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Harnessing Adaptive Evolution to Achieve Superior Mannitol Production by *Lactococcus lactis* Using Its Native Metabolism

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ABSTRACT: Mannitol can be obtained as a by-product of certain heterolactic lactic acid bacteria, when grown on substrates containing fructose. *Lactococcus lactis*, a homolactic lactic acid bacterium, normally does not form mannitol but can be persuaded into doing so by expressing certain foreign enzyme activities. In this study, we find that *L. lactis* has an inherent capacity to form mannitol from glucose. By adaptively evolving *L. lactis* or derivatives blocked in NAD⁺ regenerating pathways, we manage to accelerate growth on mannitol. When cells of the adapted strains are resuspended in buffer containing glucose, 4–58% of the glucose metabolized is converted into mannitol, in contrast to nonadapted strains. The highest conversion was obtained for a strain lacking all major NAD⁺ regenerating pathways. Mannitol had an inhibitory effect on the conversion, which we speculated was due to the mannitol uptake system. After its inactivation, 60% of the glucose was converted into mannitol by cells suspended in glucose buffer. Using a two-stage setup, where biomass first was accumulated by aerated culturing, followed by a nonaerated phase (static conditions), it was possible to obtain 6.1 g/L mannitol, where 60% of the glucose had been converted into mannitol, which is the highest yield reported for *L. lactis*.

KEYWORDS: *L. lactis*, mannitol, adaptive evolution, NADH supply, ATP

INTRODUCTION

Mannitol, a six-carbon sugar polyol that exists naturally in many plants, especially in seaweeds, has many applications in the pharmaceutical, chemical, and food industries. Clinically, mannitol is widely used in the management of cerebral edema and other conditions with raised intracranial pressure.^{1–4} In diabetic foods, due to its low glycemic index and poor intestinal absorption, it is used as a sweetener and since it is nonhygroscopic and does not promote dental decay, it is commonly used in sweets.⁵

Mannitol is mainly produced by chemical synthesis, usually from glucose/fructose mixtures; however, due to low yields, around 20%, alternative ways of producing mannitol have been sought after.⁶ In the last 20 years, numerous attempts have been made to produce mannitol by using heterofermentative LAB. Good yields and titers of mannitol have been obtained using different *Leuconostoc* and *Lactobacillus* species, which can reduce fructose to mannitol.^{7,8} The main drawback of using these organisms for producing mannitol is that fructose-containing feedstocks are needed, which puts restraints on yields and cost.⁹

Lactococcus lactis is a food-grade lactic acid bacterium (LAB) widely used in cheese production. As a homofermentative bacterium, it converts up to 95% of the sugar it metabolizes to lactate, when grown anaerobically. In the past decade, it has been demonstrated that *L. lactis* can generate mannitol from fructose-6-phosphate (F6P), a central metabolite in glycolysis.^{10,11} Using *L. lactis* to produce mannitol from other sugars besides fructose at high yield and titer could help lower mannitol production costs. A challenge, however, is that the wild-type *L. lactis* strains only produce mannitol in low

amounts. Intracellular mannitol can only be detected in lactate dehydrogenase (Ldh) deficient strains and is detectable in culture medium only when using resting cells (nongrowing cells).¹⁰ In resting conditions, the glucose-to-mannitol conversion ratio obtained for an Ldh deficient strain is around 25–33%.^{12,13} After complementing *L. lactis* with the mannitol-1-phosphatase (M1Pase) gene from *Eimeria tenella* and overexpressing the mannitol-1-phosphate 5-dehydrogenase (M1PDH) gene from *Lactobacillus plantarum*, it was demonstrated that an Ldh deficient strain could produce mannitol in growing conditions with mannitol/glucose (M/G) ratios of 42–50%.^{14,15}

Mannitol-1-phosphate (M1P) formation from F6P is catalyzed by M1PDH and consumes 1 NADH (Figure 1). The observation that mannitol only can be produced in Ldh deficient strains shows how important a sufficient NADH supply is for mannitol production. On the basis of redox considerations, in *L. lactis*, the theoretical maximum conversion ratio from glucose is 66.7% and thus there still is a large potential for increasing the mannitol yield in *L. lactis*.

In this study, rather than relying on the expression of foreign genes, we rely on adaptive laboratory evolution to achieve mannitol production in *L. lactis*. We increase mannitol yield by blocking metabolic pathways consuming NADH and carry out

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Figure 1. Illustration showing the central sugar metabolism and mannitol synthesis pathway in *L. lactis*. NADH formation and consumption as well as ATP formation is indicated. The following enzymes are involved: (1) phosphoenolpyruvate (PEP)-dependent mannitol phosphotransferase (PTS) system; (2) phosphoglucose isomerase; (3) mannitol-1-phosphate 5-dehydrogenase; (4) unknown phosphatase in *L. lactis*; (5) mannitol-specific PTS system; (6) enzymes including 6-phosphofructokinase, fructose 1,6-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase and pyruvate kinase; (7) lactate dehydrogenase; (8) α -acetolactate synthase; (9) α -acetolactate decarboxylase; (10) 2,3-butanediol dehydrogenase; (11) pyruvate dehydrogenase complex; (12) pyruvate-formate lyase; (13) acetaldehyde dehydrogenase and alcohol dehydrogenase; (14) phosphate acetyltransferase and acetate kinase.

experiments with both resting and growing cells. Finally, we obtain a strain that can convert more than 60% of the glucose into mannitol, which, as far as we know, is the highest yield reported for *L. lactis*.

