Safe ATP-dependent DNase (Epicentre, Madison, WI), which selectively hydrolyzes linear double-stranded DNA to deoxynucleotides without affecting closed circular supercoiled or nicked circular double-stranded DNAs (dsDNAs). The purified plasmids were sequenced as a pool on a 454 Genome Sequencer FLX system after preparation of a sonication library, bar coding of the library with the Roche multiplex identifier system, and emulsion PCR (emPCR) amplification (Beckman Coulter Genomics). Bar coding of the library was necessary since pools of plasmids from different isolates were sequenced simultaneously. Automatic *de novo* assembly of data resulted in 56 large (>500 bp) contigs providing ca. 135 kb of sequence data (12× average coverage). Gap closure was performed by PCR using primers designed with the Primer-Select v.8.0.2 software program (DNASTar), followed by sequencing of the PCR products (Beckman Coulter Genomics). Putative open reading frames (ORFs) were automatically predicted and annotated using the Glimmer 2.0 software program (7). Annotated DNA sequences were examined manually using the Artemis viewer (36), and BLASTX and BLASTP analyses (1) were used to examine intergenic regions for putative ORFs not identified by Glimmer, to identify putative frameshifts, and to refine the start codon assignments. The InterProScan software tool ([www.ebi.ac.uk/Tools/InterProScan](http://www.ebi.ac.uk/Tools/InterProScan)), the PFAM database (13), and the Conserved Domain Database (CDD) (23) were used to predict protein function. InterProScan predicts the occurrence of functional domains and motifs/signatures in a protein by combining 12 different databases and their relative protein signature recognition methods (55). Prediction of topology in putative alpha-helical membrane proteins, including a specification and the in/out orientation of the membrane-spanning segments, was obtained by using the TOPCONS hidden Markov model (<http://topcons.cbr.su.se/index.php>), which uses five different topology prediction algorithms (SCAMPI-seq, SCAMPI-msa, PRODIV-TMHMM, PRO-TMHMM, and OCTOPUS) to generate a consensus prediction for the protein (3). Putative ribosome binding sites (RBS) and terminator sequences were automatically identified by using the RBSFinder+TransTerm program (<http://nbc11.biologie.uni-kl.de/>). Manual identification of terminator sequences was achieved by following three main criteria: (i) a  $\Delta G$  value of less than -6, (ii) a stem-loop structure rich in GC, and (iii) a T-rich sequence following the stem-loop structure.

**Growth inhibition assays.** Bacterial sensitivity to cadmium (Cd<sup>2+</sup>), cobalt (Co<sup>2+</sup>), tetracycline, streptomycin, and kanamycin (Sigma) was assessed by using a microplate procedure. Two hundred microliters of GM17 or LM17 broth containing either CdCl<sub>2</sub> (0 to 0.3 mM), CoCl<sub>2</sub> (0 to 6 mM), tetracycline (0 to 10 µg/ml), streptomycin (10 to 500 µg/ml), or kanamycin (10 to 500 µg/ml) was inoculated at 1% with overnight cultures on 96-well microplates (Sarstedt, Newton, NC). Bacterial growth was assessed over 18 h at 30°C by measuring the absorbance at 600 nm with a GENios Plus reader (Tecan, Switzerland).

**Determination of glutamate dehydrogenase (GDH) activity.** GDH activity was determined in cell-free extracts (CFEs) as previously described (20), with some modifications. Briefly, cell cultures (200 ml) growing in mid-log phase (optical density [OD] = 0.4) were centrifuged (4,100 × g, 15 min, 4°C) and washed twice with 50 mM triethanolamine buffer (TEA) (pH 7). Pellets were resuspended in 5 ml of TEA containing lysozyme (1.6 mg/ml) and saccharose (0.2 mg/ml), incubated at 30°C for 2 h, centrifuged, and resuspended in hypotonic buffer TEA to provoke spheroplast lysis. Cell debris was eliminated by centrifugation (20,000 × g for 30 min), and the supernatant was filtered (0.45-µm filter; Millipore) and used as CFE. The CFE protein concentration was determined by the Bradford method using bovine serum albumin as the standard (4). Determination of GDH activity in CFEs was based on a modification of a colorimetric glutamic acid assay (Boehringer Ingelheim GmbH, Germany) where the reduced cofactor (NADH or NADPH), produced by GDH-mediated oxidative deamination of glutamate, reacts with iodinitrotetrazolium in the presence of diaphorase to produce a colorimetric product (formazan) that is measured by absorbance at 492 nm. A 300-µl reaction mixture consisted of 40 µl of 50 mM potassium phosphate-50 mM TEA buffer (pH 9.0) containing 1% of Triton X-100 (Promega Corporation), 20 µl of 100 mM glutamic acid, 20 µl of 2 mM iodinitrotetrazolium, 20 µl of diaphorase (1.76 U/ml), 20 µl of NAD<sup>+</sup> (17.33 mM) or NADP<sup>+</sup> (13.8 mM), and 180 µl of water. The colorimetric reaction was started by adding 30 µl of CFEs to the reaction mixture, and the changes in the NADH concentration after 1 h of incubation at 37°C were determined by measuring the *A*<sub>492</sub>. A control test lacking glutamic acid was prepared for each strain in order to subtract for nonspecific reactions producing the reduced cofactors. The GDH activity was expressed by the increase in *A*<sub>492</sub> per mg of protein of CFE and per min of reaction.

**Plasmid curing.** Plasmid curing experiments were performed by growing strains at 37°C in LM17 lacking the buffering agent β-glycerophosphate (β-GP)

TABLE 2. Primers used in this study to map gene features of *L. lactis* DPC3901 plasmids

Primer <sup>a</sup>	Sequence (5'–3')	Product size (bp)	Target	Plasmid
orfs18-21 (F)	GAAAACATCAAGAAGAACGCAGAGT	1,858	<i>orf18-21</i>	pVF18
orfs18-21 (R)	TGGCAATCGAAGCATCAAGTAGAC			
gdh (F)	GTGGGTCGACGATGCTGGTAAAGTT	822	<i>gdh</i>	pVF21
gdh (R)	TGGACGCCGCTGGAAATCAATA			
corA (F)	TTTATGTGTATGGTGACGAACGAGAAT	812	<i>corA</i>	pVF22
corA (R)	CAGCACAGCAAAAAACCCATAAGAAGAT			
ldh (F)	GTGCTGCCAAATATAGATGCCCAACA	484	<i>ldh</i>	pVF50
ldh (R)	ATGCCAAAGCGGTTAGATGATTATTACGA			

<sup>a</sup> F, forward primer; R, reverse primer. Columns indicate the primer's name and sequence, expected size of the PCR product, target gene(s), and its plasmid location.

for 50 generations before being transferred to fresh medium. The strain was plated on LM17 agar at each transfer in order to analyze plasmid profiles of individual colonies.

