Deciphering the Regulation of the Mannitol Operon Paves the Way for Efficient Production of Mannitol in *Lactococcus lactis*

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is the highest titer ever reported for *L. lactis*. Summing up, the results of our study should be useful for improving the mannitol producing capacity of this important industrial organism.

Importance

Lactococcus lactis is the most studied species of the Lactic Acid Bacteria, and it is widely used in various food fermentations. To date, there have been several attempts to persuade *L. lactis* into producing mannitol, a sugar alcohol with important therapeutic and food applications. Until now, to achieve mannitol production in *L. lactis*, with significant titer and yield, it has been necessary to introduce and express foreign genes, which precludes the use of such strains in foods, due to their recombinant status. In this study, we systematically characterize how the mannitol genes in *L. lactis* are regulated, and demonstrate how this impacts on mannitol production capability. We harness this information and manage to establish efficient mannitol production, without introducing foreign genes.

Key words

L. lactis, mannitol, *mtlR*, *mtlD*, *ccpA*

Introduction:

Lactococcus lactis (*L. lactis*) is a lactic acid bacterium (LAB) with a long history of safe use in the dairy and food industry. Due to its safe status, its well-known metabolism, and the many genetic tools that have been developed for it, *L. lactis* is regarded as an excellent production platform for food ingredients as well as for compounds with sensitive applications, such as therapeutics (1). Mannitol, a sugar alcohol, is an example of a compound that can be produced in many LAB, including *L. lactis* (2, 3). Different from heterologous fermentative LAB, which produce mannitol rely on reducing fructose, *L. lactis* and other homologous fermentative LAB (4, 5, 6) produce mannitol rely on reducing fructose-6-phosphate (F6P), which is a common intermediate in the glycolytic process. This means *L. lactis* could

theoretically produce mannitol from all carbon sources that are metabolized to F6P, which would further broaden its application as a mannitol producer. Although mannitol production in *L. lactis* has been studied to some extent during the last two decades, still many aspects of mannitol production are unclear (7, 8).

Mannitol biosynthesis in *L. lactis*, and other homofermentative lactic acid bacteria, starts with the glycolysis intermediate (F6P). The mannitol 1-phosphate dehydrogenase (M1PDH) is responsible for reducing F6P into mannitol 1-phosphate (M1P), which subsequently is dephosphorylated to mannitol by an uncharacterized phosphatase. The normal function of M1PDH is not to facilitate mannitol production, but rather growth on mannitol, and its encoding gene *mtlD* is located together with genes coding for the mannitol specific PEP (phosphoenolpyruvate)-PTS components (*mtlA* & *mtlF*) and the gene for the regulator (*mtlR*). Although *L. lactis* has the metabolic capacity for making mannitol, the relevant genes are under tight control and appear not to be expressed on glucose.

In most Gram-positive bacteria, the transcription activator MtlR controls the expression of the genes encoding M1PDH, and the D-mannitol-specific PTS components (9). MtlR from different Gram-positive bacteria have been studied and they all share domains that have different regulatory functions depending on organism. In general, MtlR contains an N-terminal helix-turn-helix motif followed by an Mga-like domain, two PTS regulation domains (PRDs), an EIIB^{Gat}-like, and an EIIA^{Mtl}-like domain (Fig. 1). There are exceptions, however, and, for instance, MtlR in *Lactobacillus casei* lacks the PRD1 and EIIB^{Gat}-like domains. There are several regulatory sites in MtlR that can be phosphorylated by Enzyme IIs (EIIs) and HPr, which can activate or inhibit the activity of MtlR. In the presence of an efficiently metabolized PTS substrate, e.g. glucose, HPr is barely phosphorylated (10), and this prevents activation of MtlR. Another regulatory mechanism, which turns off MtlR when mannitol is absent, is the mannitol specific PTS component EIIA, which can phosphorylate the EIIB^{Gat}-

73 like domain of MtlR, leading to inactivation of MtlR (11). In addition to these forms of
74 regulation the classical carbon catabolite repression (CCR) is also involved in the regulation
75 of the mannitol operon (12) and together these three regulatory mechanisms probably explain
76 why the mannitol genes normally are repressed during growth on other sugars, and why
77 mannitol is not produced.

78 There have been many efforts to achieve mannitol production in *L. lactis*. Gaspar et al.
79 (13), reported that an *L. lactis* strain deficient in lactate dehydrogenase (*ldh*) formed small
80 amounts of mannitol (0.7 mM) when growing on glucose, however, after glucose depletion
81 mannitol was metabolized. The same researchers also found that non-growing cells
82 resuspended in buffer, were able to convert 33% of the glucose metabolized into mannitol.
83 Wisselink et al. demonstrated that the MIPDH had an impact on mannitol production, but the
84 effect was only seen clearly in a lactate dehydrogenase deficient strain, even under non-
85 growing conditions (14). By heterologous overexpression of the mannitol-1-phosphatase
86 from *Eimeria tenella* and a MIPDH from *Lactobacillus plantarum* in a LDH deficient strain,
87 efficient mannitol production (9.0 g/L, 50% yield) was accomplished in growing *L. lactis*
88 cells for the first time (15). All reported cases of mannitol production using growing cells,
89 involve introduction of foreign genes in *L. lactis*, in combination with additional
90 modifications to metabolism. Since such strains are genetically engineered, their use in food
91 fermentations is precluded. To overcome this problem we recently attempted a different
92 strategy, namely adaptive evolution on mannitol, where the aim was to debottleneck mannitol
93 catabolism, in the hope that this could result in more efficient production of mannitol from
94 glucose. It was indeed possible to enhance growth on mannitol, and the mutants obtained
95 contained a mutation upstream *mtlA*, which greatly enhanced expression of the mannitol
96 genes. By further eliminating competing NADH consuming pathways, it was possible to

97 achieve mannitol production from glucose with high yield (60%) and fairly high titer (6.1 g/L)
98 using a two-stage fermentation setup (16).