METHODS

Bacterial Strains and Growth Conditions. *Lactococcus lactis* subsp. *cremoris* MG1363 and its derivatives CS4099, CS4363, Ace001, and AceM were used in this study (described in Table 1). All of these strains are plasmid-free. *L. lactis* strains were cultured in M17 broth supplemented with 1% glucose (Sigma-Aldrich) unless indicated otherwise. For laboratory adaptation, MG1363, CS4099, CS4363, and Ace001 were cultured aerobically in M17 broth supplemented with 0.5% mannitol and 2.5 μ g/mL hemin. After reaching a stationary phase, 1 mL was transferred to a 9 mL fresh medium. Laboratory adaptations were stopped after 35–40 transfers. M17 agar plates supplemented with 1% mannitol were used to screen for mutants with superior growth on mannitol, and these were characterized further in liquid medium. The following strains were isolated after adaptive evolution: MG1363-M, CS4099-M, CS4363-M, and Ace001-M. For characterizing growth in liquid medium, the strains were precultured until reaching the early exponential phase, then transferred into a startup medium at a cell density of $OD_{600} = 0.02$ and finally cultured aerobically in M17 at 30 °C. The cell density was measured using a spectrophotometer (VWR, V-1200) at 600 nm. Specific growth rates were calculated as described by Friedrich.¹⁶

Preparation and Cultivation of Resting Cells. For preparing resting cells, *L. lactis* was precultured in M17 supplemented with 0.5%

Table 1. Strains and Plasmids Used in This Work

strain and plasmid	relevant genotype	source or refs
<i>L. lactis</i> Strains		
MG1363	wild-type <i>L. lactis</i> subsp. <i>cremoris</i>	20
MG1363-M	MG1363 adapted on mannitol	this work
CS4099	MG1363 Δ^3ldh (Δldh , $\Delta ldhB$ and $\Delta ldhX$)	21
CS4099-M	CS4099 adapted on mannitol	this work
CS4363	MG1363 Δ^3ldh , Δpta , $\Delta adhE$	21
CS4363-M	CS4363 adapted on mannitol	this work
Ace001	MG1363 Δ^3ldh , Δpta , $\Delta adhE$, $\Delta butBA$	22
Ace001-M	Ace001 adapted on mannitol	this work
AceM	Ace001-M $\Delta mtlF$	this work
MC1000	<i>Escherichia coli</i> cloning host	19
Plasmid		
pG ⁺ host8	<i>E. coli</i> / <i>L. lactis</i> shuttle vector, Tet ^R , thermosensitive replicon	18
pKmtlF	used for knocking out <i>mtlF</i>	this work

glucose and 2.5 μ g/mL hemin under aerobic conditions. The cells were harvested in the early exponential phase, washed two times using PBS (pH = 7.0), and resuspended in PBS supplemented with 2%

glucose at an OD₆₀₀ of around 15.0. The suspensions were incubated at 30 °C under anaerobic conditions, and samples were withdrawn at specific time points.

Quantifying Metabolites by High-Performance Liquid Chromatography (HPLC). For quantifying the sugars and metabolites produced, HPLC (Dionex) equipped with a Shodex RI-101 refractive index detector (Showa Denko K.) and a BioRad Aminex HPX-87H column (BioRad) was used. The mobile phase was water containing 5 mM H₂SO₄. The temperature of the column oven was set to 60 °C, and a flow speed of 0.5 mL/min was used. The samples for HPLC analysis were filtered using 0.22 μm filters (Labsolute) immediately after sampling and were stored at −20 °C until use.

Molecular Techniques. *L. lactis* was made electrocompetent by growth in GM17 medium containing 2% glycine and 0.25 M sucrose and transformed by electroporation, as previously described by Holo and Nes.¹⁷ DreamTaq Hot Start DNA Polymerase (ThermoFisher Scientific) was used for PCR. For plasmid extraction from *L. lactis*, cells were first incubated with a 20 mg/mL lysozyme (Fluka, 7000U/mg) solution at 37 °C for 2 h and subsequently a Zippy plasmid mini-prep kit (Zymo Research) was used for extracting the plasmids. DNA sequencing was performed by Macrogen, South Korea.

Construction of Strains and Plasmids. All strains and plasmids used in this work are listed in Table 1. To delete the gene *mtlF*, a plasmid with a thermosensitive replicon, pG⁺host8,¹⁸ was used. gBlocks containing in total approximately 2400 bp of DNA upstream and downstream the region to be deleted were synthesized by IDT. Both pG⁺host8 and gBlocks were digested with restriction enzymes (ThermoFisher Scientific) *Bam*HI (GGATCC) and *Xba*I (TCTGA) and ligated together using T4 ligase (ThermoFisher Scientific). The ligated plasmid, designated pKmtlF, was introduced by electroporation into *E. coli* MC1000.¹⁹ To obtain the desired gene deletion in *L. lactis*, the approach described briefly below was used: pKmtlF was transferred into *L. lactis* by electroporation (2 kV). After short incubation at 28 °C, transformants were spread on GM17 plates, containing 5 μg/mL tetracycline, and incubated at 37 °C. After 2 days of incubation, the presence of big colonies indicated recombination of the plasmid into the chromosome (crossing-in) had taken place. Large colonies were then, after restreaking at 37 °C, streaked on new plates without tetracycline and incubated at 28 °C to allow for crossing out of the plasmid. Big colonies at 28 °C indicated that a crossing-out event had taken place, and this was verified by streaking the strains on plates with/without tetracycline at 37 °C and by using PCR.

