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Dynamics in Copy Numbers of Five Plasmids of a Dairy *Lactococcus lactis* Strain under Dairy-Related Conditions Including Near-Zero Growth Rates

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ABSTRACT Lactic acid bacteria can carry multiple plasmids affecting their performance in dairy fermentations. The expression of plasmid-borne genes and the activity of the corresponding proteins are severely affected by changes in the numbers of plasmid copies. We studied the impact of growth rate on the dynamics of plasmid copy numbers at high growth rates in chemostat cultures and down to near-zero growth rates in retentostat cultures. Five plasmids of the dairy strain *Lactococcus lactis* FM03-V1 were selected, and these varied in size (3 to 39 kb), in replication mechanism (theta or rolling circle), and in putative (dairy-associated) functions. The copy numbers ranged from 1.5 to 40.5, and the copy number of theta-type replicating plasmids was negatively correlated to the plasmid size. Despite the extremely wide range of growth rates (0.0003 h^{-1} to 0.6 h^{-1}), the copy numbers of the five plasmids were stable and only slightly increased at near-zero growth rates, showing that the plasmid replication rate was strictly controlled. One low-copy-number plasmid, carrying a large exopolysaccharide gene cluster, was segregationally unstable during retentostat cultivations, reflected in a complete loss of the plasmid in one of the retentostat cultures. The copy number of the five plasmids was also hardly affected by varying the pH value, nutrient limitation, or the presence of citrate (maximum 2.2-fold), signifying the stability in copy number of the plasmids.

IMPORTANCE *Lactococcus lactis* is extensively used in starter cultures for dairy fermentations. Important traits for the growth and survival of *L. lactis* in dairy fermentations are encoded by genes located on plasmids, such as genes involved in lactose and citrate metabolism, protein degradation, oligopeptide uptake, and bacteriophage resistance. Because the number of plasmid copies could affect the expression of plasmid-borne genes, it is important to know the factors that influence the plasmid copy numbers. We monitored the plasmid copy numbers of *L. lactis* at near-zero growth rates, characteristic for cheese ripening. Moreover, we analyzed the effects of pH, nutrient limitation, and the presence of citrate. This showed that the plasmid copy numbers were stable, giving insight into plasmid copy number dynamics in dairy fermentations.

KEYWORDS plasmid copy number, retentostat, theta, rolling circle, replication, segregational stability

The lactic acid bacterium *Lactococcus lactis* is extensively used as a starter culture in dairy fermentations due to its high acidification rate and contribution to flavor and texture development in the fermented product. Important traits for the growth and survival in the milk environment are encoded by genes located on plasmids. This includes genes linked to lactose utilization, casein hydrolysis, and bacteriophage and oxidative stress resistance (1, 2), as well as genes involved in flavor and texture formation (e.g., citrate uptake and exopolysaccharide production) (3, 4). Plasmids are extrachromosomal DNA

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TABLE 1 Characteristics of the plasmids in *L. lactis* FM03-V1 used in this study

Plasmid	Accession no.	Size (kb)	Replication type	Putative function(s)
pLd1	CP020605.1	8.3	Theta	Citrate uptake
pLd3	CP020607.1	4.0	RCR[a]	Unknown
pLd9	MF150537.1	15.3	Theta	Phage defense/D-lactate dehydrogenase
pLd10	MG813924.1	39.6	Theta	Exopolysaccharide production
pLd12	MG813926.1	3.3	Theta	Restriction-modification system

[a]RCR, rolling-circle replication.

molecules which can be lost and acquired and which can be exchanged within the population. Because plasmids replicate independently from the chromosome, their copy numbers (i.e., the number of plasmids per chromosome) can widely vary depending on, for instance, their replication mechanism, environmental conditions, and the genetic background of the strain carrying the plasmid. This could severely affect the total expression level of the genes they carry and the activity of the corresponding proteins.

Lactic acid bacteria encounter a wide variety of environmental conditions during industrial dairy processes, such as cheese production. pH values range from approximately 7 to 4. Lactose is abundant in milk but scarce during cheese ripening, and abundances of other nutrients, such as citrate, will change as well. This leads to high growth rates at the start of the fermentations, while growth rates severely reduce during cheese ripening. Because the plasmids carry essential functions for growth in milk, plasmids should be segregationally stable. Moreover, a constant plasmid copy number under a wide variety of conditions might be preferred for delivering a constant and predictable quality of the fermented product.

