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ManLMN is a glucose transporter and central metabolic regulator in *Streptococcus pneumoniae*

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Summary

Streptococcus pneumoniae is a common colonizer of the human nasopharynx and a leading cause of bacterial pneumonia and otitis media, among other invasive diseases. During both colonization and invasive disease *S. pneumoniae* ferments host-derived carbohydrates as its primary means of generating energy. This pathogen is adept at transporting and metabolizing a wide variety of carbohydrates. We found the highly conserved PTS ManLMN contributes to growth on glucose and is also essential for growth on a variety of nonpreferred carbohydrates, suggesting it is a multisubstrate transporter. Exploration of this phenotype revealed ManLMN is required for inducing expression of downstream metabolic genes in response to carbohydrate stimuli. We further demonstrate that ManLMN's role as a constitutively expressed transporter is likely unique and integral to pneumococcus's strategy of carbon catabolite repression (CCR). Using a selection for suppressors, we explored how ManLMN is integrated into the CCR regulatory framework in *S. pneumoniae*. We identified two hypothetical small proteins and the virulence regulator SmrC as potential mediators of CCR in connection with ManLMN. Characterization of these two hypothetical proteins revealed they influence transcriptional regulation of carbohydrate transporters. We propose a model unifying these observations in which ManLMN is a versatile surveyor of available carbohydrates in *S. pneumoniae*.

Introduction

ManLMN, encoded by the *manLMN* operon is a conserved phosphoenolpyruvate-dependent phosphotransferase system (PTS) that plays a predominant role in carbohydrate metabolism and metabolic regulation in many Gram positive species, in particular low G + C species (Abranches *et al.*, 2003). ManLMN is typically a major glucose transporter that is also capable of transporting a varying number of other carbohydrate substrates including mannose, fructose, galactose and *N*-acetyl glucosamine (GlcNAc) (Vadeboncoeur *et al.*, 2000; Jahreis *et al.*, 2008; Bidossi *et al.*, 2012). Unlike PTS systems with limited substrate specificity, *manLMN* is frequently constitutively expressed, meaning its regulation is not responsive to the presence or absence of any particular carbohydrate substrate (Jahreis *et al.*,

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Supporting information

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2008). Similar to other PTS's of the mannose class, *manLMN* encodes a fused EIIAB domain protein (ManL) responsible for phosphorylation of the incoming carbohydrate, and EIIC and EIID proteins (ManMN, respectively) constituting the permease through which the carbohydrate molecule enters the cell (Zúñiga *et al.*, 2005; Jahreis *et al.*, 2008). This complex and ManL in particular have been implicated as a mediator of CCR not only for its role in glucose transport but also as an independent regulator. Much like $EIIBA_{glu}$ from *Escherichia coli*, ManL was previously named Enzyme III because its pleotropic effects on carbohydrate metabolism set it apart from other EIIA/EIIB proteins according to what was understood of the scope of these proteins' functions (Vadeboncoeur and Gauthier, 1987). Inactivation of *manL* in *Streptococcus salivarius* positively and negatively affected the expression and activity of other PTS systems, caused general proteome changes and eliminated diauxic growth in some conditions (Vadeboncoeur *et al.*, 1983; Vadeboncoeur and Pelletier, 1997). Inactivation of *manL* in *Streptococcus mutans* caused general proteome changes, and affected transcription of over 100 genes including many carbohydrate transporters (Abranches *et al.*, 2003, 2006; Moye *et al.*, 2014b). It was also speculated that ManL may directly influence a transcriptional regulator's activity in *S. mutans* (Zeng and Burne, 2008).

Despite being conserved in all sequenced *Streptococcus pneumoniae* strains, relatively little is known about the function of ManLMN (encoded by SP_0282-4) in this organism. As the fitness of this human pathogen is dependent on fermentation of host derived carbohydrates, it is important to understand how carbon sources are acquired. Given the importance of ManLMN in related *Streptococci* we hypothesized ManLMN would play a central role in carbohydrate metabolism in *S. pneumoniae*. Studies in D39 (serotype 2) showed *manLMN* is repressed by both the global CCR regulator, catabolite protein A (CcpA) and CiaR, the response regulator of the conserved two-component system CiaRH implicated in competence, autolysis and β-lactam resistance (Mascher *et al.*, 2003; Halfmann *et al.*, 2007, 2011; Marx *et al.*, 2010; Carvalho *et al.*, 2011). In D39, inactivation of *manM* encoding the EIIC component, resulted in a mild growth defect in glucose, and more severely reduced growth in GlcNAc, mannose and galactose (Bidossi *et al.*, 2012). In contrast to D39, we found ManLMN in TIGR4 (serotype 4) to be essential for growth on five nonpreferred carbohydrates, and required to induce expression of downstream metabolic genes. We show ManLMN contributes to growth on glucose as a high affinity glucose transporter, and is required for inducer exclusion of lactose. Using a selection for suppressors that relieve the dependence on ManLMN, we identified two hypothetical proteins as putative factors mediating ManLMN-dependent regulation of nonpreferred carbohydrate metabolism gene expression.