Whole Genome Sequencing. Genomic DNA of MG1363, MG1363-M, CS4099, CS4099-M, CS4363, CS4363-M, Ace001, and Ace001-M was extracted using the Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research) according to the manufacturer's instructions. Whole genome sequencing was performed using PE150 sequencing and the DNBseq tech platform (BGISEQ),^{23,24} after short insert fragment library preparation, and 1 Gb of clean data per sample was generated. To analyze the sequencing data, map the genome, and find variations, we used Geneious Prime 2019. The program BBduk was used for trimming, to remove the adapters at the left end, for eliminating sequences of low quality at both ends, and for discarding reads shorter than 50 bp. Bowtie2 was then used for aligning the trimmed reads to the reference sequence (*L. lactis* MG1363, Genbank Accession number: NC009004) by default settings. single nucleotide variations (SNVs) were found by meeting the requirements of variant frequency no less than 90% and strand bias not exceeding 75%. Finally, the identified SNVs were compared between all mannitol adaptive strains and their parental strains.

Fermentation Optimization. AceM was precultured aerobically in M17 containing 1% glucose until reaching OD₆₀₀ = 0.5 or 4.5. Culture medium containing cells was immediately distributed into different volumes, either 25, 50, 75, or 100 mL in 100 mL flasks. Selected cultures were supplemented with 35 mM arginine (pH = 7.0) and additional glucose and shaken in a water bath at 30 °C, either at 20 or 60 rpm. As controls, vigorously aerated cultures (240 rpm) and nonaerated (static) cultures were included. Samples were withdrawn at particular time points and analyzed by HPLC.

RESULTS

Accelerated Growth on Mannitol after Adaptive Laboratory Evolution. *L. lactis* can grow slowly on mannitol, which demonstrates that all enzymes needed for mannitol catabolism are present, but perhaps some of these are poorly expressed. In an attempt to overcome the putative bottleneck, we decided to carry out an adaptive laboratory evolution experiment on mannitol to obtain mutants with accelerated growth on this particular sugar alcohol. Mannitol is a sugar alcohol, and its catabolism results in formation of one additional NADH when compared with glucose. For this reason, we decided to carry out the adaptive evolution under aerated conditions with hemin present, as this leads to more efficient NAD⁺ regeneration.²⁵ First, we adapted the wild-type *L. lactis* strain MG1363, and the adaptation lasted for 2 months. In the course of the evolution, the faster growing isolate MG1363-M was obtained and its growth characterized. As shown in Figure 2, MG1363 grows slowly ($\mu_{\max}$ = 0.067) on

Figure 2. Growth performance of MG1363 and MG1363-M on mannitol. Growth of MG1363 and MG1363-M in M17 supplemented with hemin and 0.5% of the indicated sugar. Aerated growth conditions are used. Rectangle: MG1363 grown on glucose; circle: MG1363 grown on mannitol; triangle: MG1363-M grown on mannitol.

mannitol and can reach a stationary phase after 30 h. In the M17 medium used, a two-phased growth pattern was observed due to the presence of additional metabolizable sugars (without added sugars a cell density of around 0.6 can be reached in M17). MG1363-M, however, grew much faster than MG1363 in a mannitol containing medium ($\mu_{\max}$ = 0.472), although slower than on glucose ($\mu_{\max}$ = 0.665). These results demonstrate that it is possible to increase the specific growth rate of *L. lactis* on mannitol.

Adaptation to Fast Growth on Mannitol Leads to Mannitol and Acetoin Formation from Glucose in Resting Cells under Static Conditions. MG1363-M is clearly better at metabolizing mannitol, and since the mannitol metabolism in *L. lactis* has been reported to be reversible, we suspected that the mannitol-adapted strain MG1363-M might also be better at producing mannitol from glucose. We decided to test the adapted strain under resting and static conditions to determine whether mannitol could be produced from glucose. Under static conditions, no active aeration takes place; however, slow diffusion of atmospheric oxygen into the cell suspension is possible, thus allowing for the NADH oxidase to slowly convert NADH into NAD⁺. As shown in Figure 3, MG1363-M indeed generated mannitol from glucose and approximately 4% of the glucose could be transformed into

Figure 3. Mannitol production by resting cells of MG1363 and MG1363-M. (a) Glucose consumption and products generated by MG1363 under static (no active aeration, slow diffusion of oxygen possible) conditions; (b) glucose consumption and products produced by MG1363-M under static conditions; (c) glucose consumption and products produced by MG1363-M under aerobic conditions. Experiments were conducted in duplicates, and error bars indicate standard deviations. Cells ($OD_{600} = 15.0$) were suspended with PBS buffer (pH = 7.0) supplemented with 2% glucose.