99 In this study, we further characterize the effects of those mutations and study the roles that
100 MtlR and CcpA have in regulating mannitol production from glucose. Based on the
101 knowledge accumulated we manage to establish efficient mannitol production using
102 stationary phase cells, and achieve the highest titer and yield ever reported, when relying
103 solely on native metabolism.

104 **Results**

105 **Organization of the mannitol genes in *L. lactis***

106 As mentioned above, we recently reported that adaptive laboratory evolution combined
107 with systematic elimination of NADH consuming reactions can lead to efficient mannitol
108 production, by *L. lactis*, from glucose. The strains obtained after adaptive evolution contained
109 two different mutations, C-39T and G-46T, both in the *mtlA* promoter region, which we
110 found were critical for mannitol production (16).

111 To better understand the effect of these mutations, we first examined the genetic
112 arrangement of the mannitol genes in *L. lactis* and compared our findings to those previously
113 reported for other Gram-positive bacteria. As shown in Fig. 2a, the mannitol genes appear to
114 be organized in a similar manner in all the bacteria investigated, except for *B. subtilis*, where
115 the gene encoding the regulator MtlR is distantly located from the other mannitol genes.
116 Nevertheless, we observed two distinct features for the *L. lactis* genes. The first one was that
117 the spaces between *mtlR* & *mtlF*, and between *mtlF* & *mtlD* appear longer in *L. lactis*, than in
118 other organisms. Another difference is that there is a predicted rho-independent terminator
119 between *mtlF* and *mtlD*, suggesting that *mtlD* is transcribed from its own promoter.
120 Interestingly, the gene just after *mtlD*, encoding a characterized 6S (17) RNA may also be

121 affected by the promoters upstream of *mtlD*, since no transcriptional terminator can be
122 identified between the 6S RNA gene and *mtlD*.

123 The promoter regions upstream *mtlA* and *mtlD* in *L. lactis* were also analyzed, and
124 interestingly, we found that they share more than 47% identity. Both regions have putative
125 MtlR binding sequences and *cre* sites that are well-conserved. Furthermore, as shown in Fig.
126 2b, the two mutations, C-39T and G-46T were both found to locate in the predicted putative
127 *cre* site, where C-39T also overlapped with the putative -10 element region, suggesting that
128 the mutations affect the expression of mannitol operon.

129 **Adaptive laboratory evolution on mannitol lead to mutations which enhance the *mtlA*** 130 **promoter**

131 To assess the effect of these mutations on expression from the *mtlA* promoter,
132 transcriptional fusions between the *mtlA* promoter and the reporter gene *gusA* (encoding β -
133 glucuronidase) were constructed based on the reporter vector pTD6. As shown in Table 2, on
134 glucose, a low promoter activity, no more than 0.1 MU (Miller Units), was observed for the
135 wild-type promoter. In contrast, the promoters containing the C-39T or G-46T mutations
136 were significantly stronger, in particular the C-39T mutation, which was 40 fold stronger than
137 the promoter containing the G-46T mutation. The β -glucuronidase activity measured,
138 depended on host, e.g. the activity of the C-39T promoter in the mannitol adapted strain
139 MG1363M, having the C-39T mutation in its genome, was 52.16 MU, whereas the activity in
140 the wild-type strain MG1363 only reached 15.6 MU. As MtlR is expressed together with
141 *mtlA* and the mannitol PTS genes (one transcriptional unit), the C-39T mutation in
142 MG1363M would result in higher expression of MtlR, which could in turn explain the higher
143 activity of the *mtlA* promoter in the MG1363M background. On mannitol, as expected, we

144 found a higher activity, and whereas the wild-type promoter reached 449 MU, the C-39T
145 promoter activity resulted in more than 4000 MU.

146 Based on this information, the promoter mutations in the adapted strains appear to enhance
147 the expression of *mtlA*, *mtlR* and *mtlF* and possibly *mtlD*. We previously found that deleting
148 *mtlF* in *L. lactis* had a beneficial effect on mannitol production (16), while others have found
149 that strains lacking either *mtlA* or *mtlF* perform similarly in terms of mannitol production (13).

150 **The promoters upstream *mtlA* (P_{mtlA}) and *mtlD* (P_{mtlD}) are both activated by MtlR and**
151 **drive expression of separate transcriptional units**

152 To investigate the promoter upstream *mtlD*, we inserted it into the promoter probe vector
153 pTD6, and introduced the plasmid in AceR, the *mtlR* depleted strain derived from Ace001M
154 (Ace001 adapted on mannitol, containing the C-39T mutation in *mtlA* promoter). We also
155 introduced the previously constructed *mtlA* promoter-*gusA* fusion in the same strain. As
156 shown in Fig. 3, on glucose, in the AceR background, we found that both promoters were less
157 active than in the parental strain Ace001M. In Ace001M the mannitol operon is expressed to
158 a higher level, leading to more of the activator MtlR. These findings clearly demonstrated
159 that MtlR was an activator of the promoter upstream *mtlD*. Interestingly, the promoter
160 activities were much higher for cells in stationary phase than in exponential phase and for
161 both promoters, a large induction was observed on mannitol.