Results

ManLMN is required for growth on a variety of nonpreferred carbohydrates

Deletion of *manLMN* in the TIGR4 strain of *S. pneumoniae* abrogated growth on the nonpreferred carbohydrates galactose, lactose, glucosamine (GlcN), GlcNAc and fructose. Growth was diminished on the nonpreferred carbohydrates mannose and raffinose, while growth on the preferred carbohydrates glucose, sucrose and on the glucose disaccharides

trehalose and maltose was unaffected (Fig. 1A–H, Supporting information Fig. S1A–C). Based on homology to transporters in related species, *S. pneumoniae* has at least one additional putative transporter for each of these nonpreferred carbohydrates. In additional to ManLMN, there are three predicted galactose transporters (SP_0061-3; PTS, SP_0090-2; ABC and SP_0645-7; PTS), one predicted GlcN transporter (SP_1684; incomplete PTS), one predicted GlcNAc transporter (SP_0061-3; PTS) and one predicted fructose transporter (SP_0877; PTS), encoded in the TIGR4 genome. There is only one predicted lactose transporter (SP_1185-6; PTS) (Bidossi *et al.*, 2012). We chose to analyze the contribution of three such PTS transporters to growth in these conditions. Although there are other transporters predicted to recognize these substrates, for clarity we refer to the transporters we investigated as the GlcNAc/gal PTS (SP_0061-3), the lac PTS (SP_1185-6) and the fru PTS (SP_0877). In contrast to the *manLMN* deletion strain, inactivation of the GlcNAc/gal, lac or fru PTS had no effect on growth in these conditions (Fig. 1B, C and F respectively). One explanation of these results is that ManLMN is required for growth in these conditions because it supplies the initial intracellular carbohydrate-inducers needed to induce expression of these other transporters and downstream metabolic enzymes.

The manLMN operon is constitutively expressed

Transcriptional analysis of *manL* as a representative of the *manLMN* operon revealed this operon is constitutively expressed in a variety of preferred and nonpreferred carbohydrate conditions (Fig. 2A). This included the glucose-replete rich broth THY. Constitutive expression is in contrast to the typical regulation pattern of substrate-specific transporters, which are substantially upregulated in response to growth in their cognate carbohydrate (Fig. 2A) (Deutscher *et al.*, 2006). Constitutive expression is consistent with our finding that ManLMN is essential for growth in these conditions.

In *S. pneumoniae* D39, *manLMN* is repressed by two global regulators; the conserved two-component system response regulation CiaR and the CCR regulator CcpA (Mascher *et al.*, 2003; Halfmann *et al.*, 2007, 2011; Marx *et al.*, 2010; Carvalho *et al.*, 2011). In order to determine if *manLMN* is similarly regulated in TIGR4, we analyzed transcription of *manL* in the Δ*ciaR* and Δ*ccpA* strains. Deletion of *ciaR* but not *ccpA* resulted in de-repression of *manL* in all growth media tested (Fig. 2B and Supporting information Fig. S2B and C). This difference with D39 could be due to the different growth medium used.

ManLMN is required for induction of nonpreferred carbohydrate metabolic genes

Our observation that unlike other carbohydrate transporters, *manLMN* is constitutively expressed and is required for growth on many carbohydrates led us to hypothesize that ManLMN is providing the carbohydrate-inducers needed to induce expression of substrate-specific transporters and downstream metabolic enzymes. According to this hypothesis, induction of galactose, lactose, GlcN, GlcNAc and fructose metabolism genes should be reduced if not abolished in Δ*manLMN*. To test this we compared lactose-dependent induction of endogenous β-galactosidase (BgaA) activity in WT and Δ*manLMN*. *Streptococcus pneumoniae* encodes multiple β-galactosidases but only BgaA recognizes *o*-nitrophenyl-galactopyranoside (ONPG) as a substrate (Zäahner and Hakenbeck, 2000; Kaufman and Yother, 2007; Jeong *et al.*, 2009; Cheng *et al.*, 2012). Unlike WT, which

showed over 800-fold induction of BgaA activity two hours after introduction to lactose medium, Δ*manLMN* showed no induction (Fig. 3).

In order to determine if deleting *manLMN* similarly affects induction of other metabolism operons, we constructed transcriptional reporter strains in which expression of α-galactosidase (the reporter) is controlled by different carbohydrate metabolism operon promoters. As another test for loss of lactose/galactose-dependent induction, we used the tagatose-6-phosphate operon (*lacABCD*; SP_1190-3) promoter (P_{lacA}). This promoter has been shown previously to be upregulated in TIGR4 during growth in galactose and lactose (Fleming *et al.*, 2015). To test for loss of GlcN/GlcNAc-dependent induction, we used the glucosamine-6-phosphate deaminase (*nagB;* SP_1415) promoter (P_{nagB}). Although NagB is uncharacterized in *S. pneumoniae* in a variety of other species it enables energy generation from GlcN and GlcNAc, which induce its expression (White, 1968; Bertram *et al.*, 2011; Moye *et al.*, 2014a). And to test for loss of fructose-dependent induction we used the fructose operon (*fruRCA;* SP_0875-7) promoter (P_{fruR}). This operon closely resembles the well characterized fructose-inducible *fruRCA* operon in *Lactococcis lactis* (Barriere *et al.*, 2005), and the encoded fructose-type PTS transporter (FruA) was shown to contribute to growth of serotype 2 *S. pneumoniae* in fructose medium (Bidossi *et al.*, 2012). The genomic coordinates of the exact promoter regions used are listed in Supporting information Table S1. Using these reporter constructs we analyzed temporal induction of α-galactosidase reporter activity in response to carbohydrate inducers in the WT, Δ*manLMN* and the *manLMN* complemented *in trans* strain backgrounds. In the WT background, each of these reporter constructs showed α-galactosidase activity was responsive to the predicted cognate carbohydrate(s) (Fig. 4). By two hours the P_{lacA} reporter showed a 13-fold increase in α-galactosidase activity in response to galactose or lactose (Fig. 4A and B respectively). Deletion of *manLMN* completely abolished this induction. Deletion of *manLMN* had a similar effect on GlcN- and GlcNAc-dependent induction of α-galactosidase activity in the P_{nagB} reporter strain. While in the WT background this construct showed 56-fold increase in α-galactosidase activity in response to GlcN and over 100-fold induction in response to GlcNAc, the Δ*manLMN* strain maintained uninduced levels of α-galactosidase activity over the course of the experiment (Fig. 4C and D). These results corroborate our analysis of endogenous β-galactosidase activity and are consistent with ManLMN facilitating induction of downstream metabolism operons.