Figure 4. Mannitol production by resting cell cultures of CS4099-M and CS4363-M. (a) Glucose consumption and products produced by resting cells of CS4099-M; (b) glucose consumption and products produced by resting cells of CS4363-M. Static conditions are used (no active aeration, slow diffusion of oxygen possible). Experiments were conducted in duplicate, and error bars indicate standard deviations. Cells ($OD_{600} = 15.0$) were suspended with PBS buffer (pH = 7.0) supplemented with 2% glucose.

Figure 5. Mannitol production by resting cells of Ace001-M. (a) Glucose consumption and products produced from Ace001-M; (b) the conversion ratio of all metabolites from glucose and carbon recovery in Ace001-M. Static conditions are used (no active aeration, slow diffusion of oxygen possible). Experiments were conducted in duplicate, and error bars indicate standard deviations. Cells ($OD_{600} = 15.0$) were suspended with PBS buffer (pH = 7.0) supplemented with 2% glucose.

mannitol. In contrast, the wild-type strain MG1363 only produced lactate. We also tested whether mannitol could be formed when the cells were aerated; however, as expected, no mannitol was detected, indicating that the NADH supply under these conditions was insufficient, probably due to NADH consumption by the NADH oxidase (NoxE) when oxygen was present.

Increasing the NADH Supply Is Beneficial for Mannitol Production. To enhance the NADH supply, we decided to explore an MG1363 derivative lacking lactate dehydrogenase activity (CS4099) and a derivative that

furthermore lacks the alcohol dehydrogenase and the phosphotransacetylase (CS4363). Both strains were adapted for fast growth on mannitol, and two adapted derivatives, named CS4099-M and CS4363-M (see in Methods and Supporting Information, Table 1), were isolated and further characterized in resting cell experiments. No mannitol was detected from both of the nonadapted strains CS4099 and CS4363 under resting conditions; however, under the same conditions, both of the adapted strains formed large amounts of mannitol. As shown in Figure 4, CS4099-M converted approximately 21% of the glucose to mannitol, with lactate,

Figure 6. Mannitol formation by resting cells of AceM. (a) Glucose consumption and by-products produced by AceM; (b) product/glucose ratio for mannitol and acetoin, as well as the overall carbon recovery for AceM. Static conditions are used. Experiments were conducted in duplicate, and error bars indicate standard deviations. Cells ($OD_{600} = 15.0$) were suspended with PBS buffer (pH = 7.0) supplemented with 2% glucose.

Figure 7. Overview of the by-products formed by the different strains studied in resting cell experiments and the corresponding mannitol/glucose conversion ratios. (a) comparison of product ratios obtained for different strains; (b) Mannitol yield for strains adapted on mannitol. O_2 indicates aeration. All data shown were collected from samples withdrawn prior to 15 h of cultivation.

Table 2. Overview of By-products (Yields) Formed from Glucose for All Strains Used in This Study in Resting Cell Experiments^a

resting cells	glucose	mannitol	lactate	acetoin	acetate	2,3-BDO	ethanol	recovery
MG1363	1	nd	1.07 ± 0.08	nd	nd	nd	nd	1.07 ± 0.08
MG1363-M	1	0.04 ± 0.00	0.95 ± 0.02	nd	nd	nd	nd	0.99 ± 0.02
MG1363-M, O_2	1	nd	0.46 ± 0.03	0.34 ± 0.01	0.22 ± 0.01	0.07 ± 0.01	nd	0.98 ± 0.05
CS4099	1	nd	nd	nd	nd	0.53 ± 0.06	0.36 ± 0.04	0.89 ± 0.02
CS4099-M	1	0.21 ± 0.02	0.18 ± 0.01	nd	0.12 ± 0.00	0.37 ± 0.01	nd	0.88 ± 0.03
CS4099-M, O_2	1	nd	0.13 ± 0.02	0.46 ± 0.04	0.22 ± 0.02	0.17 ± 0.02	nd	0.98 ± 0.03
CS4363	1	nd	nd	nd	nd	0.55 ± 0.05	nd	0.55 ± 0.05
CS4363-M	1	0.43 ± 0.01	nd	nd	nd	0.51 ± 0.04	nd	0.94 ± 0.02
CS4363, O_2	1	nd	nd	0.93 ± 0.01	nd	0.04 ± 0.00	nd	0.97 ± 0.00
CS4363-M, O_2	1	nd	nd	0.88 ± 0.01	nd	nd	nd	0.88 ± 0.01
Ace001	1	nd	nd	0.82 ± 0.04	nd	nd	nd	0.82 ± 0.04
Ace001-M	1	0.58 ± 0.02	nd	0.45 ± 0.06	nd	nd	nd	1.03 ± 0.04
AceM	1	0.60 ± 0.00	nd	0.41 ± 0.02	nd	nd	nd	1.01 ± 0.02

^aShown are average values $\pm$ standard deviations from at least two independent growths. The abbreviation nd indicates “not detectable” from HPLC results. All data shown here were collected from samples taken prior to 15 h of cultivation.