162 To investigate if the expression of *mtlD* could be affected by the promoter upstream *mtlA*
163 (P_{mtlA}), we fused the mutated *mtlA* promoter (C-39T) with the *mtlD* promoter, including the
164 transcriptional terminator upstream *mtlD*, and introduced the construct into the reporter
165 vector pTD6. In the Ace001M background, a low expression level was detected, only
166 1.82 ± 0.13 MU, which is similar to that found for *mtlD* promoter alone. This demonstrates
167 that *mtlD* is not part of the *mtlA*-*mtlR*-*mtlF* operon.

168 Deleting *ccpA* increases expression of the mannitol genes

169 The mannitol genes are subject to carbon catabolite repression, and this might have a
170 negative effect on mannitol production from glucose. To investigate this, we deleted *ccpA* in
171 Ace001, thereby generating Ace001C. As shown in Fig. 4, by deleting *ccpA*, the *mtlA* and the
172 *mtlD* promoters were both derepressed, and activities of 588 MU and 405 MU, respectively
173 were measured in the exponential phase (Table 3). Furthermore, we found that the wild type
174 *mtlA* promoter and *mtlA* promoter from the mannitol adapted strains had similar activities in
175 the Δ *ccpA* background, demonstrating that the mutation had affected the functionality of the
176 *cre* site (Fig. 4). Moreover, two to three fold higher promoter activity were detected in the
177 stationary phase.

**178 MtlR is needed for high-level expression of the mannitol genes, and its level can be
179 enhanced by deleting *mtlF***

180 For investigating the extent to which MtlR affects expression of mannitol genes, we
181 deleted *ccpA* in AceR, which lacks *mtlR*, thus generating AceCR. As shown in Fig. 5,
182 compared with AceR (Fig. 3), deleting *ccpA* (AceCR) only had a small positive effect on the
183 *mtlA* promoter, and the *mtlD* promoter had no activity. These results clearly demonstrate that
184 the mannitol genes in *L. lactis* are tightly regulated by MtlR. To investigate whether
185 eliminating CcpA could be beneficial for mannitol production, we deleted *ccpA* in strain
186 AceF, which harbors the C-39T promoter mutation and in which *mtlF* has been deleted; the
187 outcome was strain AceCF. As observed earlier, without *mtlF*, the activity of *mtlA* and *mtlD*
188 promoters were dramatically increased to 3787 MU and 1955 MU, respectively, in the
189 exponential phase, and increased to 6055 MU and 3664 MU in the stationary phase (Fig. 5
190 and Table 3).

191 Inactivating CcpA enables mannitol production in non-mannitol adapted strains

192 To quantify the effect of MtlR on the expression level of the mannitol genes, on mannitol
193 catabolism and on mannitol production from glucose, we overexpressed *mtlR* from a
194 promoter of intermediate strength in the non-mannitol producer Ace001 (MG1363 Δldh
195 $\Delta ldhB$ $\Delta ldhX$ Δpta $\Delta adhE$ $\Delta butBA$). As shown in Table 4, this enabled mannitol production
196 from glucose. Deleting *mtlR* in the mannitol producer Ace001M, abolished both growth on
197 mannitol and mannitol production from glucose, which indicates that MtlR has a key role in
198 both mannitol catabolism and production. AceCF and AceF were both able to produce
199 mannitol, but unable to grow on mannitol, where the latter was expected, since *mtlF* encodes
200 a critical mannitol PTS component.

201 **MtlR has no influence on the mannitol-1-phosphatase activity**

202 In *L. lactis*, two activities are needed for mannitol production to occur (Fig. 6); the first is
203 mannitol-1-phosphate dehydrogenase (*mtlD*), and the second is a phosphatase activity that
204 can convert mannitol-1-phosphate into mannitol, where the latter has not been identified. The
205 impact that MtlR has on the expression of the mannitol genes is clear, however, is it possible
206 that MtlR could serve as an MlPase or be able to increase the MlPase activity in cells, as
207 MtlR contains several phosphoryl transfer domains. For testing this, we measured the MlPase
208 activity in Ace001-*mtlR* and AceR. We finally detected similar MlPase activity in Ace001-
209 *mtlR* AceR and Ace001 (0.48, 0.49 and 0.49 respectively). Based on these results, we
210 concluded that MtlR can not be responsible for the MlPase activity in *L. lactis*.

211 **Mannitol production using two-step fermentation from different strains**

212 As deletion of *ccpA* leads to increased expression of the mannitol genes, we decided to test
213 if this could also be beneficial for mannitol production. We therefore tested AceCF and AceF
214 in the two-step fermentation setup used previously (16). As shown in Table 5, AceCF, the
215 strain lacking *ccpA* had similar performance with its parental strain AceF, both in terms of

titer and yield for mannitol. One possible explanation is that glucose metabolism is hampered to such an extent that the beneficial effect coming from overexpressing the mannitol genes is lost. For AceR-*mtlD*, a strain that overexpresses *mtlD* and lacks MtlR, surprisingly a titer of 10.1 g/L with a 55% yield was attained, which is the highest ever reported for *L. lactis*. This clearly demonstrated the key role of the mannitol 1-phosphate dehydrogenase in mannitol production. Furthermore, we also tested if the strains AceCF and AceR-*mtlD* were able to produce mannitol under semi-anaerobic growth conditions, where slow diffusion of oxygen into the culture medium was allowed (no active aeration), and indeed, slow growth and mannitol production was observed.

Discussion

In *L. lactis*, *mtlD* encodes M1PDH, which is a key enzyme in mannitol metabolism. In this study, we found that MtlR is essential for maintaining the expression of *mtlD*, thus allowing for mannitol production in non-growing or slowly growing cells.