In the WT background, the P_{fruR} reporter construct showed higher basal α-galactosidase reporter activity without fructose, and at most fourfold induction of activity in response to fructose (Fig. 4E). This induction was reduced to less than twofold by deleting *manLMN*, but was not completely abolished. This result in in contrast to the other transcriptional reporter strains, and suggests ManLMN contributes to induction of the *fruRCA* operon in conjunction with another fructose transporter. One possibility is that the higher basal level of transcription of this operon allows the fru transporter encoded by *fruA* to contribute to further induction by transporting fructose. For all three reporter constructs, induction was largely or completely restored by complementing *manLMN* expression *in trans* (Fig. 4A–E).

Growth of Δ*manLMN* is partially rescued by deleting CcpA

Analysis of the α-galactosidase reporter strains demonstrated that ManLMN is required for induction of certain nonpreferred carbohydrate metabolism genes. Because the β-galactosidase we monitored activity of (BgaA) is co-transcribed with a predicted galactose PTS system (Zäahner and Hakenbeck, 2000; Kaufman and Yother, 2007), our analysis of β-galactosidase activity further suggested that ManLMN may be required for induction of at least some substrate-specific carbohydrate transporters. Accordingly, we hypothesized that elevated expression of one of these carbohydrate-specific transporters should rescue growth and induction in Δ*manLMN* in the corresponding carbohydrate condition. CcpA, the master transcriptional CCR regulator, is known to partially repress transcription of multiple carbohydrate transport systems (Warner and Lolkema, 2003; Lorca *et al.*, 2005; Deutscher *et al.*, 2006; Carvalho *et al.*, 2011; Fleming *et al.*, 2015), and thus we hypothesize deletion of *ccpA* should partially rescue growth of Δ*manLMN*. To test this hypothesis, we determined the growth profile of the Δ*manLMN* Δ*ccpA* double mutant strain in the five conditions that require ManLMN (galactose, lactose, GlcN, GlcNAc and fructose). Deletion of *ccpA* partially rescued growth of Δ*manLMN* in galactose, lactose and GlcNAc (Fig. 5A–C respectively). Due to CcpA's known role in transcriptional repression of carbohydrate transporters, these results suggest partially de-repressed transcription of galactose, lactose and GlcNAc transporters circumvents the requirement for ManLMN for growth in these conditions. The poor growth of the Δ*manLMN* Δ*ccpA* double mutant strain in these conditions is likely due to the global mis-regulation caused by *ccpA* inactivation (Carvalho *et al.*, 2011). Growth was not rescued in GlcN nor fructose (Fig. 5D and E respectively), which is inconsistent with our hypothesis. It is possible CcpA does not repress the GlcN and fructose transporter(s). As discussed, TIGR4 encodes at least one predicted fructose PTS (SP_0877) which was shown to contribute to growth on fructose in D39. Deletion of SP_1684 encoding a predicted EIIBC GlcN transporter reduced growth of D39 in GlcN medium, again suggesting other transporters exist for these carbohydrates (Bidossi *et al.*, 2012).

To more directly test our hypothesis that expression of predicted substrate-specific carbohydrate transporters can circumvent the requirement for ManLMN, we individually deleted three PTS systems in Δ*manLMN*, and expressed them *in trans* under the control of the *manLMN* operon promoter (P_{man}). Expression of the GlcNAc/gal PTS under the control of P_{man} partially restored growth of Δ*manLMN* in galactose and nearly fully restored growth in GlcNAc (Fig. 6A and B respectively). Constitutive expression of this PTS was not able to rescue growth in GlcN or any other condition tested (Fig. 6C and *data not shown*). Similarly, expression of the lac PTS system under the control of P_{man} partially restored growth of Δ*manLMN* in galactose and fully restored growth in lactose (Fig. 6D and E respectively), but did not affect growth in any other condition tested (*data not shown*). The two PTS systems we chose are known to be repressed by CcpA in TIGR4 (Fleming *et al.*, 2015), and deletion of neither transporter in the WT background affected growth in these conditions (Fig. 1B and C). These results corroborate our interpretation of the Δ*manLMN* Δ*ccpA* strain growth, and together, support our hypothesis that defective growth of Δ*manLMN* on nonpreferred carbohydrates is due to loss of ManLMN-dependent induction of specific transport and metabolic genes. In contrast, constitutive expression of the fru PTS under the

control of P_{man} was not sufficient to rescue growth of Δ*manLMN* in fructose (Supporting information Fig. S3). However, this PTS behaves differently from the GlcNAc/gal and lac PTSs in that it has a high basal level of expression (Fig. 4E) and despite having a predicted CcpA-binding site in its promoter region, Δ*manLMN* growth in fructose could not be rescued by inactivating *ccpA*. It is unknown why the high basal level of transcription of the fructose operon is unable to promote growth of Δ*manLMN* on fructose. It is possible that the encoded fructose-type PTS system (FruA) is not a fructose transporter in TIGR4.