2,3-butanediol, and acetate as by-products. For CS4363-M, the yield more than doubled to 43% and 2,3-butanediol was the sole by-product (>50%). Under aerated conditions, CS4099-M produced lactate, acetoin, 2,3-butanediol, and acetate without any mannitol being detected. For CS4363-M, nearly 90% of the by-product formed was acetoin. Compared with cells incubated anaerobically, the dramatically decreased yield of 2,3-butanediol indicated that NAD^+ could be regenerated efficiently by the NADH oxidase.^{26–28}

Channeling More NADH into Mannitol Production by Blocking 2,3-Butanediol Formation. 2,3-Butanediol is formed from acetoin by butanediol dehydrogenase (encoded by *butBA*), which consumes one NADH. To get rid of the by-product 2,3-butanediol and thereby further boost the NADH supply for mannitol production, we relied on *L. lactis* Ace001, a strain that is derived from CS4363 by deleting *butBA*. In Ace001, when oxygen is unavailable as electron acceptor, NADH can only be oxidized into NAD^+ when mannitol is generated, and for this reason Ace001 grows poorly under

Table 3. Single-Nucleotide Variations Occurring after Mannitol Adaptation in Different Strains

strains	RP ^a	coverage	region	NC ^b	AAC ^c	protein id	product or (distance from CDS) neared product
MG1363-M	26 455	509	intergenic	C > T			(−39 bp) PTS mannitol transporter subunit IIBC
MG1363-M	30 716	656	gene CDS	C > T	A > V	WP_011834136.1	PTS mannitol transporter subunit IIA
MG1363-M	369 233	550	gene CDS	G > A	P > L	WP_011834429.1	amino acid permease
MG1363-M	1 132 975	199	intergenic	A > G			(+103) Endoglucanase and (−149) polysacchride deacetylase family protein
MG1363-M	1 779 597	421	gene CDS	C > A	L > F	WP_011835577.1	SpaH/EbpB family LPXTG-anchored major pilin
CS4099-M	26 455	522	intergenic	C > T			(−39 bp) PTS mannitol transporter subunit IIBC
CS4099-M	1 357 615	515	gene CDS	C > T	E > K	WP_011835247.1	type IV secretory system conjugative DNA transfer family protein
CS4363-M	26 448	495	intergenic	G > T			(−46 bp) PTS mannitol transporter subunit IIBC
CS4363-M	30 518	845	gene CDS	G > A	G > D	WP_011834136.1	PTS mannitol transporter subunit IIA
CS4363-M	1 488 149	633	gene CDS	-T	frameshift	WP_011835356.1	redox-sensing transcriptional repressor
CS4363-M	1 582 199	393	gene CDS	T > G	E > A	WP_011835437.1	SGNH/GDSL hydrolase family protein
Ace001-M	26 455	506	intergenic	C > T			(−39 bp) PTS mannitol transporter subunit IIBC
Ace001-M	30 716	775	gene CDS	C > T	A > V	WP_011834136.1	PTS mannitol transporter subunit IIA
Ace001-M	401 743	615	intergenic	C > T			(−79 bp) NADH oxidase
Ace001-M	1 951 535	663	gene CDS	C > A	V > F	WP_004255207.1	Fe–S cluster assembly protein SufB
Ace001-M	2 333 009	551	Gene CDS	C > A	D > Y	WP_011836032.1	acetyltransferase

^aReference position, location is based on the genome sequence of *L. lactis* MG1363 in Genbank (Accession number: NC009004). ^bNucleotide change. ^cAmino acid change.

static conditions. In resting conditions, Ace001 formed only small amounts of acetoin, most likely due to the NADH oxidase and oxygen diffusing into the cell suspension, and no mannitol was detected. After adaptation on mannitol for more than 1 month, Ace001-M was obtained (see Methods and Supporting Information, Table 1). As shown in Figure 5, the mannitol yield on glucose increased to 58% and the only by-product formed was acetoin. Intriguingly, lactate was detected after 20 h of incubation, which coincided with a decrease in mannitol concentration and the mannitol conversion ratio therefore decreased to less than 40%. We found that the presence of mannitol prevented formation of mannitol by the resting cells. For this reason, we decided to block mannitol uptake by deleting *mtlF*, encoding the mannitol specific, soluble IIA PTS component in Ace001-M.

Knocking-Out Mannitol Uptake Further Stimulates Mannitol Production. A 302bp DNA sequence that included the promoter of *mtlF* gene (soluble EIIA component of the mannitol PTS) was deleted in Ace001-M, as described in the Materials & Methods section, and the strain obtained was designated AceM. Without mannitol transport activity, AceM was unable to metabolize mannitol. AceM, however, could still convert glucose to mannitol and mannitol could be transported out of the cell. As shown in Figures 6 and 7, AceM had mannitol yield from glucose of 60%, which is the highest yield we achieved. In addition, unlike Ace001-M, when a high concentration of mannitol was present in the cell suspension, no inhibitory effect of mannitol on mannitol production was observed. Interestingly, after deleting *mtlF* (soluble EIIA component of the mannitol PTS), lactate was no longer formed.