The dairy isolate *L. lactis* FM03-V1 harbors 11 plasmids carrying genes encoding functions such as lactose and citrate utilization, oligopeptide uptake, ion transport, bacteriophage resistance, heavy metal transport, stress resistance, and polysaccharide production (O. van Mastrigt, E. Di Stefano, S. Hartono, T. Abee, and E. J. Smid, unpublished data). Of these 11 plasmids, there is one rolling-circle replicating plasmid (pLd3) and 10 theta-type replicating plasmids.

In this study, the copy number of five plasmids of *L. lactis* FM03-V1 was monitored as a function of different environmental parameters relevant in cheese manufacturing, including growth rate (0.6 to 0.0003 h^{-1}), pH (7 and 5.5), nutrient limitation (lactose or amino acid limitation), and the presence of citrate (0 or 10 mM).

RESULTS

The copy numbers were monitored for five plasmids of the dairy strain *Lactococcus lactis* FM03-V1. These five plasmids were chosen because they differ greatly in size (3.3 to 39.6 kb), have two replication types (1 rolling circle and 4 theta type), and differ in putative encoded functions (Table 1). Plasmid pLd8 carrying a lactose operon was not included in the analysis, because the cultures were lactose limited with this disaccharide acting as the main carbon and energy source. This affects the stability of plasmid pLd8 as indicated by the loss of this plasmid only during growth in M17 supplemented with glucose (van Mastrigt et al., unpublished data), which confirmed previous studies showing that the ability to ferment lactose was only maintained during propagation in the presence of lactose or galactose (5, 6). The dynamics in the plasmid copy numbers were analyzed in two separate experiments varying either the growth rate or the environmental conditions (pH, nutrient limitation, presence of citrate). The effect of the growth rate on the plasmid copy numbers was studied using chemostat and retentostat cultures to cover an extremely wide range of growth rates (0.0003 to 0.6 h^{-1}). To study the effect of environmental conditions, *L. lactis* was grown in chemostat cultivations at a constant growth rate (0.135 h^{-1}). Because retentostat cultivation involves continuous filtration of the broth, a chemically defined medium was used in all experiments. This medium contained lactose, citrate, and Bacto tryptone (hydrolyzed casein proteins) to represent the main energy, carbon, and nitrogen sources in milk. All cultivations were performed in duplicate.

Effect of growth rate. *L. lactis* FM03-V1 was grown in chemostat cultures at high growth rates (0.05 to 0.6 h^{-1}) and in retentostat cultures at extremely low growth rates (0.0003 to 0.025 h^{-1}). Despite the near-zero growth rates, the bacteria remained viable (>80%) as determined by live/dead staining with SYTO 9 and propidium iodide (7). Linear mixed-effects modeling was used to analyze the effect of growth rate on the copy numbers of five plasmids. To correct for small differences in the amount of extracted DNA, which affects the plasmid extraction efficiency (data not shown), the threshold cycle of the chromosome was added as a fixed explanatory variable in the model. The plasmid copy numbers in the retentostat cultivations were analyzed using a log transformation of the growth rate. During chemostat and retentostat cultivations, the plasmid copy numbers were not significantly affected or were only slightly affected, with those of plasmids pLd1 (citrate uptake), pLd3 (rolling circle), and pLd9 (bacteriophage resistance) increasing 1.7-, 3.5-, and 1.6-fold, respectively, at near-zero growth rates (Fig. 1; Table 2).

Notably, the largest plasmid, pLd10 (39.6 kb), was completely lost in retentostat culture 1, and its copy number was significantly decreased (4.5-fold) in retentostat culture 2 compared to that in chemostat cultures. This shows that plasmid pLd10 was segregationally unstable at low growth rates, and the decreased copy number might be caused by the loss of the plasmid in a portion of the population. In general, the copy numbers of theta-type replicating plasmids were negatively correlated with the plasmid size ($R^2 = 0.921$) (Fig. 2), suggesting that bigger theta-type plasmids are less stable due to their lower copy number. Extrapolation of the linear relation between the size and copy number of theta-type replicating plasmids to the remaining six plasmids of *L. lactis* FM03-V1 gives a total plasmid content of 1.74 Mb, which is 72% of the amount of chromosomal DNA (2.43 Mb).