**Measurement of bacterial growth rate and acidification capacity.** The growth rates and acidification capacities of *L. lactis* DPC3901 and of its pVF18/pVF22-cured derivative (*L. lactis* DPC3901-c2) were compared with those of cheese starter strains *L. lactis* subsp. *cremoris* DPC4268 and *L. lactis* subsp. *lactis* SK1 as previously described (12). Briefly, growth rate parameters (maximum growth rate,  $\mu_{max}$ , and generation time, *G*) were determined after growth in LM17 at 30°C, whereas the acidification capacities (starter activity) were evaluated, based on final pH, after growth in 11% reconstituted skim milk (RSM) over a 5-h incubation time through a simulated cheddar cheese-making temperature profile (32°C for 70 min, 40°C for 190 min, and 32°C for 40 min).

**PCR analysis.** Detection of specific genes within the plasmid complement of *L. lactis* DPC3901 and its cured derivatives, DPC3901-c1 and DPC3901-c2, was obtained by PCR analysis using the primers listed in Table 2.

**Nucleotide sequence accession numbers.** The complete nucleotide sequences of plasmids pVF18, pVF21, pVF22, and pVF50 have been submitted to the EMBL/GenBank database and are available under accession numbers JN172910, JN172911, JN172912, and JN225497, respectively.

## RESULTS

**Plasmid DNA sequencing.** Sequencing of the 16S rRNA gene revealed a subsp. *lactis* genotype for *L. lactis* DPC3901, which was also classified in the biovar diacetylactis due to its ability to ferment citrate during growth on KMK medium (data not shown). *L. lactis* DPC3901 was previously found to harbor four plasmids greater than 10 kb in size and to be completely resistant to a number of 936- and c2-type phages (11). Consequently, the entire plasmid complement of *L. lactis* DPC3901 was sequenced to investigate its contribution to the technological capacities of the strain. By using a PCR-based gap closure strategy, the complete circular sequences of plasmids pVF50 (53,876 bp), pVF22 (22,166 bp), pVF21 (21,728 bp), and pVF18 (18,977 bp) were obtained (Fig. 1; see also Tables S1 to S4 in the supplemental material). The GC content of these plasmids ranged from 33.6 to 35.1%, which is similar to that of other lactococcal plasmids (42). Sequence analysis suggests that all plasmids could replicate according to a theta mechanism, since each one carries an origin of replication (*ori*), located upstream of the *rep* gene encoding the replication initiator protein, containing highly conserved structural motifs typical of theta replicons (data not shown) (39). The most significant genetic features of *L. lactis* DPC3901 plasmids are discussed below.

**Genes from pVF18, pVF21, and pVF50 suggest a plant-based origin for *L. lactis* DPC3901.** pVF18 contains 25 genes encoding predicted proteins with no obvious dairy-associated functions, eight of which (*orf8*, *orf11*, *orf18* to *orf21*, *orf23*, and *orf25*) have been rarely if ever described for *Lactococcus*. Some

of these genes encode products whose functions are irrelevant to bacteria growing in milk but important to those associated with or striving to become established within the natural plant ecosystem. An example is *orf11*, which encodes a putative polysaccharide deacetylase (432 amino acids [aa]) belonging to a family of conserved proteins (PFAM01522) involved in hydrolysis of the plant cell wall. Members of this family include the chitooligosaccharide deacetylase NodB from *Rhizobium* (15), chitin deacetylases from *Saccharomyces cerevisiae* (27), and microbial endoxylanases (26). These last enzymes share sequence similarity with the NodB deacetylase (26), whose activity enables *Rhizobium* to associate symbiotically with leguminous plants, thus allowing them access to mineral nitrogen necessary for their growth. The *nodB* gene is plasmid encoded in *Rhizobium* sp. NGR234, and this was suggested to be the result of a recent HGT from *Agrobacterium* (15). Orf11-like proteins are highly conserved in *Enterococcus faecium*, *Staphylococcus aureus*, and some *Ruminococcus* and *Clostridium* members, but no homologues have been found to date in *Lactococcus*. Similarly, homologues of the product of *orf25* can be found in various environmental subspecies of *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Corynebacterium*, *Bacillus*, *Geobacillus*, and *Clostridium* but not in *Lactococcus* or other milk-derived bacteria. Orf25 is a 140-aa protein containing the conserved barrel domain typical of cupins (PFAM07883). The functions of these proteins range from isomerase and epimerase activities involved in the modification of cell wall carbohydrates in bacteria and plants to nonenzymatic germins and plant seed storage proteins, which provide the major nitrogen source for the developing plant (10).

Traces of a putative plant origin can also be linked to pVF50 and pVF21. pVF50 is largely similar to the plant-derived lactococcal plasmid pGdh442 (47), with which it shares five gene modules spanning more than 30 kb of sequence and exhibiting 97 to 100% homology at both the DNA and protein levels. One of these shared gene blocks was so far exclusive to pGdh442 and is composed of three genes encoding membrane components of Na<sup>+</sup> and K<sup>+</sup> transport systems, which are virtually identical in pVF50 (*nah*, *orf21*, and *orf22*) and in pGdh442 (*orf15* to *orf17*) but share only 41 to 45% identity to other homologues found in *Clostridium* (47). Also, pVF21 carries a region 5.6 kb long and containing the genes *gdh*, *tnp-IS1216*, *cadA*, and *cadC*, which was so far unique to pGdh442 (47). In particular, the glutamate dehydrogenase (GDH) activity encoded by the *gdh* gene is rare in *L. lactis* and so far has been identified only in strains of plant and animal origin (47, 48). Additionally, pVF21 carries a gene (*orf15*) predicted to