Constitutive expression of the lac PTS in place of ManLMN abolishes CCR of lactose metabolism

Having established that *manLMN* required for induction of other metabolic genes, we next wanted to determine if ManLMN serves a unique role in CCR. To address this question we asked if the strain constitutively expressing the lac PTS, Δ*manLMN* Δ*lac PTS* P_{man}-*lac PTS*, is capable of lactose inducer exclusion. We monitored the activity of the endogenous extracellular β-galactosidase (BgaA) as a reporter for lactosedependent induction. The strains were grown in rich medium (baseline activity), washed and then introduced to glucose CDM (noninducing condition), lactose CDM (inducing condition), or glucose + lactose CDM (CCR-repressing condition), for 1 or 2 h. Over time, the WT strain showed induction of β-galactosidase activity only in the inducing condition (Fig. 7A). In contrast the strain constitutively expressing the lac PTS showed equivalent levels of induction of β-galactosidase activity in both the inducing condition and the CCR condition (Fig. 7B). This result suggests that this strain is unable to restrict transport through the lac PTS in the presence of a preferred carbohydrate. Conversely, the Δ*lac PTS* P_{man}-*lac PTS* strain which constitutively expresses the lac PTS but still has ManLMN, was able to inducer exclude lactose in the CCR-repressing condition (Supporting information Fig. S4A). These results suggest ManLMN is notable not only for its expression pattern and impact on nonpreferred carbohydrate gene expression, but also for contributing directly or indirectly to inducer exclusion.

ManLMN supports growth in glucose

Consistent with ManLMN playing a role in CCR, the Δ*manLMN* Δ*ccpA* strain had a pronounced growth defect on glucose compared to the *manLMN* and *ccpA* single deletion strains (Supporting information Fig. S5), suggesting CcpA and ManLMN are functionally related. This result could also indicate that ManLMN is a glucose transporter, despite Δ*manLMN* having no growth defect in glucose. CcpA in related species is known to activate transcription of glucose transporters (Warner and Lolkema, 2003; Lorca *et al.*, 2005; Deutscher *et al.*, 2006). Thus, the absence of *manLMN* and reduced expression of other glucose transporter(s) could have a compounded negative effect on growth of Δ*manLMN* Δ*ccpA* in glucose. We reasoned that although Δ*manLMN* has no growth defect at the high concentration of glucose we routinely use (0.5%), it may exhibit a difference in growth when glucose is limiting. To test this hypothesis we compared growth of WT and Δ*manLMN* in decreasing glucose concentration. The Δ*manLMN* strain showed altered growth dynamics fromWT in 0.25% glucose (Fig. 8A) and failed to grow in 0.03% and below, whileWT maintained growth in concentrations as low as 0.003% (Fig. 8B and C). This result suggests that ManLMN may function as a high affinity glucose transporter.

To more directly test if ManLMN is capable of transporting glucose, we compared glucose uptake by WT and Δ*manLMN* using a radio-labelled carbohydrate transport assay in a glucose-limiting condition (0.007% glucose). Following growth in glucose rich medium, WT transported glucose with an appreciable rate, whereas glucose transport was undetectable in Δ*manLMN* (Fig. 9). These results support the conclusion that ManLMN recognizes glucose as a substrate and has a relatively high affinity for glucose compared to the other glucose transporter(s) expressed under these conditions.

In other species, the EIIA and/or EIIB components of major glucose PTS transporters have been shown to have CCR-regulatory activities (Abranches *et al*., 2003; Aké *et al*., 2011). The *manL* gene encodes the dual domain EIIAB protein of the ManLMN transporter. To determine if ManL fulfills a function independently of the rest of this system, we tested if a Δ*manLMN* strain having *manL* complemented *in trans* was sufficient to rescue growth in nonpreferred carbohydrates and restore induction of the α-galactosidase reporter strains. In all cases, we found ManL was insufficient to restore these phenotypes (Supporting information Fig. S6 and *data not shown*).

ManLMN's high affinity for glucose may be crucial for CCR

Thus far we have demonstrated that ManLMN is required for growth and induction of some metabolic genes in a variety of nonpreferred carbohydrate replete conditions. We have also shown that ManLMN is required for transport and growth in glucose-limited but not glucose-replete conditions. By constitutively expressing the lac PTS with and without ManLMN present, we showed that ManLMN is also required for lactose inducer exclusion. One model which unifies these growth, induction, transport and CCR phenotypes is that ManLMN is a multisubstrate transporter in TIGR4 as it is in related species. We hypothesize that by having a higher affinity for glucose over nonpreferred carbohydrate substrates such as lactose, ManLMN may be able to selectively transport glucose, thereby contributing to CCR. As a rudimentary way of testing this hypothesis, we compared growth of WT to the lac PTS constitutively expressed strains (Δ*manLMN* Δ*lac PTS* P_{man}-*lac PTS*, and Δ*lac PTS* P_{man}-*lac PTS*) on decreasing concentrations of lactose. Strikingly, while WT showed a markedly decreased growth rate at 0.03% lactose and failed to grow at 0.003% lactose, the strains constitutively expressing the lac PTS were able to grow in all conditions tested except for 0% lactose (Fig. 10A–D and Supporting information Fig. S4B–E). These results suggest that constitutive expression of the lac PTS enables detection of low lactose concentrations that WT (which according to this model is relying on ManLMN) otherwise cannot detect. This result is in contrast to our finding that WT grows robustly in low glucose conditions, provided ManLMN is constitutively expressed. Although these results are consistent with a model in which ManLMN has a higher affinity for glucose than for lactose, ManLMN's ability to transport non-glucose substrates has yet to be shown in *S. pneumoniae*.