Effects of environmental conditions. To study the effects of pH, nutrient limitation, and the presence of citrate in the medium on plasmid copy numbers, *L. lactis* FM03-V1 was grown in chemostat cultures at a constant growth rate (0.135 h^{-1}) at pH 5.5 and 7, with and without supplementation of citrate and under lactose and amino acid limitation. These environmental parameters were chosen to represent dairy environments during fermentation. Milk contains approximately 10 mM citrate (8). Milk is acidified from approximately pH 7 to 5 (9) by lactic acid bacteria, and amino acids or lactose might be limiting depending on the lactose uptake kinetics and the proteolytic capacity of the starter culture. Linear mixed-effects models were used to determine the effect of each parameter on the copy number of each plasmid. The copy numbers of plasmids pLd1 (citrate uptake) and pLd10 (exopolysaccharide [EPS] production) were significantly lower at low pH (2.0- and 1.9-fold, respectively), while the copy number of plasmid pLd3 (rolling circle) was lower under amino acid limitation (2.2-fold) (Fig. 3; Table 3). The copy numbers of the remaining two plasmids were not significantly affected under any of the tested conditions.

DISCUSSION

To assess the dynamics in the copy numbers of five plasmids, *Lactococcus lactis* FM03-V1 was grown in environments that reflect the conditions encountered during dairy fermentations. The copy numbers of plasmids were studied at near-zero growth rates using retentostat cultivation. Differences in plasmid copy numbers were limited (<4-fold) compared to the extremely wide range of growth rates (>1,000-fold). This shows that the replication rate of the plasmids is strictly coupled to the growth rate. It has been demonstrated that the plasmid replication rate is controlled by negative regulatory systems responding to fluctuations in the copy number, as extensively reviewed by del Solar et al. (10) and Chattoraj (11). More specifically, these systems respond to fluctuations in plasmid concentrations. Because the cell volume slightly increases at near-zero growth rates (7), a constant plasmid concentration implies a slightly higher number of plasmids per cell in bigger cells. Since these bigger cells still contain only one chromosomal copy, increased cell volumes could explain the increased copy numbers of plasmids pLd1, pLd3, and pLd9 at near-zero growth rates.