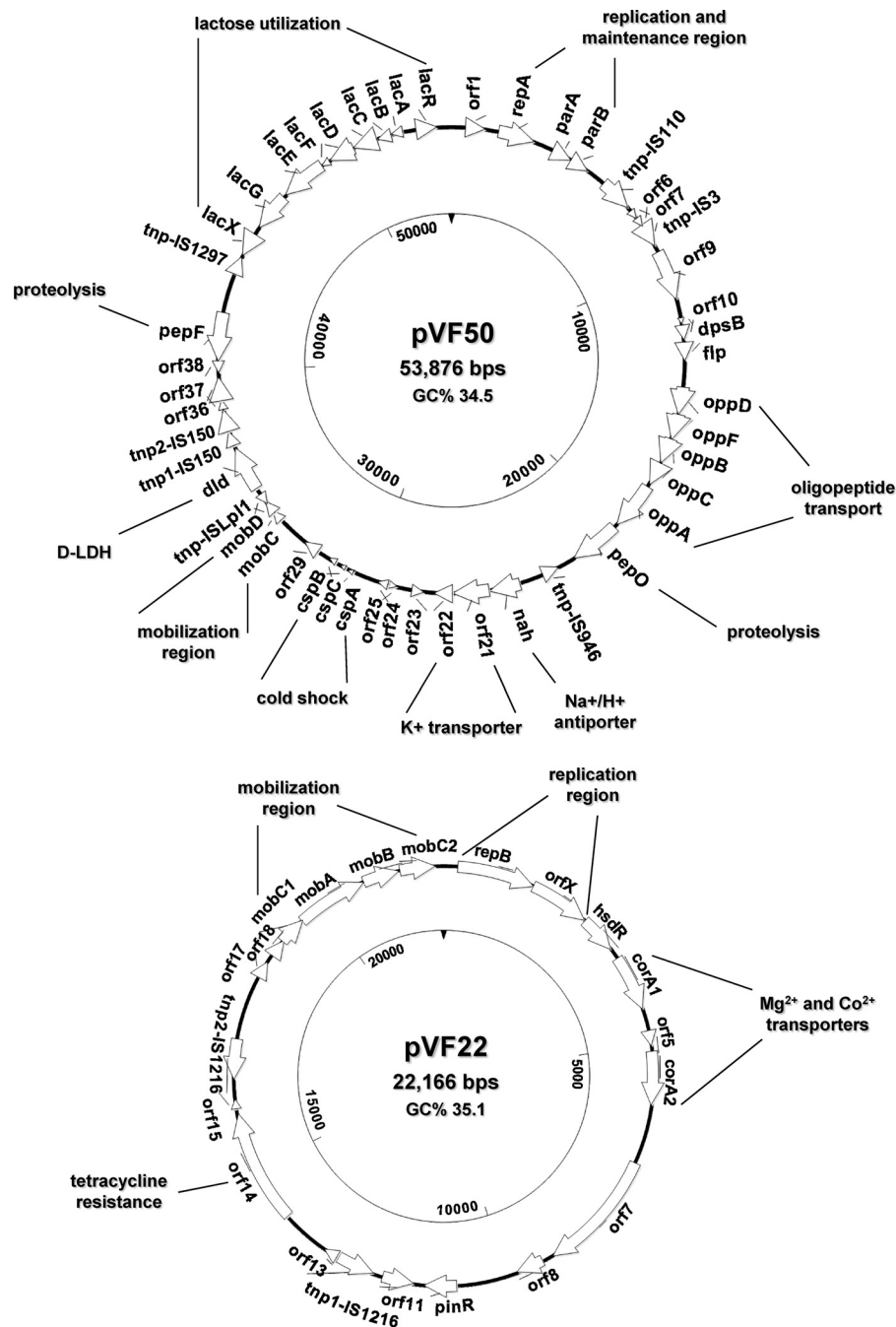


FIG. 1. Physical and genetic maps of plasmids pVF50, pVF22, pVF21, and pVF18 of *L. lactis* DPC3901. Position and orientation of genes are indicated by arrows. Inner circles show the nucleotide numbering.

encode a 105-aa hypothetical protein identical to its only database match, encoded by the plasmid pKF147A, isolated from an *L. lactis* plant strain (41). Finally, pVF21 encodes a hypothetical protein (Orf5) of 365 aa with no homologues in *Lactococcus* or other lactic acid bacteria (LAB) and with the most similar matches (27 to 32%) being proteins from two *Bacillus cereus* strains. Orf5 contains the conserved motifs (DGQHR, QR, and FxxxN) typical of the highly divergent DGQHR domain (TIGR03187), and although the function of proteins containing this domain is unknown, several

members of this family have been annotated as putative bacteriophage proteins.

**Functional  $\text{Co}^{2+}$  transport mechanisms in *L. lactis* DPC3901 plasmids.** Cobalt ( $\text{Co}^{2+}$ ) is an essential component of many enzymes and must be transported into cells in appropriate amounts when needed (34). In *L. lactis* DPC3901, pVF22 carries two copies (*corA1* and *corA2*) of the *corA* gene (COG0598), which encodes the principal transporter of  $\text{Mg}^{2+}$  in prokaryotes but also functions in the uptake of  $\text{Co}^{2+}$  (28). Additionally, pVF18 carries a putative polycistronic operon



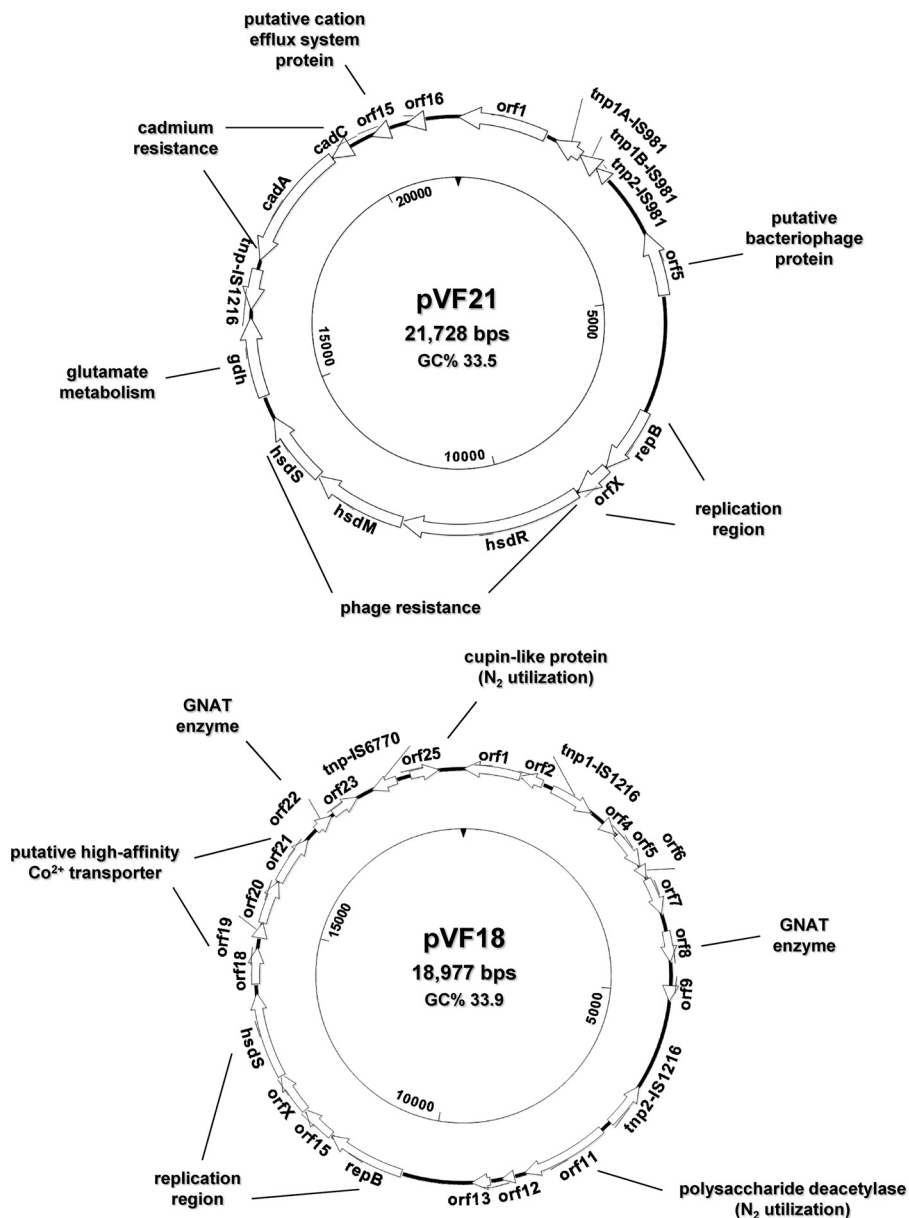


FIG. 1. —Continued.