A selection for suppressors identifies factors that relieve dependence on ManLMN

We showed a Δ*manLMN* strain constitutively expressing the lac PTS cannot inhibit lactose transport (inducer exclude) in the presence of glucose (Fig. 7). This phenotype did not result merely from constitutive expression of the lac PTS, but rather from constitutive expression of this PTS in the absence of ManLMN, since the Δ*lac PTS* P_{man}-*lac PTS* strain which still

has ManLMN was able to inducer exclude lactose (Supporting information Fig. S4A). Accordingly, we hypothesize that glucose transport by ManLMN has a regulatory influence on the activity of non-glucose transporters. We chose to use a selection for suppressors to address this hypothesis. By identifying mutations that relieve the necessity of ManLMN for growth on nonpreferred carbohydrates, we anticipated we would uncover factors that regulate the activity and/or expression of non-glucose transporters.

For the selection, approximately 10^8 cells of glucose-grown Δ*manLMN* were plated on CDM agar plates with 0.5% final concentration of galactose, lactose, GlcN, GlcNAc or fructose. Suppressor colonies were picked 2–5 days after plating, colony purified and tested for their ability to grow in the selection condition. An average of six suppressors from each condition were whole genome sequenced to identify the location of mutation(s) contributing to the growth phenotype.

A complete list of the mutations identified is available in Supporting information Table S2. The vast majority of suppressor mutants sequenced had more than one polymorphism compared to its parental Δ*manLMN* strain. In order to predict which mutation(s) are responsible for rescuing growth of Δ*manLMN*, we compiled a list of all genes and operons that were mutated in at least two independent isolates. The location, frequency and predicted consequence of these mutations are listed in Table 1. The majority (~60%) of these reoccurring mutations are expected to activate the expression or activity of carbohydrate transporters including the GlcNAc/gal PTS and the lac PTS. The prevalence of these mutations and the ability of the corresponding isolates to grow on GlcNAc/galactose and galactose/lactose corroborates our previous results, and suggests our selection was technically sound.

Mutations in *smrC*, encoding the LysR-family transcriptional regulator, SmrC, were the second most common type of mutation (Table 1). SmrC is considered to be a critical virulence regulator that is highly active during infections in mice (Lau *et al.*, 2001; Mahdi *et al.*, 2013, 2014). Mutations in *smrC* arose from selection on multiple carbohydrates suggesting SmrC has a global regulatory influence on carbohydrate metabolism. We recovered suppressors with mutations mapping to both the DNA-binding and ligand-binding domains of SmrC. It is unclear if the mutations we uncovered cause a loss or gain of function, although the lack of nonsense or frame-shift mutations suggests that a complete loss of SmrC function does not answer the selection. Neither the DNA recognition sequence nor the ligand(s) recognized by SmrC's ligand binding domain are known.

Two hypothetical small proteins identified in suppressor screen as putative carbohydrate regulators

A small number of suppressor mutations are predicted to affect the expression or function of hypothetical proteins. From the GlcNAc condition, we recovered four suppressors with mutations mapping to SP_0451, encoding a 73 amino acid hypothetical protein (Table 1 and Supporting information Fig. S7A). The −35 signal of the predicted SP_0451 promoter was made nonconsensus by two separate mutations (Supporting information Fig. S7A). These mutations likely decrease transcription from this promoter. The missense mutation affecting the start codon is expected to abolish translation, and the internal nonsense mutation will

result in a truncated product (Supporting information Fig. S7A). The SP_0451 gene and putative amino acid sequence encode no conserved domains and no homologs in other species except for some closely related *Streptococci*. Similar to SP_0451, we uncovered a start codon mutation in SP_1473 encoding another hypothetical protein (Supporting information Table S2). The predicted product of SP_1473 is an 83 amino acid protein that is highly homologous to YnzC, encoded by the last gene of a three-gene operon present in many Gram positive species. In *B. subtilis* this operon is repressed by the SOS regulator, LexA (Kawai *et al.*, 2003). The function of YnzC in *B. subtilis* is not known, but YneA (encoded by first gene in the operon) is involved in preventing cell division during the SOS response (Kawai *et al.*, 2003). Neither *yneA* nor the second gene in this operon are conserved in *S. pneumoniae*. We chose to pursue characterization of the hypothetical proteins encoded by SP_0451 and SP_1473 in order to determine their roles in carbohydrate regulation.

All four suppressor isolates containing a SP_0451 mutation rescued growth to a similar extent in GlcNAc medium (Supporting information Fig. S7B). This result suggests the SP_0451 mutations are responsible for the rescue phenotype. Although these suppressors were isolated in GlcNAc medium, all four showed similar abilities to rescue growth in galactose and lactose as well (Supporting information Fig. S7C and D). As the mutations occurring in this locus are expected to negatively affect expression or function of the encoded small protein, we made marked deletions of SP_0451 in the WT and Δ*manLMN* backgrounds. The single deletion strain grew like WT in all conditions tested (Fig. 11A–C). As expected, deletion of SP_0451 rescued growth of Δ*manLMN* in GlcNAc, galactose and lactose to a similar extent as the SP_0451 suppressor mutations (Fig. 11A–C). This result suggests the protein encoded by SP_0451 represses galactose, lactose and GlcNAc metabolism.

Similar results were obtained for SP_1473. The start codon suppressor mutation (*galactose suppressor #1-1*) was able to partially restore growth in lactose and fructose, and very mildly restored growth in GlcNAc in addition to its selection condition, galactose (Supporting information Fig. S8A–E). Deletion of SP_1473 rescued growth of Δ*manLMN* in galactose and lactose media, but not in GlcNAc or fructose media (Fig. 12A and B, and data not shown). The inability to grow in GlcNAc or fructose was unexpected, but may be due to a spontaneous mutation acquired in the capsular biosynthesis gene *cps4E*. Mutations in Cps4E like the one we recovered (Gly125Asp) are known to result in an acapsular phenotype (Shainheit *et al.*, 2015). We have not explored the possible connection between ManLMN, SP_1473 and capsule metabolism further.