The phosphatase responsible for dephosphorylating mannitol-1-phosphate remains to be found

Although several studies have shown that *L. lactis* is able to convert M1P into mannitol, the responsible enzyme has not been identified (13, 18). It has been suggested that a mannitol specific PTS component could be involved in this, e.g. the EIIBC^{mtl}, as it is able to transfer phosphoryl group between M1P and mannitol. However, Ramos and coworkers showed that neither EIIBC^{mtl} nor EIHA^{mtl} are responsible for the M1Pase activity in *L. lactis* (13). In this study, we clarified that the other phosphoryl transferring protein MtlR also is not responsible for the M1Pase activity. Inducer expulsion, which is a unique mechanism only found in few Gram-positive bacteria, including *L. lactis*, could be the explanation. Inducer expulsion is the process where intracellular sugar phosphates are dephosphorylated and exported when

240 preferred sugars, like glucose are present (19, 20, 21, 22, 23). In our case, when cells are
241 incubated with glucose, M1P accumulates, is dephosphorylated to mannitol, and transported
242 out of the cell. Interestingly, in *L. lactis* a 10 kDa sugar-phosphatase having a broad substrate
243 specificity was characterized and found to be responsible for inducer expulsion (24, 25). If
244 this enzyme is the M1Pase, we probably could overcome the bottleneck in mannitol
245 production by enhancing its expression, thus avoiding heterologous gene expression (15).

246 **CCR is relieved when cells reach stationary phase**

247 CcpA is a global transcriptional regulator, which is responsible for CCR in Gram-positive
248 bacteria. It functions as a complex with P-Ser-Hpr, and exerts CCR by binding to the *cre* site
249 upstream of various genes. In this study, we validated that CcpA serves as a strong repressor,
250 preventing expression of the mannitol genes when glucose is present. We characterized
251 several mutants adapted to growth on mannitol, and in one of these a mutation was found in
252 the *mtlA* promoter region (C-39T mutant). In the C-39T mutant, the *mtlA* promoter had
253 become more active, both on glucose but especially on mannitol. However, after inactivating
254 the *ccpA* gene, the C-39T promoter displayed a similar activity as the wild type promoter.
255 This indicates that the C-39T mutation had an influence on the functionality of the *cre* site in
256 this promoter. In stationary phase cells, the mannitol genes appeared to be upregulated
257 around 50-fold as compared to the exponential phase. This provided an explanation for why
258 the two-step fermentation approach used previously resulted in a high mannitol production in
259 the presence of glucose, which promotes catabolite repression (16). This de-repression could
260 be due to a decreased level of P-Ser-Hpr in the cells, and it has been reported that Hpr mainly
261 exists in the dephosphorylated form in stationary phase cells (26). We speculate that a
262 decreased level of CcpA could also contribute to the observed behavior, however, to clarify
263 this further studies are needed, eg., where the transcriptome of stationary phase cells is
264 scrutinized.

265 **Expression of a 6S RNA gene located downstream *mtlD*, is influenced by the *mtlD***
266 **promoter**

267 van der Meulen et al. characterized a 6S RNA gene located 105 bp downstream of *mtlD* (17).
268 Interestingly, it was shown that the expression of this gene was upregulated by 3 fold after
269 deletion of the *ccpA* gene, and by growing the cells in galactose or cellobiose, repression
270 could also be relieved. Although there is a predicted promoter and a *cre* site upstream of this
271 gene, there is no predicted transcriptional terminator between this gene and *mtlD*, suggesting
272 that increased expression of the *mtlD* gene could positively affect the expression of this 6S
273 RNA gene. In addition, it was also shown that several noncoding RNAs and protein coding
274 genes are regulated by this 6S RNA, and it was deduced to be active when CCR is relieved
275 during stationary phase and/or growth on alternative carbon sources (17).

276 ***L. lactis* MtlR is different from homologs in other firmicutes**

277 In this study, we substantiated that MtlR is indeed a transcriptional activator, which plays
278 an important role for the expression of mannitol genes, and we characterized a mutant *mtlA*
279 promoter found in a mannitol adapted strain, which grows fast on mannitol. The mutated
280 *mtlA* promoter retained an activity of around 40 MU in the absence of mannitol, which
281 appears to be due to less carbon catabolite repression, as the mutation is located in the
282 predicted *cre* site. In *B. subtilis*, MtlR binds to an incomplete inverse repeat sequence
283 upstream of the *mtlA* promoter (11). In *L. lactis*, a similar inverse repeat sequence can also be
284 found upstream of both *mtlA* and *mtlD* (Fig. 2b). Interestingly, these inverse repeat sequences
285 are likely to form rho-independent terminators, as they contain a GC rich harpin following by
286 several Ts. From a protein alignment between studied MtlRs from other Gram-positive
287 bacteria, we found that *L. lactis* MtlR is much more similar to the *S. mutans* MtlR
288 (supplementary table 1). However, in one study it was claimed that MtlR in *S. mutans* was