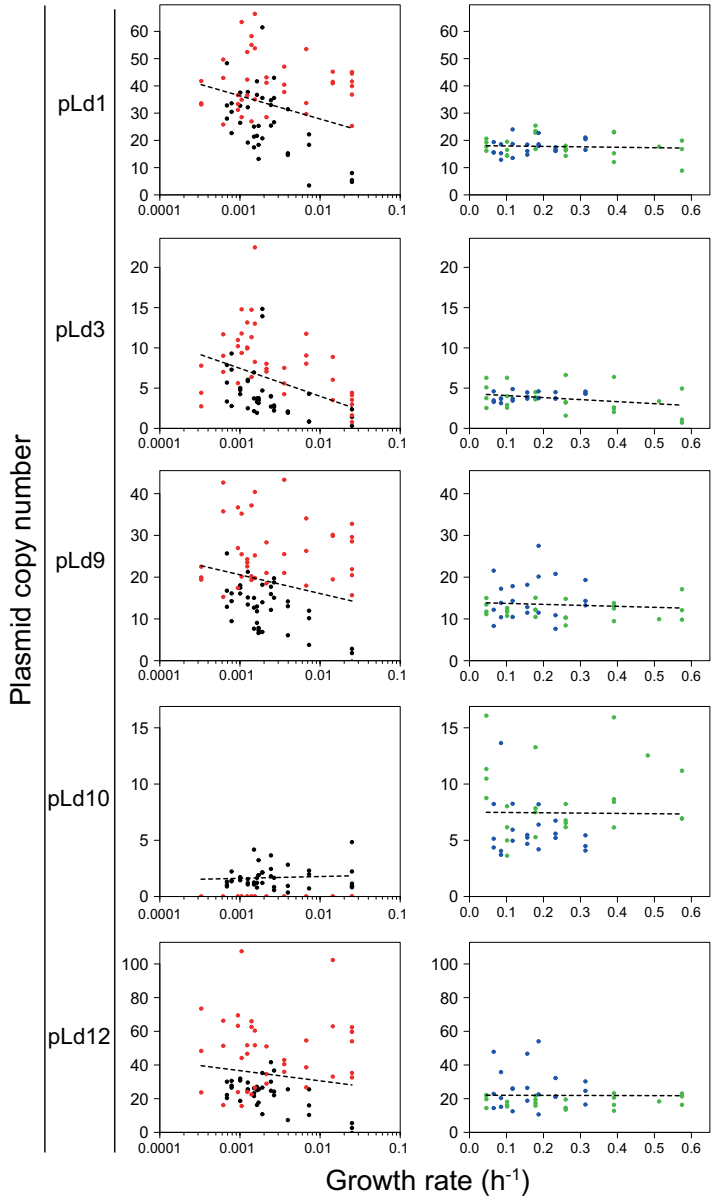

FIG 1 Copy numbers of five plasmids of *L. lactis* FM03-V1 in retentostat (left) and chemostat (right) cultures. Red, black, blue, and green represent independent retentostat cultures 1 and 2 and chemostat cultures 3 and 4, respectively. Every point represents the average plasmid copy number per DNA extraction calculated from triplicate qPCR measurements. These plasmid copy numbers were corrected for slight changes in the amount of DNA extracted, which significantly affected the copy number measurement of most plasmids (Table 2). The dashed lines represent predictions calculated from a linear mixed-effects model as explained in Materials and Methods.

Despite the strict regulation of the plasmid copy numbers, plasmids can still be lost under specific conditions, as illustrated by the complete loss of plasmid pLd10 in retentostat culture 1 and the significantly decreased apparent copy number in retentostat culture 2, which might be caused by the loss of the plasmid in a portion of the population. This plasmid carries a large *eps* gene cluster. Exopolysaccharide (EPS) production is an expensive process in terms of energy usage, and we speculate that the extreme energy limitation in retentostat cultivation might have selected for cells that did not produce EPS due to loss of plasmid pLd10. The energy advantage of cells that have lost plasmid pLd10 enabled these cells to grow at lower substrate availabilities, resulting in the dominance of these cells in the retentostat cultures.

TABLE 2 Significance of effects of DNA extraction and the growth rate on the copy numbers of five plasmids of *L. lactis* FM03-V1 during retentostat and chemostat cultivations

		Fixed factors[a]								
		Extraction			Growth rate			PCN as predicted by model[b]		
Cultivation	Plasmid	df	*t* value	*P* value	df	*t* value	*P* value	Minimum	Maximum	Fold change
Retentostat (0.0003–0.025 h^{-1})	pLd1	77	5.5	4.4×10^{-7}	77	−3.3	0.0016	24.5	40.5	1.7
	pLd3	77	2.6	0.011	77	−4.5	2.0×10^{-5}	2.6	9.1	3.5
	pLd9	77	6.0	7.1×10^{-8}	77	−2.9	0.0056	14.3	22.7	1.6
	pLd10				40	0.52	0.61	1.5	1.8[c]	1.2
	pLd12	77	4.3	5.2×10^{-5}	77	−1.6	0.12	28.2	39.6	1.4
Chemostat (0.05–0.6 h^{-1})	pLd1	42	4.1	1.9×10^{-4}	42	−0.44	0.66	17.2	18.0	1.0
	pLd3	42	4.6	3.8×10^{-5}	42	−2.0	0.057	2.9	4.2	1.5
	pLd9	42	3.2	0.0029	80	−0.54	0.59	12.6	13.8	1.1
	pLd10	42	3.8	4.2×10^{-4}	42	−0.095	0.92	7.3	7.5	1.0
	pLd12				42	−0.05	0.96	21.8	22.0	1.0

[a]Statistical analyses were performed with R by regression with a linear mixed-effects model as explained in Materials and Methods.
[b]PCN, plasmid copy number. The model was used to predict the minimum and maximum number of plasmid copies in the tested range and the maximum fold change was calculated.
[c]Higher predicted plasmid copy number at higher growth rates.

The mode of replication has been suggested to be a major factor in the segregational stability of plasmids in *L. lactis*, with theta-type replication plasmids being more stable (12). In this study, a theta-type replicating plasmid (pLd10) was lost in one of the retentostat cultivations, while the rolling-circle replicating plasmid was maintained. Plasmid pLd10 has a relatively low copy number compared to those of the other plasmids, which increases its chance to be lost during cell division. We found that the copy number of theta-type replicating plasmids was negatively correlated to the plasmid size. A similar relation was also found for plasmids of *Bacillus thuringiensis* YBT-1520 (13) and suggests a lower stability for larger plasmids due to their lower copy number.

The amount of chromosomal DNA in the extraction could affect the plasmid extraction efficiency and thereby plasmid copy number measurement. Therefore, the threshold cycle of the chromosomal DNA was included as fixed explanatory variable in the linear mixed-effects models to correct for small changes in the amount of extracted DNA. This variable appeared to be significant in most analyses (Tables 2 and 3) and improved the reliability of the results. This demonstrates that to obtain reliable results with the described copy number determination method, standardization of the amount of cells in the DNA extraction and/or correction for small changes in the amount of extracted DNA, as implemented in the current study, is required.