(*orf19* to *orf21* [*orf19-21*]) that may play a role in the high-affinity and regulated uptake of  $Co^{2+}$ . Two 4-bp overlaps between *orf20* and its neighbor genes, coupled with the presence of putative promoter sequences upstream of *orf19* and of a strong rho-independent terminator ( $\Delta G = -7.9$  kcal/mol) downstream of *orf21* (Fig. 2a), suggest that *orf19*, *orf20*, and *orf21* are transcribed as a single mRNA. This three-gene operon is uncommon in *L. lactis* and so far has been found only on the recently sequenced plasmid LkipL4726 from *Leuconostoc kimchii* IMSNU 11154 (30). *orf19* encodes a small protein (75 aa) of unknown function exhibiting 92% identity to its only significant match, a hypothetical protein from LkipL4726 (30). *orf20* and *orf21* encode proteins containing features typical of the ATPase and permease components of ATP-binding cassette (ABC) transporters (Fig. 2b and c). Interestingly, ho-

mologs of Orf20 are widespread in *Lactococcus*, whereas Orf21 has a single lactococcal match on pGdh442 (47). A putative involvement in  $Co^{2+}$  uptake for the ABC transporter encoded by *orf20-21* is suggested by the presence in Orf20 of a conserved domain (cd03225) found in the CbiO component of CbiMNOQ, which is the most widespread transport system for the high-affinity uptake of  $Co^{2+}$  in prokaryotic genomes (34). To verify the functionality of these mechanisms, the growth of *L. lactis* DPC3901 in the presence of increasing concentrations of  $Co^{2+}$  was compared to that of *L. lactis* MG1363, which lacks any high-affinity  $Co^{2+}$  uptake system but possesses a chromosomally encoded CorA transporter. *L. lactis* DPC3901 can grow relatively well in the presence of 4 mM  $Co^{2+}$  and tolerate concentrations as high as 6 mM (Fig. 3a). In contrast, *L. lactis* MG1363 growth is affected by the presence of 1 mM

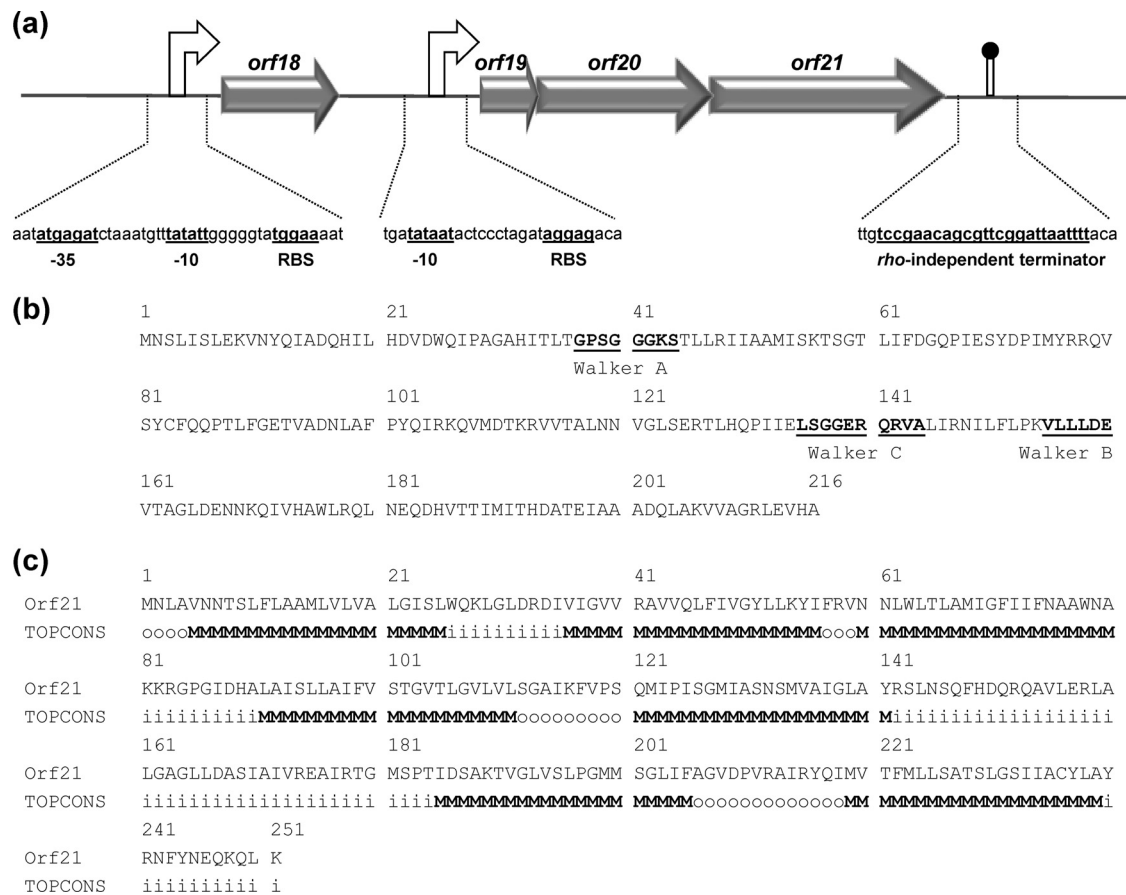


FIG. 2. Sequence analysis of the putative polycistronic operon *orf18-21*. (a) Putative regulatory elements of the *orf18-21* operon. The ribosome binding site (RBS),  $-10$  and  $-35$  promoter sequences, and *rho*-independent terminator signal are shown in bold and underlined. (b) Locations of the conserved domains typical of ABC transporter ATPases in Orf20: the Walker A (consensus GxxGxGKS/T, where x is any residue) and Walker B (consensus hhhDE, where h is a hydrophobic residue) motifs involved in ATP binding and the Walker C (consensus LSGGQ/R/KQR) motif involved in ATP hydrolysis are shown underlined. (c) TOPCONS consensus prediction of membrane topology for Orf21, showing seven transmembrane motifs (i, inside; M, transmembrane; o, outside) typical of ABC transporter permeases.

$\text{Co}^{2+}$  and is totally inhibited by concentrations of and above 2 mM (Fig. 3b). Despite its importance for bacterial metabolism,  $\text{Co}^{2+}$  uptake must be tightly regulated to avoid toxic effects (34). Results from the growth curves are indicative of the ability of *L. lactis* DPC3901 to maintain the intracellular  $\text{Co}^{2+}$  to levels that are compatible with cell life. To further confirm these indications, pVF18 and pVF22 were sequentially cured from *L. lactis* DPC3901 in order to first remove *orf19-21* and then the *corA* genes. Growth curve analysis showed that the pVF18-cured derivative, named DPC3901-c1 ( $\Delta\text{orf19-21 corA}^+$ ), could not grow at  $\text{Co}^{2+}$  concentrations higher than 1 mM (Fig. 3c), whereas the derivative cured of both pVF18 and pVF22, DPC3901-c2 ( $\Delta\text{orf19-21 } \Delta\text{corA}$ ), tolerated  $\text{Co}^{2+}$  in a fashion similar to that of the parent strain (Fig. 3d). These results confirm the involvement of the *orf19-21* genes in mediating uptake and homeostasis of the potentially toxic metal  $\text{Co}^{2+}$  in *L. lactis* DPC3901.