not needed for expression of the mannitol genes, and that this was due to a long insertion in the *mtlR* gene (27). Despite of this, in most other studies of MtlR in firmicutes, it has been shown that MtlR indeed acts as transcriptional activator for the mannitol operon (28, 29, 30, 31, 32, 33). MtlR generally consist of two parts. The first part is the helix-turn-helix Mga domain, which was first characterized in the *S. pyogenes* virulence gene regulator Mga, where it is responsible for binding to its DNA target. The second part consists of PRD domains and EIIA^{Mtl}-like domains (as well as EIIB^{Gat}-like domains in some firmicutes), which regulate the activity of MtlR through their phosphorylation states. In general, it has been found that the PRD1 domain has little effect on the activity of MtlR, whereas the phosphorylation status of the PRD2 domain greatly affects activity, where phosphorylation activates MtlR. Furthermore, dephosphorylation of the EIIA^{Mtl}-like and EIIB^{Gat}-like domains has positive effects on the activity of MtlR. Interestingly, the *L. casei* MtlR is only regulated by its EIIA^{Mtl}-like domain (9). By sequence alignment with other characterized MtlR and domain prediction, *L. lactis* MtlR appears structurally similar to *L. casei* MtlR, which does not have PRD1 and EIIB^{Gat}-like domains (supplementary Figure 1, 2). However, the histidine in the PRD2 domain is conserved in *L. lactis* MtlR. Moreover, in *B. subtilis*, it was shown that regulation of the mannitol operon mainly takes place via phosphorylation of MtlR, and that CcpA mediated carbon catabolite repression does not play a significant role, as expression of mannitol genes was not significantly affected in a *ccpA* deficient strain (12). Differently, in *L. lactis*, we have shown CcpA is a strong repressor of the mannitol genes, while MtlR seemingly is still active in the presence of glucose. In *G. stearothermophilus* and *L. casei*, phosphorylation in the EIIA^{Mtl}-like domain by P~EIIB^{Mtl} inhibits the activity of MtlR (9, 34). Several studies have shown that mannitol production can be enhanced by deleting *mtlF*, and it has been concluded that this is due to prevention of re-uptake of the mannitol produced (16, 35, 13). In this study, we detected a four-fold higher MtlR activity in a *mtlF* (encoding

314 EIIA^{Mtl}) deficient strain, where EIIB^{Mtl} always is in an unphosphorylated state because of the
315 absence of its phosphoryl group donor EIIA^{Mtl}. This suggests that the EIIA^{Mtl}-like domain in
316 *L. lactis* MtlR might be functional, and that phosphorylation of the EIIA^{Mtl}-like domain could
317 negatively affect the activity of *L. lactis* MtlR (Fig. 7). If so, deleting the *mtlF* gene not only
318 inhibits uptake of mannitol from the medium, but also to enhance the M1PDH activity by
319 activating MtlR, thus stimulating mannitol production in *L. lactis*.

320 **Deleting CcpA is not a good strategy for enhancing mannitol production in *L. lactis***

321 In this study, we have shown that there is an important role for MtlR in mannitol
322 production. However, the sole role of MtlR appears to be in activating expression of the *mtlD*
323 gene. In an attempt to improve mannitol production from glucose, we deleted the *ccpA* gene,
324 which resulted in high constitutive expression of the *mtlD* promoter. Unfortunately, we found
325 that both titer and yield did not change when deleting *ccpA*. In *L. lactis*, when *ccpA* is deleted,
326 glycolysis is hampered (36, 37, 38). Since PTS uptake of glucose requires
327 phosphoenolpyruvate (PEP), a glycolytic intermediate, this will have a direct effect on
328 mannitol production, which is what we indeed observed. It appears that a more promising
329 strategy for relieving carbon catabolite repression would be to inactivate the CcpA binding
330 site upstream of *mtlA* and *mtlD*, without reducing the activities of these promoters, which
331 perhaps could be accomplished by classical mutagenesis followed by screening.

332 In our previous study, to achieve a high mannitol titer, we relied on a two-stage production
333 setup, where we first accumulated biomass and then subsequently used this biomass for
334 producing mannitol (16). In this study, we found out that the success of this approach was
335 mainly due to the relief of CCR and high expression of *mtlD* in stationary phase cells. To
336 bypass the CCR, we tested whether overexpression of *mtlD*, the mannitol-1-phosphat
337 dehydrogenase, would enable anaerobic growth and mannitol production, however, this was

not the case. It appears that the mannitol-1-phosphatase is a bottleneck in mannitol production, and irrespective of a high mannitol-1-phosphate dehydrogenase activity, anaerobic NAD⁺ regeneration will remain hampered as long as the phosphatase activity is limiting, and the only way to achieve a high mannitol productivity is using dense cell suspensions. We also speculated whether the cells could be starving for ATP, since mannitol production is ATP demanding. To ameliorate the latter, we supplemented the cell suspensions with arginine, that can be metabolized via the arginine deiminase pathway leading to ATP formation (39). This indeed had a beneficial effect, however, only a small one. Boosting the inherent mannitol-1-phosphatase activity, e.g. by mutagenesis, thus appears to be a first priority if a successful mannitol production in *L. lactis* is to be established.

Materials and methods

Bacterial Strains and Growth Conditions

Lactococcus lactis subsp. *cremoris* MG1363 and its derivatives were used in this study (described in Table 1). *L. lactis* strains were cultured in M17 broth supplemented with 1% glucose (Sigma Aldrich) at 30°C, with shaking at 240 rpm. When needed 5 µg/ml tetracycline was used for selection, and 2 µg/ml in physiological growth experiments. The optical density of cell suspensions was measured using a spectrophotometer (Shimadzu) at 600 nm. Resting cells preparation and two-step fermentation were performed as described previously (16).

Methods and tools for bioinformatics analysis

The information about mannitol genes in different bacteria was obtained from NCBI with accession number: NC_009004 (*L. lactis*, MG1363), NC_000964 (*Bacillus subtilis*, str. 168), U18943 (*Geobacillus stearothermophilus*, ATCC 7954), U53868 (*Clostridium acetobutylicum*, ATCC 824) and AE014133 (*Streptococcus mutans*, UA159). The bioinformatics analysis was performed using different webtools: BDGP (40) and SAPPHERE

362 <https://sapphire.biw.kuleuven.be/> for promoter prediction; De Novo DNA
363 (<https://www.denovodna.com>) for predicting ribosome binding site; Arnold
364 (<http://rssf.i2bc.paris-saclay.fr/toolbox/arnold>) for predicting transcriptional terminators.