Scrutinizing the Genomes of the Mannitol-Adapted Strains. As shown in Table 2, mannitol formation was only detected for mannitol-adapted strains. In *L. lactis*, F6P is reduced by mannitol-1-phosphate 5-dehydrogenase (*mtlD* encoded) to M1P and subsequently M1P is dephosphorylated by an unidentified phosphatase. Due to its involvement in both mannitol catabolism and mannitol synthesis, we speculated that the mannitol-1-phosphate dehydrogenase locus could have mutated in the mannitol-adapted strains, thereby resulting in a

higher expression level or perhaps a more active enzyme. We therefore sequenced *mtlD* and its 3' and 5' flanking sequences in AceM and Ace001. However, no mutations were revealed and we could conclude that there were other reasons for the increased mannitol production capacity of the evolved strains. To find the underlying explanation for the enhanced growth on mannitol, as well as the superior ability to transform glucose into mannitol, for the adapted strains, we sequenced their genomes as well as that of the parent strains. As shown in Table 3, we found, in total, 16 single-nucleotide variations (SNVs) in all mannitol-adapted strains, whereof 13 were unique, including 8 in protein coding regions and 3 in intergenic regions. Interestingly, all 4 mannitol-adapted strains had mutations upstream of *mtlA* (EIIBC, the membrane-spanning component of the mannitol PTS) and 3 of them (except CS4099-M) had mutations in the *mtlF* (soluble EIIA component of the mannitol PTS) coding region, strongly indicating that the enhanced growth on mannitol of the adapted strains was linked to changes in mannitol uptake. The two SNVs detected in *mtlF* (IIA) were in the coding region, and resulted in amino acids changes, G54D and A120V, respectively. The two SNVs detected upstream of *mtlA* appeared to be in the promoter region, 39 bp and 46 bp upstream of the *mtlA* (EIIBC) start codon, resulting in nucleotide changes, C-39T and G-46T, respectively. To evaluate if the two SNVs in the upstream region of *mtlA* gene were in the *mtlA* promoter, the online promoter detection tool BDGP (www.fruitfly.org/seq_tools/promoter.html) was used and demonstrated that both of the mutations indeed were in the *mtlA* promoter (complementary Table 2), leading to altered expression of *mtlA*. To substantiate this prediction, we cloned the region containing the mutations in a promoter probe vector, allowing us to correlate promoter activity to β -glucuronidase activity. We found that promoter activity had increased by a factor of 25 (G-46T) and 800 (C-39T). In addition to these mutations, we also detected SNVs in genes encoding an amino acid permease, an SpaH/EbpB family LPXTG-anchored major pilin, a type IV secretory system conjugative DNA transfer family protein, a Redox-sensing transcriptional repressor, an SGNH/GDSL hydrolase family

Figure 8. Comparison of the effect of aeration and arginine supplementation on mannitol production in AceM. (a) Yield and titer of mannitol achieved in AceM cultures initiated at a low cell density of OD₆₀₀ = 0.5. (b) Growth curves for AceM cultures initiated at a cell density of OD₆₀₀ = 0.5. (c) Yield and titer of mannitol achieved in AceM cultures initiated at a high cell density of OD₆₀₀ = 4.5. H indicates a high shaking speed (60 rpm), and L indicates a low shaking speed (20 rpm); 240 rpm indicates that the shaking speed used was 240 rpm; static refers to cultures not being actively aerated; slow diffusion of oxygen is possible; Numbers after H/L indicate the volume of medium used in 100 mL flasks; Ar after numbers indicates addition of 35 mM arginine. Yields are calculated on the basis of data collected at different time points during the experiment and shown with standard deviations.

protein, an Fe–S cluster assembly protein SufB, and finally in the gene encoding an Acetyltransferase. All of the SNVs in gene CDS regions either lead to changes in amino acids or to frame shifts. Two SNVs in intergenic regions were also detected, one near the gene encoding the NADH oxidase and another between genes encoding an endoglucanase and a member of the polysaccharide deacetylase family.

Using a Two-Step Fermentation Setup To Improve Mannitol Production. None of the mannitol-adapted strains were able to form significant amounts of mannitol from glucose under growing conditions. When inoculated in M17 broth containing glucose, Ace001-M formed 2–4 mM mannitol and AceM formed 4–8 mM mannitol after several days of incubation and little growth took place. However, the high mannitol yield observed for resting cells indicated a great potential for mannitol production. We speculated that the ATP supply could be a limiting factor for cell growth and continuous mannitol production under anaerobic conditions, since mannitol production consumes ATP. To address the poor growth observed under static conditions, AceM was precultured aerobically to a high cell density and then the cultures were switched to static conditions. To change the NADH level, different amounts of oxygen were supplied to the cells, and to boost the supply of ATP, arginine was added, since ATP is formed when arginine is metabolized through the arginine deiminase pathway.²⁹ As shown in Figure 8, when starting from a low cell density, OD₆₀₀ of only 0.5, the amount of mannitol formed varied from 3.4 to 11.8 mM, depending on whether the cultures were aerated or not, and 5.0–14.5 mM, when arginine was supplied. The conversion ratios ranged from

14–58%. Supplying oxygen had a beneficial effect on cell growth and mannitol production, however, a negative effect on mannitol yield. Interestingly, small amounts of mannitol (1.62–1.81 mM) were also detected when AceM was cultured under aerobic conditions. When arginine was added, this resulted in an increase in the final pH from 7.16 to 7.74, compared with 6.65–6.94 without adding arginine (data not shown), which most likely was an effect of the NH₄⁺ produced in the arginine deiminase pathway. When a higher initial cell density was used (OD₆₀₀ of 4.5), higher titers of mannitol were obtained, ranging from 8.24–33.4 mM, depending on the aeration and whether or not arginine was supplied, and here the mannitol yield ranged from 41 to 63% (Figure 8). Interestingly, when compared with the low cell density experiments, a more stable mannitol titer was observed for the aerated cultures; however, supplying oxygen in this case still had a negative effect on the mannitol yield.