**Antibiotic resistance in *L. lactis* DPC3901.** pVF22 carries a gene (*orf14*) encoding a TetS-like tetracycline resistance protein (cd04168) identical to those found on pKL0018 from the fish pathogen *L. garvieae* (22) and on pK214 from an *L. lactis* isolate from raw milk soft cheese (49). This protein functions

through ribosomal protection, and its gene is typically found on mobile genetic elements, such as transposons or plasmids. In pVF22, *orf14* is inserted within a genetic context resembling a transposon-like structure since it is flanked by two *IS1216* transposases and by genes responsible for site-specific recombination (*pinR* and *orf17*) and for conjugal transfer (*mobC*, *ABC*<sub>2</sub>). The presence of this *tet(S)*-like gene on a conjugative plasmid would pose concerns regarding the use of *L. lactis* DPC3901 in foods for human consumption due to the potential spread of antibiotic resistance genes from food-borne LAB to pathogenic organisms (49). However, antibiotic resistance genes have been removed from probiotic strains by using methods that did not genetically modify the organism and retained the original probiotic characteristics (35). We took a similar approach and cured pVF22 from the pVF18-cured derivative of *L. lactis* DPC3901 (*L. lactis* DPC3901-c1) (Fig. 4a). The strain lost pVF22 after ca. 100 generations but retained pVF50 and pVF21, as also confirmed by PCR (Fig. 4b). Compared to the parent strain, the new derivative (DPC3901-c2) exhibited unaltered acidification capacities and slightly better growth rates (Table 3), probably as a result of the loss of two metabolically burdening plasmids.

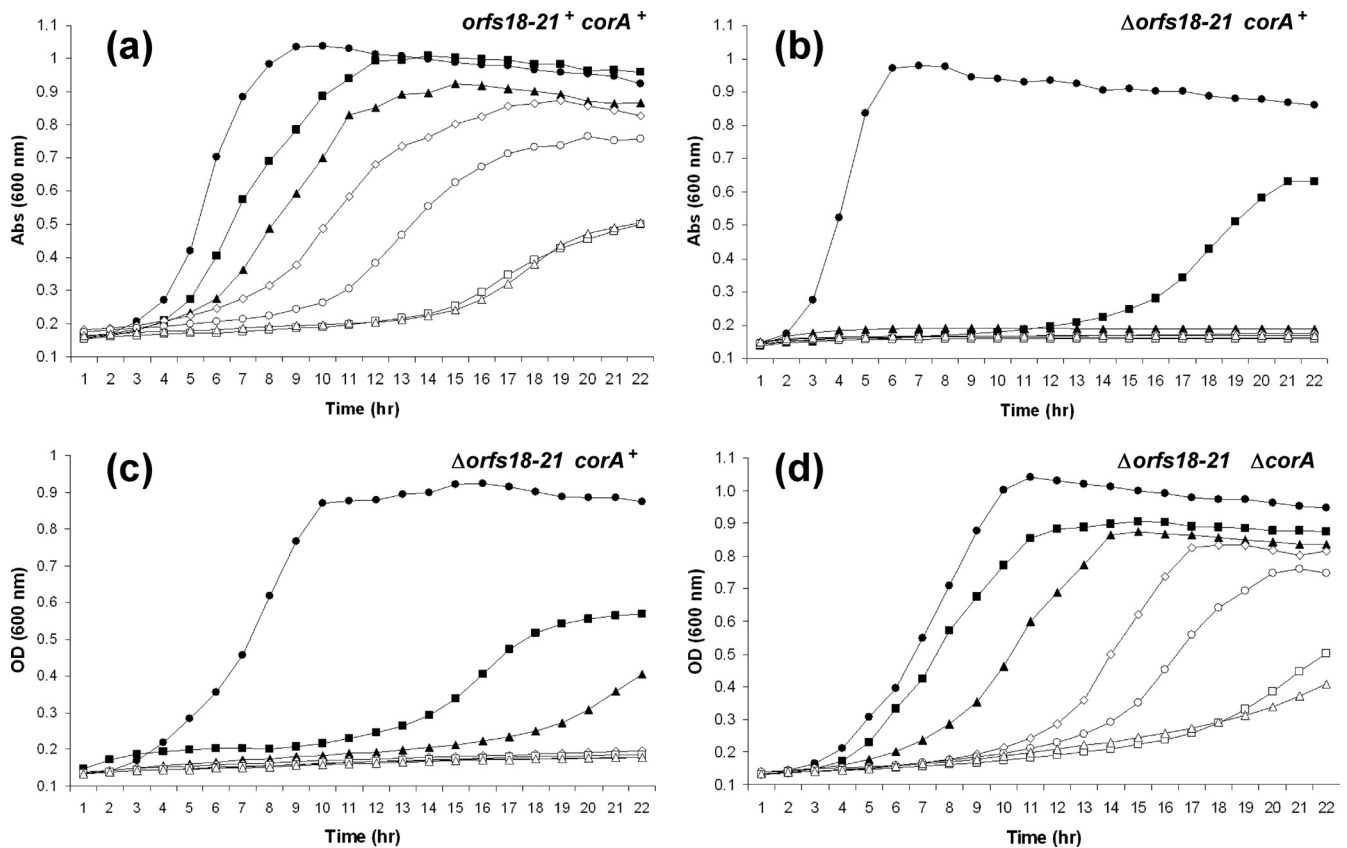


FIG. 3. Growth curves of *L. lactis* DPC3901 (a), MG1363 (b), DPC3901-c1 (c), and DPC3901-c2 (d) in medium containing 0 (●), 1 (■), 2 (▲), 3 (◇), 4 (○), 5 (□), or 6 mM (△) cobalt chloride. The minus or plus superscript is indicative of the absence or presence, respectively, of the *orf18-21* and *corA* genotypes in each strain or derivative. Abs, absorbance.

Putative determinants for antibiotic resistance were also found on pVF18, which contains two genes (*orf8* and *orf23*) encoding putative GCN5-related *N*-acetyltransferases (GNAT) (cd04301). These enzymes catalyze the transfer of an acetyl group to the cognate substrate and are thought to be implicated in a variety of functions, ranging from antibiotic (aminoglycosides) resistance to regulation of cell growth and development (54). To test their possible involvement in antibiotic resistance, *L. lactis* DPC3901 and the sensitive strain *L. lactis* MG1363 were grown in the presence of increasing concentrations of streptomycin and kanamycin (data not shown). Both strains were similarly sensitive to both aminoglycosides, which suggests that the GNAT-like proteins encoded by pVF18 are not involved in resistance to these antibiotics. Interestingly, these proteins have rarely been found in *Lactococcus* but are conserved in *Streptococcus*, *Clostridium*, *Enterococcus*, and *Staphylococcus*.