365 **Molecular techniques**

366 Electrocompetent cells were prepared using the protocol of Holo & Nes (41), using GM17
367 medium containing either 0.5% (for strains AceCF & AceCR) or 2% glycine and 0.25 M
368 sucrose. The medium was filtered. Electroporation was performed as as previously described
369 by Holo and Nes using MicroPulser Electroporator (Bio-Rad) (41). Phusion DNA
370 Polymerase (Thermofisher Scientific) was used for PCR amplifications. Zippy plasmid
371 miniprep kit (Zymo Research) was used for extracting the plasmids from *L. lactis* after a pre-
372 treatment with 20mg/mL lysozyme (Fluka, 7,000U/mg) at 37°C for 2 hours. FastDigest
373 restriction enzymes Sall, SacI and BamHI were obtained from Thermofisher Scientific.

374 **Deletion of *mtlR* in strain Ace001M**

375 To delete *mtlR* in the strain Ace001M, pG⁺host8, a plasmid with a thermosensitive
376 replicon was used. Two synthetic DNA fragments, representing the upstream and
377 downstream regions of *mtlR*, and able to overlap were ordered from Integrated DNA
378 Technologies: a 985 bp fragment upstream of *mtlR* into which a SalI restriction site had been
379 introduced in the *mtlR* distal end, and a 993 bp fragment downstream of *mtlR*, with a BamHI
380 restriction site added in the *mtlR* distal region. After fusing the two fragments they were
381 inserted into pJET1.2 using CloneJET PCR Cloning Kit by blunt ligation as described in the
382 manual and transformed into *E. coli* DH10B. The insert was excised as a SalI/BamHI
383 fragment and introduced into the corresponding sites in pG⁺host8 using *E. coli* MC1000 as
384 host. The resulting plasmid, designated pKmtlR (pG⁺host8/*mtlR*_{up}-*mtlR*_{down}), was

subsequently used to delete *mtlR* in Ace001-M in the same manner as described previously (16). The Ace001-M derivative with deleted *mtlR* was designated AceR.

Construction of strains with deleted *ccpA*

The same approach used to delete *mtlR* was taken for deleting *ccpA*. In this case a 978 bp fragment upstream of *ccpA* equipped with a SalI restriction site and a 984 bp fragment downstream of *ccpA* with a BamHI site were used. The subsequent cloning steps were identical to the ones mentioned above. For obtaining the strains with deleted *ccpA*, we relied on the slow growth phenotype of *ccpA* mutants (36), and for this reasons small colonies were selected and investigated. Deletion of *ccpA* was done for strains AceF, Ace001 and AceR, and the derivatives were designated AceCF, Ace001C and AceCR respectively.

Overexpression of *mtlD* and *mtlR*

An expression cassette consisting of a promoter, a ribosome binding sequence and *mtlD* was generated using a nested PCR approach. First, a fragment containing the entire *mtlD* gene was amplified using primers 5'-ATTTCGGGAGACACATCTGGC-3' and 5'-GTGTTCTCGCTTCGCATCAG-3'. For the second PCR a forward primer containing the 20% TPI promoter sequence (42) and a ribosome binding sequence was used 5'-CACGCGTCGACATAGATTAGTTTATTCTTGACACTACAAGCTAAATGTGGTATAATCCCATAGATATACTAGGTAAGTAATAAAATATTCGGAGGAATTTTGAAATGAAAAAAGCAGTACATTTTGGTGCAGGAAAT -3' together with the following reverse primer 5'-ATCCGGAGCTCATATTCTCTGTCTACTTGCTGTCAT -3'. The same approach was used for generating an expression cassette for *mtlR*: first, a fragment containing the entire *mtlR* gene was amplified using primers 5'-AAGAAGAATTCACGGCAATA -3' and 5'-ATCGCTGAAACATAATTTGAG -3'. For the second PCR the following primers were used: 5'-

409 CACGCGTCGACATAGATTAGTTTATTCTTGACACTACAAGCTAAATGTGGTATAA
410 TCCCATAGATATACTAGGTAAGTAATAAAATATTCGGAGGAATTTTGAAATGTTT
411 TTAACAAGTCGTGAG -3' & 5'-
412 ATCCGCTCGAGCTAATCACCATACTGTTTAACAGC -3'. All primers are ordered from
413 IDT company. The amplified fragments were digested with restriction enzymes and inserted
414 into pTD6 and the constructs introduced into Ace001 and verified by Sanger sequencing
415 (Macrogen).

416 **Quantifying mannitol production by High-Performance Liquid Chromatography** 417 **(HPLC)**

418 For quantifying glucose and mannitol, we used an HPLC equipped with a BioRad Aminex
419 HPX-87H column (BioRad) and a RI-101 detector (Shodex). The mobile phase consisted of 5
420 mM H₂SO₄ at a flow rate of 0.5 ml/min. The column oven temperature was set to 60°C. The
421 samples for HPLC analysis were filtered using 0.22 µm filters (Labsolute) and loaded to
422 HPLC immediately after sampling at appropriate cells density.

423 **M1Pase activity assay**

424 For detecting M1Pase activity, exponentially growing culture samples were quenched in
425 wet ice. The cells were washed with ice-cold MES buffer (50 mM MES, 10 mM MgCl₂,
426 pH=7.0), and resuspended to a density of OD₆₀₀=50.0. For permeabilizing the cells, 12.5 µl
427 0.1% SDS and 25 µl CHCl₃ was added. After vigorous vortex for 10 s and equilibrium at
428 30°C for 5 min, an appropriate volume of M1P (Sigma Aldrich) was added to a final
429 concentration of 5 mM to initiate the reaction, meanwhile, for negative controls, the same
430 volume of MES buffer was added. After 1 h incubation at 30°C, cells were centrifuged and
431 the supernatant was filtered with 0.22 µm filters (Labsolute). Finally, the filtered supernatant

was immediately analyzed by HPLC to determine formation of mannitol. MIPase activity was described as the amount of mannitol (mM) formed per hour.