DISCUSSION

Mannitol Adaptation Facilitates Both Mannitol Uptake and Mannitol Production. The reduction of F6P to M1P is catalyzed by mannitol-1-phosphate dehydrogenase (M1PDH), which is encoded by *mtlD*. Since the M1PDH activity in *L. lactis* is very low, different approaches have been used to enhance this enzyme activity to obtain mannitol production in *L. lactis*. One research group managed to enhance mannitol production by overexpressing *mtlD* from *L. plantarum* in a lactate deficient *L. lactis* strain and achieved a 25% mannitol yield with resting cells.¹² Gaspar et al. overexpressed native *mtlD* and *E. tenella mtlP* in *L. lactis* and

achieved a 42% mannitol yield with growing cells, which demonstrates the importance of *mtlP* in mannitol production.¹⁴ Neves et al. detected M1PDH activity in *L. lactis* when it was grown on mannitol,¹¹ and this inspired us to carry out laboratory adaptative evolution in an attempt to enhance the M1PDH activity. We found that *L. lactis* indeed could metabolize mannitol better after adaptive evolution and also that resting cells could convert glucose into mannitol. We expected that the change in behavior was linked to the changed expression of M1PDH; however, sequencing did not reveal any mutations in *mtlD* or in its vicinity. In other studies where *mtlD* was overexpressed, mannitol production in resting cells was observed, which suggests that this enzyme is important.¹²

To help find the underlying cause for the observed behavior, we performed full genome sequencing. Our sequencing data identified, in total, 11 SNVs in the mannitol adapted strains and surprisingly one locus was mutated in all 4 adapted strains and another in three of the adapted strains. One of these was *mtlF* (EIIA), where the mutations occurred in the CDS region, leading to changes in amino acids, G54D and A120V, respectively. *L. lactis* takes up mannitol by a phosphoenolpyruvate (PEP)-dependent mannitol phosphotransferase system (PTS).^{30–33} In general, PEP-PTS has two general components, enzyme I (EI) and HPr, and several sugar-specific enzyme IIs (EIIs) complex, consisting of EIIBC proteins.^{34,35} In *L. lactis*, *mtlF* encodes the mannitol specific EIIA of the mannitol PEP-PTS, which is responsible for transferring the phosphoryl group from Hpr to the mannitol-specific EIIBC.^{36,37} The identified SNVs in the *mtlF* CDS region indicate a change in EIIA protein structure that could facilitate phosphoryl group transfer between Hpr and EIIBC in the mannitol-adapted strains. Two other interesting mutations were found upstream *mtlA* (EIIBC), either 46 or 39 bp from the first codon, respectively. We found that these mutations resulted in a stronger *mtlA* promoter and since *mtlA* encodes the membrane-embedded component of the mannitol PTS (EIIBC), through which mannitol is transported,^{36,37} the enhanced growth on mannitol, as well as the enhanced production of mannitol from glucose, most likely is caused by these mutations.

Some of the remaining SNVs could also be involved in the accelerated growth observed on mannitol of the adapted strains. The SNV found in the gene encoding an amino acid permease could facilitate uptake of nutrients from the medium and in this facilitate growth on slowly metabolized mannitol. SNVs in the gene encoding a redox-sensing transcriptional repressor and in the vicinity of the gene encoding NADH oxidase may help cells adapt to the high NADH/NAD⁺ ratio on mannitol.

High NADH Supply Is Key To Obtaining a High Mannitol Yield. In *L. lactis*, two steps are needed for converting F6P into mannitol. F6P is first reduced into mannitol-1-phosphate by M1PDH, which is then dephosphorylated by an unknown sugar phosphatase (SP) into mannitol. From glucose, one NADH and one ATP are consumed in the process. For the wild-type MG1363, it has been demonstrated that a lactate dehydrogenase deficient strain has higher intracellular M1PDH and SP enzyme activity, but despite the presence of 90 mM of intracellular mannitol, mannitol is not secreted.¹⁰ When the lactate dehydrogenase is disrupted, the main sink for NADH is gone and the NADH/NAD⁺ ratio increases^{38,39} and in such mutants the NADH is oxidized in a different way, which leads to formation of ethanol, 2,3-butanediol and other products.⁴⁰ In our study, we

also observed this for our mannitol-adapted lactate dehydrogenase-deficient strain but not for the nonadapted strain, and the latter concurs with previous findings.¹⁰ To achieve high mannitol production in a lactate dehydrogenase-deficient strain, Wisselink et al. found that it was necessary to overexpress *mtlD* and after doing so managed to achieve 25% conversion of glucose into mannitol in resting cells.¹² To further enhance mannitol production, we eliminated ethanol and 2,3-butanediol formation and achieved a 58% conversion. Our results clearly illustrate the importance of a sufficient NADH supply for high-yield mannitol production. We also demonstrated that no mannitol was produced under aerobic conditions, where the NADH oxidase regenerates NAD⁺.