**pVF21 confers potential flavor-forming and phage resistance capability to DPC3901.** pVF21 carries a *gdh* gene encoding a 448-aa protein with homology to the NAD(P)-dependent glutamate dehydrogenase (GDH) enzyme of pGdh442 (46). GDH enzymes catalyze the reversible oxidative deamination of glutamate to  $\alpha$ -ketoglutarate and ammonium by utilizing NAD, NADP, or both as a cofactor. Thus, we tested the functionality of the *gdh* gene of pVF21 by using *L. lactis* IL1403 as a GDH-negative reference strain. No GDH activity was regis-

tered for *L. lactis* IL1403, whereas *L. lactis* DPC3901 exhibited NAD<sup>-</sup>- and NADP<sup>+</sup>-dependent specific activities of 0.028 and 0.01, respectively, which are similar to those reported for an *L. lactis* subsp. *cremoris* strain used in the manufacture of Gouda and cheddar-type cheeses (20). These results suggest that pVF21 encodes a functional GDH enzyme providing *L. lactis* DPC3901 with the potential to produce aroma compounds from glutamate catabolism and/or to synthesize glutamate from  $\alpha$ -ketoglutarate and ammonia.

pVF21 also carries three genes (*hsdRMS*) encoding a restriction-modification (R/M) system of type I, which is one of the many molecular mechanisms developed by *L. lactis* strains to defend themselves against bacteriophage (phage) attack (25). The *hsdR* gene encodes a restriction enzyme (1,025 aa) containing conserved helicases-related domains (cd00046; COG0610), while the 537-aa product of *hsdM* is a typical *N*<sup>6</sup>-adenine methyltransferase (pfam02384). Both proteins are virtually identical (99%) to the HsdR and HsdM enzymes encoded by genes on the lactococcal plasmid pAH82 and exhibiting low restriction efficiency (2 logs) against the small isometric-headed phage 712 (31). In contrast, the HsdS specificity protein (414 aa) of pVF21 shares only 38% identity with the homolog encoded by pAH82. Low similarities among HsdS subunits are common and reflect the large variability existing within their target recognition domains (TRDs), which impart

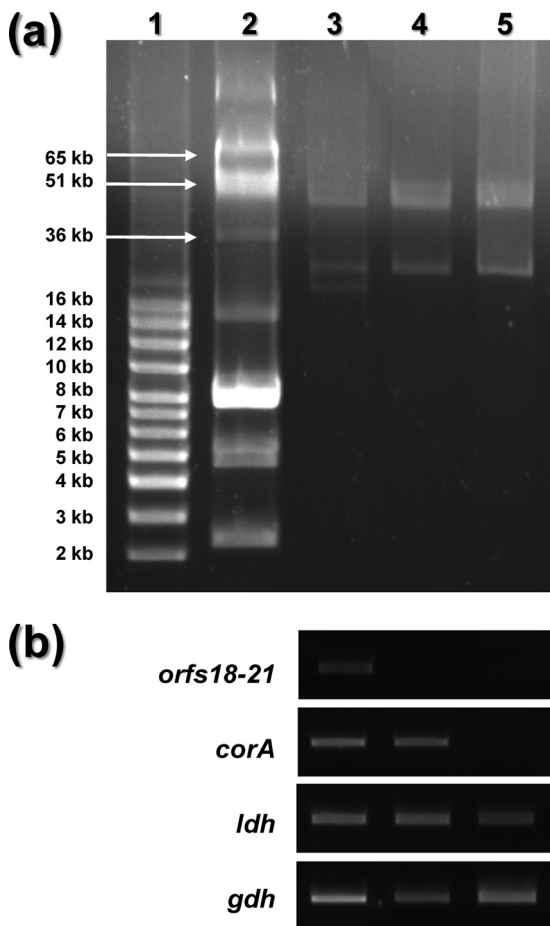


FIG. 4. (a) Plasmid DNA profiles of *L. lactis* DPC3901 (lane 3) and its cured derivatives DPC3901-c1 (pVF18<sup>-</sup>; lane 4) and DPC3901-c2 (pVF18<sup>-</sup> pVF22<sup>-</sup>; lane 5). The plasmid profile of *L. lactis* DRC3 (lane 2) provides reference plasmids of large sizes (36, 51, and 65 kb) as a complement to the 2- to 16-kb molecular size ladder (lane 1). (b) PCR-based detection of specific gene features in the plasmid complement of *L. lactis* DPC3901, DPC3901-c1, and DPC3901-c2.

target sequence specificity to both restriction and modification activities (25).

**pVF50 contributes to adaptation of *L. lactis* DPC3901 to the dairy environment.** pVF50 shares over 30 kb of nearly identical sequence with pGdh442, which in the latter plasmid include the adjacent modules *orf55-64* (*orf55* to *orf64*) and *orf1-3* (*orf1* to *orf3*) and three flanking modules spanning *orf15* to *orf23* (47). pGdh442 contains 20 IS elements that have probably mediated the acquisition of its numerous gene modules (47) and can therefore promote their transfer to plasmids of a different organism. Adjacent modules in pVF50 exhibit a swapped organization compared to their arrangement in pGdh442, thus supporting the hypothesis that these gene blocks can be mobilized by the flanking IS elements (47).

Genes on pVF50 related to growth on milk components have certainly facilitated the adaptation of *L. lactis* DPC3901 to the dairy environment. The strain's ability to grow well in milk and to utilize lactose is afforded by the lactose phosphotransferase operon (*lacXGEFDCBA*), which is negatively regulated by the product of the divergently transcribed *lacR* gene

(52, 53). The *lacXGEFDCBAR* region of pVF50 is 98 to 100% identical to that present on pSK11L at both the DNA and protein levels (42). A further contribution to lactose utilization in *L. lactis* DPC3901 might also be afforded by the *ldd* gene (*contig2.gene14*), which is predicted to encode a D-lactate dehydrogenase (D-LDH) of 559 aa and might function in D-lactate utilization under aerobic conditions (42). pVF50 also encodes the Opp system for the uptake of oligopeptides generated from casein degradation by the cell wall proteinase (51) and the oligoendopeptidases PepO and PepF, which are able to hydrolyze oligopeptides of 5 to 35 residues (5). These genotypes should endow *L. lactis* DPC3901 with the ability to optimally utilize milk caseins as a nitrogen source. The *opp* and *pepO* genes are part of a region, containing the *flp* operon and flanked by transposases of the IS3 and IS6 families, that is 98% identical to the homologous region on pGdh442 (*orf55-64*) (47). The presence of these determinants led us to evaluate the strain's ability to grow and acidify milk under conditions resembling the manufacture of cheddar cheese. Growth rates and acidification capacity of *L. lactis* DPC3901 were slightly lower than those of the widely used cheese starters *L. lactis* DPC4268 and *L. lactis* SK1 but still sufficient for cheese manufacturing (Table 3).

pVF50 is the only *L. lactis* DPC3901 plasmid to contain a replication and maintenance region composed of a typical lactococcal theta-type *ori* site (39), a *repA* gene, and two partition genes (*parA* and *parB*) that are likely to provide pVF50 with a maintenance system counteracting plasmid loss at cell division. This region is identical to homologous regions present on the plant-derived lactococcal plasmids pKF147A (41) and pGdh442 (47) and on pSK08 from *L. lactis* ML3 (accession no. AF300944).