β -glucuronidase assays

Strains were cultured in M17 broth supplemented with 1% of the indicated sugar and 2 μ g/ml tetracycline. Cells were harvested after reaching $OD_{600}=1.0$ or in the stationary phase ($OD_{600}=4.5$, for strains containing plasmid was $OD_{600}=4.0$), and quenched on wet ice followed by centrifugation at 4°C. Harvested cells were washed in ice cold PBS and resuspended in ice-cold Z buffer (0.06 M Na_2HPO_4 , 0.04 M NaH_2PO_4 , 0.01 M KCl, 0.001 M $MgSO_4$, 0.05M β -mercaptoethanol, pH=7). Cells were appropriately diluted and the cell density was measured at OD_{600} . For permeabilization, 12.5 μ l 0.1% SDS and 25 μ l $CHCl_3$ were added, and the suspension vortexed vigorously. After equilibrium at 30°C for 5 min, 100 μ l of 4 mg/ml PNPG was added, and the time needed for developing a yellow color recorded. To stop the reaction 800 μ l Na_2CO_3 was added. Absorbance at 420 nm and 550 nm were measured. Promoter strength was calculated as $1000 \times (OD_{420} - 1.75 \times OD_{550}) / (\text{time} \times OD_{600} \times \text{ml sample})$ and describe as Miller units.

Two-step fermentation procedure for producing mannitol

For producing mannitol, a two-step fermentation setup was used. First, cells were cultivated in M17 supplemented with 1% glucose at 30°C, 240 rpm until the cell density reached $OD_{600}=4.5$ (for strains containing plasmid was $OD_{600}=4.0$). Then cells were shifted to 20 rpm shaking while at the same time, glucose and arginine were added to a final concentration of 2% and 35 mM. Samples were taken at appropriate times and loaded to HPLC immediately, to determine glucose consumption and mannitol production. Mannitol yield was calculated by the mannitol conversion ratio from glucose during stationary period.

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585

586 **Tables and Figures**587 **Table 1. Strains and plasmids used in this work**

Strain and plasmid	Relevant genotype	Source or reference
Strains		
MG1363	Wild-type <i>L. lactis</i> subsp. <i>cremoris</i>	(19)
MG363-M	MG1363 adapted on mannitol	(16)
Ace001	MG1363 Δ^3ldh , Δpta , $\Delta adhE$,	(43)

	<i>ΔbutBA</i>	
Ace001-M	Ace001 adapted on mannitol	(16)
AceF	Ace001-M <i>ΔmtlF</i> (previously addressed AceM)	(16)
AceR	Ace001-M <i>ΔmtlR</i>	This work
Ace001C	Ace001 <i>ΔccpA</i>	This work
AceCF	Ace001-M <i>ΔmtlF</i> , <i>ΔccpA</i>	This work
AceCR	Ace001-M <i>ΔmtlR</i> , <i>ΔccpA</i>	This work
Ace001-mtlR	<i>mtlR</i> overexpressed Ace001, 20%TPI promoter	This work
Ace001-mtlD	<i>mtlD</i> overexpressed Ace001, 20%TPI promoter	This work
MC1000	<i>E.coli</i> cloning host	(44)
DH10B	high-efficiency competent <i>E. coli</i> host	
Plasmids		
pTD6	pTD6 A derivative of pAK80 containing a <i>gusA</i> reporter gene	(45)
pPmtlA^{WT}	pTD6 with wild type <i>mtlA</i> promoter preceding <i>gusA</i> reporter gene	This work
pPmtlA^{mut}	pTD6 with C-39T mutant <i>mtlA</i> promoter preceding <i>gusA</i> reporter gene	This work
pPmtlA^{mutII}	pTD6 with G-46T mutant <i>mtlA</i> promoter preceding <i>gusA</i>	This work

	reporter gene	
pPmtlD	pTD6 with <i>mtlD</i> promoter	This work
	preceding <i>gusA</i> reporter gene	
pPmtlAD	pTD6 with fused <i>mtlA</i> and <i>mtlD</i> promoter preceding <i>gusA</i>	
	reporter gene	
pJET1.2	high-efficiency cloning	ThermoFisher Scientific
	plasmid for blunt-ended ligation	
pG⁺host8	<i>E. coli/L. lactis</i> shuttle vector, Tet ^R , thermosensitive replicon	(46)
pKmtlR	Used for knocking out <i>mtlR</i>	This work
pKCcpA	Used for knocking out <i>ccpA</i>	This work

588

589 **Table 2. Effect of mutations in the mannitol operon promoter on expression level**

Sugars	Host	P _{mtlA,WT}	P _{mtlA,C-39T}	P _{mtlA,G-46T}
Glucose	MG1363	0.01±0.01	15.6±0.9	0.43±0.01
Glucose	MG1363M	0.06±0.01	52.16±2.0	1.17±0.11
Mannitol	MG1363M	449±5	4415±56	4183±34

590 **Notes:** P_{mtlA,WT}, wild type *mtlA* promoter; P_{mtlA,C-39T}, *mtlA* promoter containing the C-39T
591 mutation; P_{mtlA,G-46T}, *mtlA* promoter containing the G-46T mutation. Data were collected at
592 exponential phase and the promoter activities were shown as Miller Units. All treatments
593 were duplicated and deviations was indicated.