Mannitol Uptake Has a Negative Effect on Mannitol Production. *L. lactis* is capable of utilizing mannitol as a carbon source, although it only supports slow growth. Neves et al. demonstrated that the mannitol generated from glucose could be taken up rapidly after glucose had been depleted.¹¹ In our study, we observed that a high concentration of mannitol in the supernatant appeared to inhibit mannitol production and the mannitol-adapted strains could metabolize mannitol before glucose was exhausted. For this reason, we disrupted mannitol uptake. Gaspar demonstrated a complete abolishment of mannitol uptake in a lactate dehydrogenase-deficient *L. lactis* strain when *mtlF* (soluble EIIA component of the mannitol PTS) was deleted, which supports the notion that mannitol is solely taken up by a PEP-PTS system.¹³ Similarly, in this study, after *mtlF* (EIIA) was deleted, the cells could no longer metabolize mannitol and a more stable mannitol production was obtained. It is perhaps not surprising that elimination of mannitol uptake is needed for achieving mannitol production, as concurrent mannitol uptake and secretion result in a futile cycle eliminating the ATP needed for PTS uptake of glucose and for glycolysis to proceed (phosphofructokinase uses ATP).

Intriguingly, we detected that lactate was formed rapidly in Ace001 after incubation for nearly 20 h. This was unexpected as this strain lacks lactate dehydrogenase activity.²¹ However, in some studies, lactate formation has still been reported despite an absence of lactate dehydrogenase activity⁴¹ and this indicates that unknown pathways may be in operation. In the AceM strain, however, no lactate could be detected, even after 70 h of incubation.

Mannitol Production Leaves No ATP Behind for Cell Growth. In other studies, both mannitol-1-phosphate dehydrogenase and mannitol-1-phosphate phosphatase have been shown to be essential for obtaining efficient mannitol production and the overexpression of only one of these enzymes does not lead to high titers in growing cells.¹⁵ After introducing *E. tenella mtlP* (mannitol-1-phosphate phosphatase) and overexpressing *mtlD* (mannitol-1-phosphate dehydrogenase), *L. lactis* could produce mannitol efficiently, with more than 40% yield of mannitol under growing conditions.^{14,15} In our study, we only observed mannitol secretion for Ace001-M and AceM and both of these strains grew poorly. We speculated that the reason for the slow growth was due to inhibition of glycolysis, since glycolysis can be inhibited by a high NADH/NAD⁺ ratio, which leads to slower ATP formation, which would hamper growth.^{38,42} In AceM cells, formation of one acetoin leads to formation of two ATP and two NADH, and to achieve redox balance, 2 mannitol could be formed for each acetoin formed. Since formation of two moles of mannitol would consume the ATP formed via acetoin

production, there is no ATP left for growth or maintenance. In *L. lactis*, arginine is catabolized through the arginine deiminase pathway, generating NH_3 , CO_2 , and ATP.²⁹ To alleviate the NADH burden and provide extra ATP to the cells, we attempted to optimize the fermentation by aeration and supplying arginine. We found that aeration had a beneficial impact on mannitol titer; however, the yield decreased, due to some of the NADH being oxidized by the NADH oxidase. When arginine was added, we observed higher mannitol titers and mannitol yields. We speculate that the extra ATP supplied by arginine metabolism would allow cells to grow slowly but the flux would still be limited by a hampered glycolysis.

An advantage of the approach used here, where acetoin is generated, is that the need for pH control is obviated. In our batch fermentations, we achieved 6.1 g/L mannitol produced from glucose with a 60% mannitol yield. To the best of our knowledge, this is the first example of *L. lactis* secreting mannitol, while growing, without relying on any foreign genes. Due to the latter, in some countries, AceM is considered as food grade. We also achieved the highest Mannitol yield reported this far, around 63% (Supporting Information, Table 4), which is very close to the theoretical value of 66.7%, when acetoin is the sole by-product.

Great Potential for Improvements. For biosynthesis of mannitol, heterofermentative LABs have been well studied and good yields and titers have been reported. However, when using *L. lactis* as a production platform, it becomes possible to use different sugars for producing mannitol.⁹ This could lower the price of the feedstock and simplify the purification process. In this study, we successfully improved mannitol production by *L. lactis*, by using a combination of adaptive evolution and metabolic rewiring, thereby overcoming bottlenecks in metabolism and increasing the supply of NADH. The strains we have developed contain no foreign genes and are considered food-grade in many countries. The mutations that we introduced to enhance NADH supply and to limit formation of by-products could in principle also be obtained via traditional mutagenesis and screening, which would make such a strain non-GMO worldwide.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c00532>.

Growth performance of mannitol-adapted strains and their parental strains in M17 supplemented with 0.5% mannitol (Table 1); bioinformatics analysis for *mtlA* gene (Table 2); data pertaining to Figure 8a, yield and titer of mannitol achieved in AceM cultures initiated at a low cell density ($\text{OD}_{600} = 0.5$) (Table 3); data pertaining to Figure 8c, yield and titer of mannitol achieved in AceM cultures initiated at a high cell density ($\text{OD}_{600} = 4.5$) (Table 4) (PDF)

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Notes

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