## DISCUSSION

It is thought that plant-associated strains are the original source of many dairy strains since they can transfer from forage plants and meadow grasses to milk via cattle (37). Recently, some evidence of such an origin was suggested based on an analysis of chromosome length that grouped five *L. lactis* subsp. *cremoris* strains, including *L. lactis* MG1363, with plant strains, such as *L. lactis* KF147, and on the observation that remnants of genes for degradation and metabolism of plant-derived carbohydrates can also be found in the *L. lactis* MG1363 genome (18). The authors observed a reductive evolution in dairy strain genomes compared to those of the plant

TABLE 3. Comparison of growth rate parameters and acidification capacities of *L. lactis* DPC3901 and its pVF18/pVF22-cured derivative (DPC3901-c2) with those of selected lactococcal cheese starters<sup>a</sup>

<i>L. lactis</i> strain	Growth rate		Starter activity (pH)	
	$\mu_{\max}^b$ (h <sup>-1</sup> )	Generation time (h <sup>-1</sup> )	11% RSM	GM17
DPC3901	0.90 (± 0.08)	1.17 (± 0.10)	5.37 (± 0.18)	6.06 (± 0.04)
DPC3901-c2	0.99 (± 0.10)	1.09 (± 0.12)	5.25 (± 0.01)	5.90 (± 0.05)
SK1	1.18 (± 0.01)	0.85 (± 0.01)	4.91 (± 0.24)	5.67 (± 0.03)
DPC4268	1.10 (± 0.13)	0.92 (± 0.11)	4.78 (± 0.13)	5.41 (± 0.01)

<sup>a</sup> Values are means ± SD.

<sup>b</sup>  $\mu_{\max}$ , maximum growth rate.



TABLE 4. Novel genes not found before in *L. lactis* and rare genes with homologues on plant-associated lactococcal strains

Plasmid	Gene(s)	Product(s)	Predicted function/role(s)	Best homolog found in:	% identity
Novel in <i>L. lactis</i>					
pVF18	<i>orf11</i>	Polysaccharide deacetylase	Hydrolysis of cell wall carbohydrates in plants; utilization of mineral nitrogen	<i>Enterococcus faecium</i> PC4.1	99
pVF18	<i>orf19-orf20-orf21</i>	Hypothetical protein; ABC transporter, ATPase and permease components	Putative high-affinity Co <sup>2+</sup> transporter; heavy metal homeostasis	<i>Leuconostoc kimchii</i> IMSNU 11154, LkipL4726	92–99
pVF18	<i>orf25</i>	Cupin domain containing isomerase/epimerase or germin/plant storage protein	Modification of cell wall carbohydrates in plants; utilization of mineral nitrogen	<i>Streptococcus uberis</i> 0140J	92
pVF21	<i>orf1</i>	Primosomal protein N'	DNA replication factor	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC 8293	37
pVF21	<i>orf5</i>	DGQHR domain-containing hypothetical protein	Putative bacteriophage protein	<i>Bacillus cereus</i> G9842	32
pVF50	<i>tmp-IS110</i>	Transposase, IS110 family	DNA integration/recombination	<i>Streptococcus pneumoniae</i> SP19-BS75	75
Rare in <i>L. lactis</i> (single hit)					
pVF18	<i>orf8, orf23</i>	GCN5-related N-acetyltransferase	Regulation of cell growth and development	<i>Enterococcus faecium</i> ; <i>Weissella paramesenteroides</i> ATCC 33313	98; 84
pVF18	<i>orf24</i>	Transposase, IS30 family	DNA integration/recombination	<i>Enterococcus faecalis</i> TUSoD Ef11	100
pVF21	<i>gdh</i>	Glutamate dehydrogenase	Reversible oxidative deamination of glutamate	<i>Lactococcus lactis</i> , pGdh442	99
pVF21	<i>orf15</i>	Hypothetical protein LLKF_p0013	Putative cation efflux system protein	<i>Lactococcus lactis</i> KF147, pKF147A	99
pVF22	<i>orf9</i>	Hypothetical protein	Putative glycopeptide antibiotic resistance protein	<i>Streptococcus salivarius</i>	98
pVF22	<i>orf13</i>	Hypothetical protein	Unknown	<i>Lactococcus garvieae</i> , pLK0018	99
pVF50	<i>nah</i>	Na <sup>+</sup> /H <sup>+</sup> antiporter	Exchange of sodium/hydrogen ions	<i>Lactococcus lactis</i> , pGdh442	100
pVF50	<i>orf21</i>	Putative K <sup>+</sup> transporter	Uptake of potassium	<i>Lactococcus lactis</i> , pGdh442	99
pVF50	<i>orf22</i>	Putative K <sup>+</sup> transport system TrkA	Uptake of potassium	<i>Lactococcus lactis</i> , pGdh442	99

isolates, which they interpreted as part of the adaptation to growth in milk. This process involves the acquisition of genes that are mostly plasmid encoded.

Plasmids are capable of mediating extensive HGT and recombination, and this enables strains to acquire traits conferring a selective advantage to colonize specific biotopes (25). Some of these traits can be so specific to a particular niche as to be reasonably considered equivalent to traceability markers, thus enabling the plasmid complement to be used as a sort of bar-coding system of the organisms' origin. Retrieval of strains containing such traceable traits is feasible within raw milk cheeses, where the absence of pasteurization allows the transfer of plant-derived bacteria to cheese via the cow-milk network. In this study, the four-plasmid complement of the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar diacetylactis

DPC3901 was found to encode a number of gene functions not previously described for *Lactococcus* and generally typical of bacteria associated with the plant ecosystem (Table 4). These include activities of modification of the plant cell wall, a functional system for the high-affinity and regulated uptake of Co<sup>2+</sup>, and several other transporters with a confirmed or predicted role in the uptake of a variety of metal ions, which have single lactococcal homologues on plant-derived plasmids. These genotypes are irrelevant to bacteria growing in milk but important to those associated with or striving to become established within the natural plant ecosystem. Therefore, their presence in a dairy strain may be inferred to be a genetic trace of the previous niche inhabited by the organism, where they conferred on the host a real colonization advantage. The plant origin hypothesis is supported by a number of other observa-

tions. First, most of these plant-based origin gene markers are found on pVF18, which lacks any trait favoring colonization of the dairy environment and, interestingly, was the first plasmid to be cured from the host. Additionally, pVF50 and pVF21 share vast and highly homologous regions with the plant-derived lactococcal plasmid pGdh442 (47), which also encodes phenotypes (i.e., glutamate dehydrogenase and Na<sup>+</sup> and K<sup>+</sup> transporters) so far unique to pGdh442 in *L. lactis* (47, 48). This is indicative of HGT or recombination occurring between pVF50/pVF21 and pGdh442, which again suggests that *L. lactis* DPC3901 could have originally occupied a grass plant niche before transferring to the dairy environment. Also, the GDH enzyme of pGdh442 was shown to function mainly in glutamate biosynthesis, and this capacity has been suggested to be important for bacteria living on plants, since it allows more efficient elimination of the high levels of ammonia present in this environment (46). Therefore, the presence of this genotype further supports a plant origin for *L. lactis* DPC3901.