In addition to the effect of growth rate on the plasmid copy number, we analyzed the effects of several environmental parameters, which commonly change during dairy

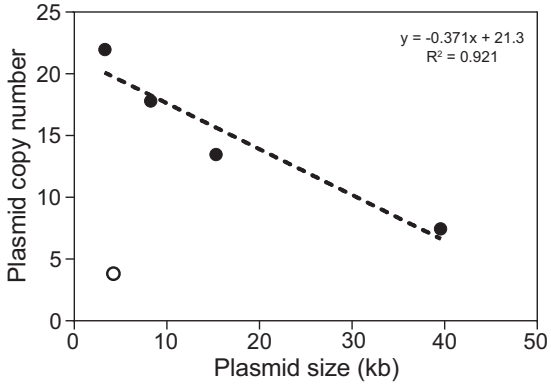

FIG 2 Correlation between the copy number and the size of the plasmids. Filled circles, theta-type replicating plasmids; open circle, rolling-circle replicating plasmid. Plasmid copy numbers were calculated at a growth rate of 0.2 h^{-1} on the basis of the obtained model for chemostat cultivations.

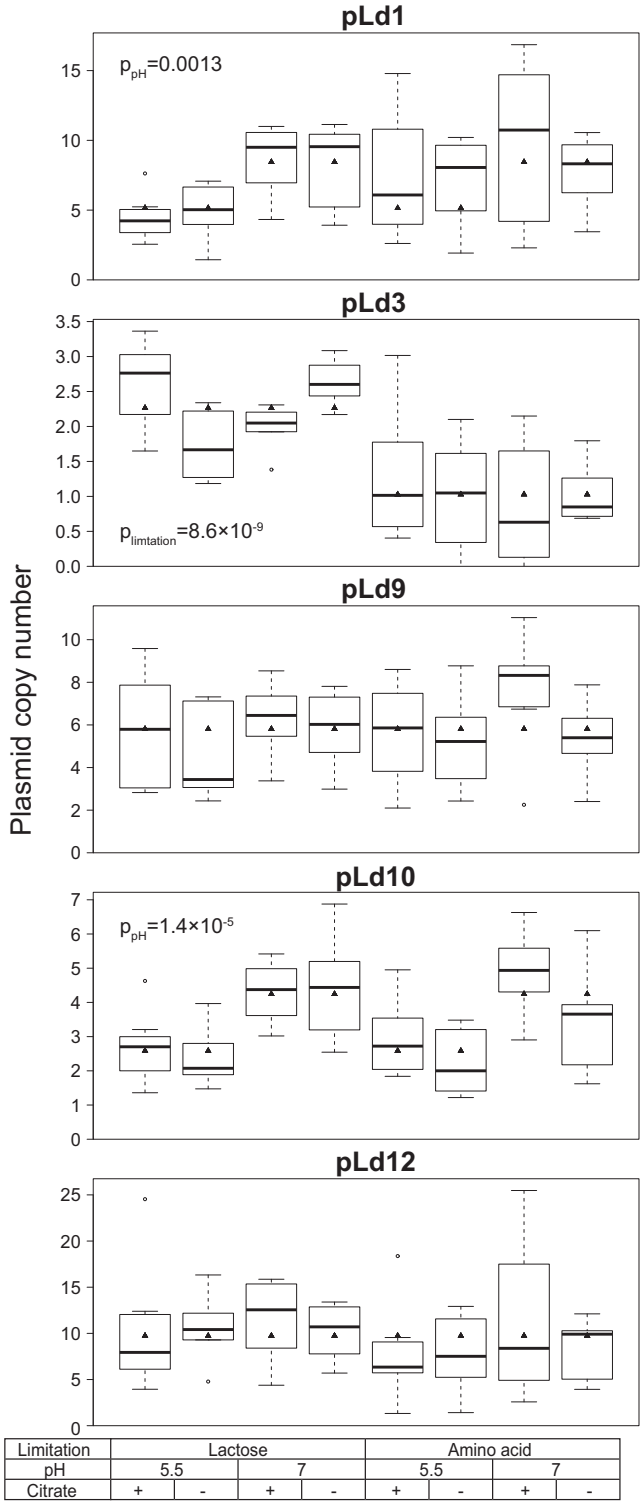

FIG 3 Copy numbers of five plasmids of *L. lactis* FM03-V1 during chemostat cultivation at a constant growth rate (0.135 h^{-1}) under different conditions: pH 5.5 or 7, under lactose or amino acid limitation, and with (+) or without (−) supplementation of citrate. Boxplots were made with the average plasmid copy numbers per DNA extraction, which were calculated from triplicate qPCR measurements. These plasmid copy numbers were corrected for slight changes in the amount of DNA extracted, which significantly affected the copy number measurements of most plasmids (Table 3). Triangles represent the predictions of linear mixed-effects models as explained in Materials and Methods. *P* values of significant effects are indicated in the plots.

TABLE 3 Significance of effects of DNA extraction, pH, and nutrient limitation on the copy numbers of five plasmids of *L. lactis* FM03-V1 during chemostat cultivation at a constant growth rate (0.135 h^{-1}) with and without supplementation of citrate[a]

	Extraction			pH[b]				Nutrient limitation[c]			
Plasmid	df	*t* value	*P* value	df	*t* value	*P* value	Fold change	df	*t* value	*P* value	Fold change
pLd1	50	8.7	1.4×10^{-11}	50	−3.4	0.0013	2.0				
pLd3	54	7.0	3.4×10^{-9}					54	−6.8	8.6×10^{-9}	2.2
pLd9	56	9.8	1.0×10^{-13}								
pLd10	55	8.5	1.4×10^{-11}	55	−4.8	1.4×10^{-5}	1.9				
pLd12	56	6.5	2.2×10^{-8}								

[a]Statistical analysis was performed with R by regression with a linear mixed-effects model as explained in Materials and Methods. Only significant effects are shown.
[b]Bacteria were grown at pH 5.5 or 7.
[c]Bacteria were grown under lactose or amino acid limitation.