594

595 **Table 3. Overview of *mtlA* and *mtlD* promoter strength in different regulatory**
 596 **conditions**

Sugar	Strains	Promoters	Miller units	
			Exponential	Stationary
Glucose	Ace001	<i>mtlD</i>	0.00±0.00	0.00±0.00
Glucose	AceR	<i>mtlD</i>	0.01±0.01	0.01±0.00
Glucose	Ace001M	<i>mtlD</i>	2.38±0.10	122±5
Mannitol	Ace001M	<i>mtlD</i>	871±46	1699±137
Glucose	Ace001	<i>mtlA</i> ^{WT}	0.03±0.00	0.05±0.01
Glucose	Ace001	<i>mtlA</i> ^{Mut}	15.6±1.1	40.0±0.4
Glucose	AceR	<i>mtlA</i> ^{Mut}	14.5±0.9	41±2
Glucose	Ace001M	<i>mtlA</i> ^{Mut}	44.3±2.2	2273±164
Mannitol	Ace001M	<i>mtlA</i> ^{Mut}	4078±230	7539±687
Glucose	Ace001C	<i>mtlA</i> ^{WT}	588±15.7	1880±86
Glucose	Ace001C	<i>mtlA</i> ^{Mut}	523±13.1	1842±48
Glucose	Ace001C	<i>mtlD</i>	405±6.6	894±12
Glucose	AceCF	<i>mtlA</i> ^{WT}	3787±232	6055±351
Glucose	AceCF	<i>mtlA</i> ^{Mut}	3413±70	6375±308
Glucose	AceCF	<i>mtlD</i>	1955±65	3664±88
Glucose	AceCR	<i>mtlA</i> ^{Mut}	39.0±3.5	52.2±1.5
Glucose	AceCR	<i>mtlD</i>	0.00±0.00	0.06±0.01

597

598

599 **Table 4. Effect of overexpressing/deleting *mtlR* in different *L. lactis* backgrounds**

Strain	Genotype	Growth on mannitol	Mannitol production
Ace001	MG1363 Δldh , $\Delta adhE$, Δpta , $\Delta butBA$	-	-
Ace001M	Ace001 adapted on mannitol	+	+
AceF	Ace001M $\Delta mtlF$	-	+
Ace001-mtlR [†]	<i>mtlR</i> overexpressed Ace001	-	+
AceR	Ace001M $\Delta mtlR$	-	-
Ace001C	Ace001 $\Delta ccpA$	+	+
AceCF	AceF $\Delta ccpA$	-	+
AceCR	AceR $\Delta ccpA$	-	-

600 **Notes:** For assessing if growth is possible we use the following criterium: Cell density
 601 attained in M17 containing mannitol should be above OD₆₀₀ > 0.9 after 24 hours of
 602 incubation (M17 without added sugar/mannitol supports growth to approximately OD₆₀₀
 603 around 0.8.). For assessing mannitol production, if more than 0.001 g/L mannitol is formed,
 604 we interpret this as an ability to produce mannitol from glucose.

605

606 **Table 5. The effects of deleting CcpA on mannitol production**

Condition	Strains	Mannitol produced (g/L)	Yield (%)	Biomass (OD ₆₀₀)
TSF	AceF	6.08	0.6	-
TSF	AceCF	5.92	0.59	-
TSF	AceR-mtlD [†]	10.1	0.55	-
AG	AceF	2.18	0.41	1.38
AG	AceCF	0.62	0.34	0.65
AG	AceR-mtlD [†]	0.71	0.34	0.47

607 TSF, two-step fermentation; AG, Anaerobic growth, 35 mM arginine were added; the
608 biomass and yield were measured and calculated after 7 days incubation.

609 **Figure 1. Protein structure of the characterized mtlR from *B. subtilis*, *G.***
610 ***stearothermophilus* and *L. casei*.** The phosphorylation sites were indicated with the amino
611 acid and their position.
612

624

625 **Figure 3. Testing the effect of *mtlR* on expression of mannitol genes.** (a) The effect on
626 *mtlD* promoter expression. (b) The effect on *mtlA* promoter expression, WT, wild type *mtlA*
627 promoter; Mut, *mtlA* promoter with C-39T mutation. The carbon source is indicated with G
628 (1% glucose) or M (1% mannitol). The host is indicated with Ace001, AceR, Ace001M. Cells
629 harvested in exponential phase are indicated with red color, while cells harvested in
630 stationary phase are indicated with blue color. Experiments were carried out in duplicates,
631 and standard deviations are indicated with error bars.

632

633 **Figure 4. The activity of *mtlA* and *mtlD* promoters in Ace001C.** WT, wild type *mtlA*
634 promoter; Mut, *mtlA* promoter with C-39T mutation. Cells are grown in 1% glucose and
635 harvested at exponential phase (red color), stationary phase (blue color). Experiments were
636 carried out in duplicates and standard deviations indicated by error bars.

637

638 **Figure 5. The activity of *mtlA* and *mtlD* promoters in AceCF and AceCR.** WT, wild type
639 *mtlA* promoter; Mut, *mtlA* promoter with C-39T mutation. Cells were growing in 1% glucose
640 and harvested at exponential phase (red color), stationary phase (blue color). Experiments
641 were carried out in duplicates and standard deviations are indicated with error bars.

642

643 **Figure 6. Scheme of mannitol production in *L. lactis*.** PEP-PTS, phosphoenolpyruvate
644 (PEP)-dependent glucose phosphotransferase system (PTS); F6P, fructose-6-phosphate;
645 M1PDH, mannitol-1-phosphate 5-dehydrogenase; M1P, Mannitol-1-phosphate.

Figure 7. The proposed phosphoryl group transfer route in *L. lactis* MtlR.