The ability of the Δ*manLMN* Δ*SP_0451* and Δ*manLMN* Δ*SP_1473* strains to grow in multiple nonpreferred carbohydrate conditions suggests SP_0451 and SP_1473 have pleiotropic effects on carbohydrate metabolism. Based on the prevalence of carbohydrate transporter mutations in our suppressor isolates, we hypothesized that the proteins encoded by SP_0451 and SP_1473 may function to diminish the expression and/or activity of non-glucose carbohydrate transporters. The effect could be transcriptional or posttranscriptional. To test the former possibility, we compared transcript levels of the GlcNAc/gal and lac PTS system genes in WT, Δ*SP_0451* and Δ*SP_1473* in the glucose-based rich medium, THY,

which is a condition in which non-glucose transporters are robustly repressed (Kaufman and Yother, 2007; Carvalho *et al.*, 2011; Fleming *et al.*, 2015). Both Δ*SP_0451* and Δ*SP_1473* showed significant de-repression of the GlcNAc/gal PTS operon (Fig. 13A). This result corroborates our previous analysis of the Δ*manLMN* Δ*SP_0451* strain, and suggests that deletion of SP_0451 rescues growth of Δ*manLMN* in GlcNAc and galactose by transcriptionally de-repressing the GlcNAc/gal PTS operon. Although Δ*SP_1473* also resulted in de-repression of this PTS operon, the magnitude was less than that seen in the Δ*SP_0451* strain, which may explain why the Δ*manLMN* Δ*SP_1473* strain cannot grow on GlcNAc (Supporting information Fig. S8).

Deletion of SP_0451 resulted in modest but significant de-repression (~twofold) of the lac PTS (Fig. 13B). Given that the lac PTS likely has a high affinity for lactose, it is plausible that twofold de-repression of this system is sufficient to jump-start growth in lactose medium. Deletion of SP_1473 did not affect transcription of the lac PTS (Fig. 13B). It is possible that SP_1473 represses additional PTS genes for these carbohydrates that were not examined. These results suggest that SP_0451 and SP_1473 function primarily as transcriptional repressors, although whether direct or indirect is not known.

Discussion

Numerous studies have established a strong link between carbohydrate metabolism and pneumococcal persistence and virulence (Hava and Camilli, 2002; Chapuy-Regaud *et al.*, 2003; Hava *et al.*, 2003; King *et al.*, 2004; Iyer *et al.*, 2005; Iyer and Camilli, 2007; Dalia *et al.*, 2010; Limoli *et al.*, 2011; Marion *et al.*, 2011; van Opijnen and Camilli, 2012; Singh *et al.*, 2014). In all stages of colonization and invasive disease, fermentation of host-derived carbohydrates likely represents the primary mode of energy generation for *S. pneumoniae* (Lamblin *et al.*, 2001; King *et al.*, 2006; Burnaugh *et al.*, 2008; Yesilkaya *et al.*, 2008, 2009; King, 2010; Buckwalter and King, 2012). All *S. pneumoniae* serotypes examined encode a great deal of machinery anticipated to aid in harvesting and metabolizing these carbohydrates (Tettelin *et al.*, 2001; Bidossi *et al.*, 2012). This includes a preponderance of predicted carbohydrate transporters (Tettelin *et al.*, 2001; Bidossi *et al.*, 2012). Despite encoding 29 known and predicted carbohydrate transporters, we found a single PTS, ManLMN, to be essential for growth of TIGR4 *S. pneumoniae* in five nonpreferred carbohydrates under the conditions tested. These observations support a model in which ManLMN acts as a constitutively expressed environmental carbohydrate surveyor, whose activity is required for subsequent induction of downstream metabolic genes and possibly other transporters. It is notable that some of these carbohydrates are among the most prevalent in host airway glycoconjugates (Lamblin *et al.*, 2001; King *et al.*, 2004, 2006; Monzon *et al.*, 2006; Burnaugh *et al.*, 2008; Buckwalter and King, 2012). This circumstance suggests that ManLMN plays a critical role in colonization and lung infection. Indeed, inactivation of *manL* was shown to be detrimental to nasopharyngeal colonization and lung invasive disease in mice (van Opijnen and Camilli, 2012). We demonstrate that induction of multiple operons involved in the metabolism of these carbohydrates is abolished in a Δ*manLMN* strain. We further show that this dependence on ManLMN for growth can be relieved for particular carbohydrates by constitutively expressing other, cognate PTS systems encoded in the genome. Together these results suggest that Δ*manLMN's* inability to

grow in these nonpreferred carbohydrate conditions is the result of failure to induce expression of required metabolic enzymes, potentially including other carbohydrate transporters.

We showed ManLMN was required for transport and growth in growth-limiting glucose concentrations, but was dispensable for growth in excess glucose. This result suggests ManLMN functions as a high affinity glucose transporter in *S. pneumonaie*. In contrast, a strain constitutively expressing the lac PTS in place of *manLMN* displayed a heightened ability to detect growth-limiting lactose concentrations compared to WT (in which *manLMN* is constitutively expressed). We found this strain constitutively expressing the lac PTS in the absence of ManLMN to be unable to inducer exclude lactose (evidenced by induction of β-galactosidase activity). Loss of CCR in this strain is likely not because of an inability to detect the glucose as a nonlimiting amount of glucose was used. Interestingly, constitutive expression of the lac PTS in the presence of ManLMN had minimal effect on lactose inducer exclusion, suggesting ManLMN is required for CCR of lactose metabolism. Adding to our model, we propose ManLMN serves both as a conduit for multiple carbohydrate-inducers, and a critical CCR-regulator. The relative affinity ManLMN has for each of its substrates could determine the hierarchy of most-preferred to least-preferred carbohydrate.