fermentations: pH, nutrient limitation, and the presence of citrate. In general, their effects on the plasmid copy numbers were very small (maximum 2.2-fold), signifying the stability of the plasmid copy numbers. Comparisons of the plasmid copy numbers in the chemostat cultures at different dilution rates (Fig. 1) and under different environmental conditions (Fig. 3) showed that the determined copy numbers in the latter chemostat cultures were approximately 2-fold lower. This can be explained by the slightly larger amount of chromosomal DNA that was extracted on average in these samples (chromosomal threshold cycle of 12.5 to 15 compared to 15 to 17), because we only corrected for the amount of chromosomal DNA within experiments, i.e., conditions analyzed in the same linear mixed-effects model, and not between experiments. This demonstrates the importance of standardization of the amount of cells in the DNA extraction for comparisons between experiments.

The copy number of plasmid pLd1 carrying the *citQRP* operon required for citrate uptake decreased 2.0-fold at low pH, while the nutrient limitation and the presence of citrate did not have a significant effect. In contrast, earlier studies (3) revealed a slight increase (1.4-fold) in the copy number of plasmid pLd1 at pH 5.5 compared to that at pH 7. This discrepancy shows that such small differences in plasmid copy number (<5 plasmid copies) cannot be reproducibly found with the applied technique and should be considered not significant. This is signified by the relatively small changes compared to the increase in citrate utilization rate at low pH in the presence of citrate, which can mount up to 65-fold (3).

In conclusion, plasmid copy numbers were remarkably stable under different environmental conditions relevant for cheese manufacturing, which leads to a constant and predictable product quality in industrial processes.

MATERIALS AND METHODS

Strain and growth medium. For chemostat and retentostat cultivations, *Lactococcus lactis* subsp. *lactis* bv. diacetylactis FM03-V1 (3) was precultured as previously described (7). The chemically defined media that were used for all cultivations contained 0.5% or 2% (wt/wt) lactose, 0 or 10 mM $(NH_4)_3$ citrate, and 0.1 or 1% Bacto tryptone (3) and were prepared in 20-liter batches. The effect of growth rate was studied in a chemically defined medium containing 0.5% lactose, 10 mM $(NH_4)_3$ citrate, and 1% Bacto tryptone.