Computational and functional analyses showed that *L. lactis* DPC3901 carries a functional pVF18-encoded system for the regulated uptake of Co<sup>2+</sup> in addition to a *corA* gene duplication on pVF22. Two CorA transporters are uncommon in *L. lactis* and are likely to enhance the strain's ability to take up Co<sup>2+</sup> in environments (e.g., the soil) where its presence in trace amounts might be growth limiting for the strain (34). However, this also increases the strain's sensitivity to high concentrations of environmental Co<sup>2+</sup>. Our analyses showed that the presence of pVF18 and of its Orf19-21 transport system endows *L. lactis* DPC3901 with the ability to maintain Co<sup>2+</sup> homeostasis by probably allowing a regulated efflux outside the cell. Sequence analysis suggests that Orf20 and Orf21 form an ABC transporter and that Orf20 contains a domain also found on CbiO from the high-affinity and widespread Co<sup>2+</sup> transport system in prokaryotes, CbiMNQO (34). The integral membrane protein CbiM and the cobalt-binding periplasmic protein CbiN have been shown to be the minimal requirements for a functional CbiMNQO system (34). Thus, Orf20 could be the counterpart of the ATPase-like protein CbiO, whereas Orf21 functionality could resemble that of CbiM, which also contains seven transmembrane helices. Orf19 is probably a cytoplasmic protein but lacks any conserved Co<sup>2+</sup> binding motifs (50), which discards the option that it might be a CbiN homolog. CbiMNQO transporters are generally controlled on the level of translation initiation by B12 riboswitch elements (34). In pVF18, expression of the *orf19-21* operon could be controlled by *orf18*, which encodes a transcriptional regulator belonging to the XRE family (cd00093). Putative promoter sequences but no termination signal could be identified for *orf18* (Fig. 3a), suggesting that it could be cotranscribed with *orf19-21* and possibly regulate the activity of this operon, thus endowing *L. lactis* DPC3901 with the ability to maintain Co<sup>2+</sup> homeostasis. To date, the *orf19-21* operon has been found only on plasmids from *Leuconostoc kimchii* IMSNU 11154 (30) and *L. lactis* DPC3901 (this study). Interestingly, a recent and comprehensive comparative and functional analysis of Co<sup>2+</sup> transporters in prokaryotic genomes retrieved no homologues of *cbi* genes in both *Leuconostoc* and *Lactococcus* (34). Thus, bacteria from these genera could have recently evolved a novel plasmid-encoded system dedicated to Co<sup>2+</sup> uptake and homeostasis. *Leuconostoc kim-*

*chii* IMSNU 11154 was isolated from a traditional Korean fermented food made from cabbage (30), whereas *L. lactis* DPC3901 derives from a raw milk cheese, where the absence of pasteurization preserves the natural milk microflora, mostly derived from grass plants (37). LkipL4726 and pVF18 hosts could therefore have inhabited natural environments, where perhaps trace amounts of Co<sup>2+</sup> have promoted the development of an appropriate high-affinity system. The observation that lactococcal homologs of the Orf20-21 transporter can be found only on the plant-derived pGdh442 (47) further supports the link between *L. lactis* DPC3901 and the plant environment.

Assuming that *L. lactis* DPC3901 originated from a plant niche, its transfer to the dairy niche probably occurred some time ago, since the strain carries an entire plasmid (pVF50) specialized for growing and becoming established in milk. The *lac* operon, as well as the Opp system and the oligoendopeptidases PepF and PepO, should endow *L. lactis* DPC3901 with the ability to optimally utilize lactose and milk caseins, respectively, thus resulting in a fast milk coagulation phenotype (51–53). This was confirmed, since *L. lactis* DPC3901 performed similarly to other starters under simulated cheddar cheese-making conditions (Table 3). pVF50 also carries a ParAB maintenance system that, in *L. lactis*, has so far been found only on pSK08, pCI2000, pGdh442, and pKF147A (17, 41, 47). These plasmids share with pVF50 a large size (over 50 kb) and a low copy number, which make them more at risk of being lost during cell division. The maintenance system presumably increases their stability within the host by ensuring their faithful segregation at each cell division. In the case of pVF50, this would allow the stable maintenance of lactose and peptide utilization abilities that are essential to *L. lactis* DPC3901 for a successful adaptation to the dairy environment. *L. lactis* DPC3901 is also well prepared to cope with attacks by lytic phages, which is one of the major threats for lactococci in milk and a major concern for the dairy industry since it affects strains' ability to perform efficiently in large-scale fermentations (25, 31). *L. lactis* DPC3901 was previously shown to be completely resistant to a number of 936- and c2-type phages (11), and the identification of a type I R/M system on pVF21 (this study) provides the genetic basis for this resistance. Type I R/M systems are particularly effective defense mechanisms, since the result is phage inactivation without compromising cell viability and also because novel specificities can be generated by recombinational shuffling of TRDs between different HsdS subunits, thus enabling the host to restrict a wider range of phages and to cope with the evolution of phage variants (31, 38). *L. lactis* DPC3901 also has the potential to produce aroma compounds from glutamate catabolism via a functional GDH enzyme encoded by pVF21. Indeed, this enzyme is identical to that encoded by pGdh442, which was shown to be capable of stimulating glutamate conversion to aroma compounds in *L. lactis* strains that had acquired the plasmid by conjugation (45). Finally, it is noteworthy that although *L. lactis* DPC3901 is capable of fermenting citrate, no citrate permease gene was found in its plasmid complement as has generally been reported for diacetylactis strains (40). It can be hypothesized that this genotype is chromosomally encoded in this strain, alongside the citrate lyase and other genes involved in citrate breakdown (19).

The capacities of phage resistance coupled with the starter

and flavor-forming potential identified on the plasmid complement of *L. lactis* DPC3901 render this strain an all-round candidate for the preparation of starter formulations with enhanced robustness and novel technological attributes. However, the presence of *tet*(S) on the mobilizable pVF22 poses concerns regarding the use of this strain in foods for human consumption due to the potential transferability of antibiotic resistance genes from food-borne LAB to pathogenic organisms in the gut (49). Genes conferring resistance to tetracycline, erythromycin, and vancomycin are being detected more frequently in lactococci, enterococci, and lactobacilli isolated from fermented meat and milk products, probably as a consequence of improper veterinary practices (8, 14, 49). We have thus created an antibiotic resistance gene-free derivative of *L. lactis* DPC3901 via plasmid curing, which retains all the important technological properties of the parent strain and can be safely employed to generate novel starter formulations with improved protection against phage attack and the ability to diversify and widen the flavor profile of the final product.

To conclude, we have found plasmid-encoded markers that potentially trace *L. lactis* subsp. *lactis* biovar diacetylactis DPC3901 back to a plant origin and provide for the first time the genetic basis to support the concept of the plant-milk transition for *L. lactis* strains.

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