By selecting for suppressor mutations that relieve the dependence on ManLMN for growth on nonpreferred carbohydrates, we reasoned we could elucidate mechanisms through which ManLMN influences CCR. As expected, we recovered many suppressors with putatively activating mutations in predicted or previously characterized carbohydrate transporters or their regulators, including the GlcNAc/gal PTS, a β-glucoside PTS, the lac PTS, the fru PTS, the raffinose ABC transporter and a GntR-family regulator (SP_0058) of the GlcNAc/gal PTS operon (Fleming *et al.*, 2015; Leprohon *et al.*, 2015). The SP_0058-encoded repressor was previously shown to participate in CCR of this operon, suggesting our selection was suitable for identifying factors involved in CCR (Fleming *et al.*, 2015).

We isolated four suppressors on galactose medium with mutations in or near the lac PTS operon. One of these mutations mapped to the region between the predicted promoter and the first gene of gene of this operon, potentially increasing transcription by altering a regulatory site. Interestingly, the three remaining mutations occurred within the coding regions of the lac PTS components, $EIIA^{lac}$ and $EIICB^{lac}$. Two of these mutations are expected to result in aspartic acid substitutions of two different well conserved alanine residues in $EIIA^{lac}$ (Ala31Asp and Ala53Asp). We hypothesize these are gain of function mutations, however, they are not proximal to the active, phosphorylation, metal coordination or trimer stabilization sites in $EIIA^{lac}$. It is possible these mutations increase transport through the lac PTS by improving the interaction of $EIIA^{lac}$ with the PTS-active form of HPr (HPr-His~P), or increase stability of the $EIIA^{lac}$ protein. The final mutation in this operon is expected to cause a glycine substitution of a conserved valine residue in the EIIB domain of $EIICB^{lac}$ (Val466Gly). Val466 is proximal to the EIIB phosphorylation site suggesting this mutation may improve the phosphorylation dynamics between $EIICB^{lac}$ and $EIIA^{lac}$ and/or $EIICB^{lac}$ and its carbohydrate substrate. It is also possible that one or more of the sites mutated in $EIIA^{lac}$ and $EIICB^{lac}$ are critical for a currently uncharacterized method of direct

CCR-inhibition of this lac PTS. Overall, we hypothesize these mutations increase the activity or stability of the lac PTS.

Similar to the lac PTS, the multiple mutations we recovered in the fru PTS are anticipated to cause amino acid substitutions in all domains of the encoded multidomain fructose-type PTS, EIIABC^{fru}. Although we could not definitively show that this system transports fructose, the fact that suppressor isolates with fru PTS mutations can grow on fructose and the frequency with which these mutations arose in fructose medium suggest that the encoded transporter is involved in fructose transport. Unlike all the other PTS systems identified in this selection, all six of the fru PTS suppressor mutations mapped to the coding region and not to classic transcriptional regulatory regions. The nature of the EIIABC^{fru} mutations and the high basal activity in our *fruRCA* promoter α-galactosidase transcriptional strain favour the idea that this PTS requires a specific posttranslational modification or interaction to be active. This conclusion is further supported by our observation that constitutive expression of this transporter was not sufficient to rescue growth of Δ*manLMN* in fructose medium. Fructose PTS systems in a variety of species have been shown to generate fructose-1-phosphate during transport (Wen *et al.*, 2001; Barriere *et al.*, 2005). Another possible explanation is that the fru PTS may generate a different form of phosphorylated fructose (fructose-1-phosphate versus fructose-6-phosphate), that what is needed to restore growth to Δ*manLMN*.

Suppressor mutations identified in our selection also implicated two hypothetical small proteins in carbohydrate regulation. We showed deletion of the genes encoding these proteins partially rescued growth of Δ*manLMN* in multiple carbohydrate conditions. Transcriptional analysis suggested these small proteins repress expression of carbohydrate transporters, however, these results do not fully explain the growth phenotypes of these strains. It will be interesting to explore these proteins further to determine what other genes they regulate, and the mechanism of their regulation.

PTS transporters in other species have been shown to act as carbohydrate sensors. Perhaps the most well studied example is PTS^{MPo} in *Listeria monocytogenes*. PTS^{MPo} serves as a constitutively expressed glucose sensor, and is responsible for inducing expression of *manLMN*, encoding the major glucose transporter in this species (Aké *et al.*, 2011). In this system, EIIB^{MPo} has a regulatory influence over ManR, the transcriptional activator of the *manLMN* operon. Our growth, transcriptional induction, transport and CCR data suggest ManLMN may play a similar but more extensive role in *S. pneumoniae*. ManLMN's function as a multisubstrate transporter is widely conserved in other species. Inactivation of *manLMN* commonly results in upregulation of other carbohydrate transporters (Vadeboncoeur *et al.*, 1983; Vadeboncoeur and Pelletier, 1997; Abranches *et al.*, 2003, 2006; Moye *et al.*, 2014a,b). *Streptococcus pneumoniae* is set apart by its strict dependence on this transporter for growth in a multitude of carbohydrate conditions. It remains to be seen, however, if ManLMN functions as a multisubstrate transporter in *S. pneumoniae*. ManLMN undoubtedly has a profound effect on nonpreferred carbohydrate metabolism whether it be due to its transport and/or regulatory activities.