Chemostat and retentostat cultivations. Chemostat and retentostat cultivations were performed in duplicate as described previously (7). For the chemostat cultivations, the bacteria were grown at dilution rates between 0.05 and 0.6 h^{-1}. The stirring speed was set at 300 rpm, the temperature was kept constant at 30°C, and the pH was controlled at 5.5 or 7 by the automatic addition of 5 M NaOH. The headspace was flushed with nitrogen gas at a rate of 0.1 liter/min to maintain anaerobic conditions. Samples were taken after reaching steady-state conditions, which were considered to be achieved after a minimum of five volume changes at which the optical density remained constant. Conditions for the retentostat cultivations were the same as for the chemostat cultivations, except that a stirring speed of 400 rpm was used and the pH was set always at 5.5. After a steady state was achieved in a chemostat cultivation at a dilution rate of 0.025 h^{-1}, retentostat cultivation was initiated by the insertion of a serializable polyethersulfone cross-flow filter (Spectrum Laboratories, USA) in the effluent.

Plasmid copy number determination. The plasmid copy numbers were determined in two steps: (i) DNA extraction followed by (ii) quantitative PCR (qPCR) analysis. DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany) as previously described (3). Subsequently, chromosomal and plasmid DNAs were quantified with qPCR using specific primer sets (Table 4). The PCRs took place in 20-μl reaction mixtures containing 10 μl iQ SYBR green supermix (Bio-Rad, USA), 2 μl purified DNA, 1 μM

TABLE 4 Primers used for plasmid copy number determination by qPCR

Target	Target gene	Primer sequence (5′→3′)		Amplicon size (bp)
		Forward	Reverse	
Chromosome	*hisB*, BSR25_0747[a]	TCAAGCGGACATTAGTACAGGTATTGGTT	CGAGCTTATTACCTAATGCTTCGCTGATAC	194
pLd1	*citP*, BSR25_2464	CATTGCTATGCCAATCATGG	TTCCCGAGGATAACTGTTGG	132
pLd3	BSR25_2483	ATGGTCTAACTCACGGCTTCGCAATA	GTTAGAACGTCGTGAGAGCGTCTTAGAT	136
pLd9	pLd9_6	CAACGTGACCGGACGATCCAGATA	GGTTGTGCGTGACGTTGAATCTGATAA	92
pLd10	*mobB*, pLd10_21	AACTGCCATAAATTCCAGCACCGATTAAA	ATTGTCTGCCTGTTCTGTTTCTTAGTTGTC	193
pLd12	*repB*, pLd12_1	GCGTTTAGAATAGGCTTCTTGTTTGCTAGA	ATGCAAAGCCCTTATACTAAGCTACTGTCT	198

[a]The gene *hisB*, located with one copy on the chromosome, was targeted to quantify the amount of chromosomal DNA.

forward primer, and 1 μM reverse primer. The reactions were started with an initial denaturation step at 95°C for 5 min, followed by 39 cycles of 95°C for 30 s and 64°C for 30 s, and finalized with a melting curve step in which the temperature was raised from 65 to 95°C in steps of 0.5°C every 5 s to check for nonspecific amplification. The fluorescence of SYBR green was monitored, indicating the amount of double-stranded DNA (dsDNA). The threshold cycles in the qPCR were determined by regression using the Bio-Rad software CFX manager 3.1, which corrects for small differences in PCR efficiencies. DNA extractions were performed in duplicate and triplicate for chemostat and retentostat samples, respectively. Chromosomal and plasmid DNAs of each DNA extraction were quantified in triplicate and averaged for statistical analysis.

The plasmid copy number (PCN) was calculated from the threshold cycles for chromosomal (C_{Tc}) and plasmid (C_{Tp}) DNA using the equation

$$PCN = 2^{C_{Tc}-C_{Tp}} \quad (1)$$

in which an efficiency of 2 was used because the threshold cycles were determined by regression, which already corrects for slightly lower or higher efficiencies.

Statistical analysis. To determine the effects of variables (growth rate and environmental conditions) on the plasmid copy numbers, statistical analyses were performed with R using the nlme package (14). Linear mixed-effects models (15) were fitted to the data to correct for the nested structure of the data: different conditions were analyzed within the same cultivation and at least three DNA extractions were performed on the same sample. Therefore, DNA extraction nested within cultivation was used as a random categorical variable. For the fixed explanatory variables, either growth rate or pH, citrate, nutrient limitation, and their interactions were used. For retentostat cultivations, the growth rate was log transformed to make samples taken at high and low growth rates equally important. The determined plasmid copy numbers significantly correlated to the threshold cycle of the chromosome, which is a measure of how much chromosomal DNA was extracted. Therefore, the threshold cycle of the chromosome was added as a fixed explanatory variable to all models to correct for small changes in the amount of extracted DNA. First, a linear mixed-effects model (LME) with all fixed explanatory variables was compared with a generalized least-squares model (GLS) by restricted maximum likelihood estimation (REML) to see if the LME was a better model, indicating that cells had more similar plasmid copy numbers within cultivations than between cultivations. Subsequently, the fixed explanatory variables of the LME containing all explanatory variables were optimized by a repetitive process of fitting a full model, dropping all allowable terms in turn, comparing the models with the maximum likelihood, dropping the least significant term, and repeating the whole process until all terms were significant (P value of <0.05) (15). The growth rate was never dropped as a fixed factor. Finally, the final model was made by REML, and residual errors in final models were checked for homogeneity.

Accession number(s). The sequences of plasmids pLd10 and pLd12 of *L. lactis* FM03-V1 have been deposited in GenBank under the accession numbers MG813924 and MG813926.

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REFERENCES

1. Górecki RK, Koryszewska-Bagińska A, Gołębiewski M, Żylińska J, Grynberg M, Bardowski JK. 2011. Adaptative potential of the *Lactococcus lactis* IL594 strain encoded in its 7 plasmids. PLoS One 6:e22238. https://doi.org/10.1371/journal.pone.0022238.
2. Siezen RJ, Renckens B, van Swam I, Peters S, van Kranenburg R, Kleerebezem M, de Vos WM. 2005. Complete sequences of four plasmids of *Lactococcus lactis* subsp. *cremoris* SK11 reveal extensive adaptation to the dairy environment. Appl Environ Microbiol 71:8371–8382. https://doi.org/10.1128/AEM.71.12.8371-8382.2005.
3. van Mastrigt O, Mager EE, Jamin C, Abee T, Smid EJ. 2018. Citrate, low pH and amino acid limitation induce citrate utilization in *Lactococcus lactis* biovar diacetylactis. Microb Biotechnol 11:369–380. https://doi.org/10.1111/1751-7915.13031.
4. van Kranenburg R, Marugg JD, van Swam II, Willem NJ, de Vos WM. 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol Microbiol 24:387–397. https://doi.org/10.1046/j.1365-2958.1997.3521720.x.
5. McKay LL, Baldwin KA, Zottola EA. 1972. Loss of lactose metabolism in lactic streptococci. Appl Microbiol 23:1090–1096.
6. Okulitch O, Eagles BA. 1936. Cheese-ripening studies: the influence of the configurational relations of the hexoses on the sugar-fermenting abilities of lactose acid streptococci: a preliminary note. Can J Res 14b:320–324. https://doi.org/10.1139/cjr36b-036.
7. van Mastrigt O, Abee T, Lillevang SK, Smid EJ. 2018. Quantitative physiology and aroma formation of a dairy *Lactococcus lactis* at near-zero

growth rates. Food Microbiol 73:216–226. https://doi.org/10.1016/j.fm.2018.01.027.
8. Garnsworthy PC, Masson LL, Lock AL, Mottram TT. 2006. Variation of milk citrate with stage of lactation and *de novo* fatty acid synthesis in dairy cows. J Dairy Sci 89:1604–1612. https://doi.org/10.3168/jds.S0022-0302(06)72227-5.
9. Fox PF, McSweeney PLH. 2017. Cheese: an overview, p 5–21. *In* McSweeney PLH, Fox PF, Cotter P, Everett DW (ed), Cheese: chemistry, physics and microbiology, 4th ed, vol 1. Academic Press, San Diego, CA.
10. del Solar G, Giraldo R, Ruiz-Echevarría MJ, Espinosa M, Díaz-Orejas R. 1998. Replication and control of circular bacterial plasmids. Microbiol Mol Biol Rev 62:434–464.
11. Chattoraj DK. 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol Microbiol 37:467–476. https://doi.org/10.1046/j.1365-2958.2000.01986.x.
12. Kiewiet R, Kok J, Seegers JFML, Venema G, Bron S. 1993. The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. Appl Environ Microbiol 59:358–364.
13. Zhong C, Peng D, Ye W, Chai L, Qi J, Yu Z, Ruan L, Sun M. 2011. Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520. PLoS One 6:e16025. https://doi.org/10.1371/journal.pone.0016025.
14. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2016. nlme: linear and nonlinear mixed-effects models. http://CRAN.R-project.org/package=nlme. Accessed 5 February 2018.
15. Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. Mixed effects models and extensions in ecology with R. Springer, New York, NY.