There is a precedence for centralized modes of carbohydrate transport regulation in *S. pneumoniae*. Many serotype strains encode a number of ABC carbohydrate permeases that apparently lack an ATP-binding cassette protein (Marion *et al.*, 2011; Bidossi *et al.*, 2012). It was shown that a single ATP-binding cassette protein, MsmK, was capable of powering transport though multiple ABC permeases (Marion *et al.*, 2011). ManLMN's effect on growth and regulation in nonpreferred carbohydrate conditions is reminiscent of MsmK's role in ABC transport. Both systems are amenable to instigating CCR of nonpreferred carbohydrate transporters. By sequestering MsmK and/or prioritizing its interaction with a particular ABC permease, nonpreferred carbohydrate transport could be restricted when needed. Similarly, we imagine ManLMN's high affinity for glucose and its effect on other carbohydrate transporters (such as the lac PTS) will enable prioritization of preferred carbohydrate transport.

It is noteworthy that MsmK was shown to be essential for transport of sialic acid; another prevalent carbohydrate on human airway glycoconjugates. Together ManLMN and MsmK may play central roles in regulating carbon source utilization by *S. pneumoniae* within its natural niche, the human nasopharynx. Both the diversity and low abundance of free carbohydrates in the nasopharynx likely favour multifunctioning carbohydrate transporters that are inherently cost-saving yet able to enact wide-sweeping metabolic regulation, such as CCR.

Experimental procedures

Bacterial strains and culture conditions

Unless otherwise noted, *S. pneumoniae* TIGR4 (serotype 4) and isogenic mutants (Table 2) were grown at 37°C and 5% carbon dioxide in Todd Hewitt broth with 0.5% yeast extract (THY; BD Biosciences), chemically defined medium with varying carbohydrates [CDM (Kloosterman *et al.*, 2006)] or on 5% sheep's blood agar plates (Northeast laboratory services). All liquid culture conditions were supplemented with 300 U/ml of catalase (Worthington Biochemicals). Antibiotics were used at the following concentrations for selection of mutants: chloramphenicol (4 μg ml^{-1}), spectinomycin (200 μg ml^{-1}), and erythromycin (0.1 μg ml^{-1}).

Generation of mutants

All strains used in this study are listed in Table 2. Marked deletion strains were created by transforming WT or other strains with a PCR product encoding the desired mutation. The *manLMN* unmarked deletion strain was created by natural transformation using the CHESHIRE cassette system, as described previously (Bricker and Camilli, 1999).

Complementation *in trans* of PTS deletions was achieved using the CHESHIRE cassette system. In all cases, the neutral locus SP_1773 was replaced with *manLMN, manL* only or the genes encoding one of the predicted substrate specific PTS systems (lactose; SP_1185-1186, GlcN/GlcNAc; SP_0061-0063 and or fructose; SP_0877) under the control of the *manLMN* promoter. Selection and curing of the CHESHIRE cassette were performed as described previously (Weng *et al.*, 2009; Fleming *et al.*, 2015).

The α-galactosidase transcriptional reporter strains were created by inserting the *lacABCD, nagB* or *fruRCA* promoter region directly upstream of the start of the *agaA* open reading frame. Each promoter construct consisted of the predicted −35, −10 and RBS elements from the operon of interest, any predicted regulator binding sites, an outward-reading chloramphenicol cassette with a bidirectional transcriptional terminator for selection of transformants, and regions of homology to the target locus.

Splicing by overlap extension (SOE) PCR was used to generate the DNA constructs for these purposes, as described previously (Horton *et al.*, 1990). All primers used for SOE PCRS are listed in Supporting information Table S3. All mutations generated in this study were confirmed by Sanger sequencing by Eton Bioscience (http://www.etonbio.com).

Growth analysis

Mid-exponential THY cultures of each strain were washed once and back diluted to an optical density at 600 nm (OD_{600}) of 0.01 in CDM with 0.5% of either glucose, galactose, lactose, GlcN, GlcNAc, fructose, mannose, raffinose, sucrose, maltose or trehalose, with catalase (300 U ml^{-1}). For WT and the *lac PTS* complemented *in trans* strains, growth was also compared in a series of lactose concentrations ranging from 0.5% to 0%. OD_{600} was monitored in 96-well plates using a BioTek Synergy HT plate reader (BioTek Instruments), over the course of 15 h of incubation at 37°C and ambient CO_2 levels without aeration. At least six biological replicates from at least two different days were assayed for each strain.

Expression analysis

Transcription of *manL* as a representative of the *manLMN* operon during mid-exponential growth was assessed by quantitative reverse transcriptase PCR (qRT-PCR). WT, Δ*ccpA* and Δ*ciaR* cells were grown to mid-exponential phase in THY. WT cells were additionally grown in CDM with one of the following carbohydrates; 0.5% glucose, galactose, lactose, GlcN, GlcNAc, fructose, mannose or raffinose. Two milliliter samples were stored at −80°C in RNAprotect cell reagent (Qiagen) according to the manufacturer's instructions, for a minimum of 12 h before isolating total RNA by Trizol-chloroform extraction. RNA was further purified using the RNAeasy mini kit (Qiagen), supplemented with treatment with the TURBO DNA-*free* kit (Ambion, Life Technologies). No more than 1 μg of the purified RNA was used to template a single cycle reverse transcriptase reaction with the iScript cDNA Synthesis Kit (Bio-Rad). Reactions lacking reverse transcriptase were included for each sample to assess for residual genomic DNA contamination. The resulting cDNA was prepared for qPCR reactions in IQ SYBR green Supermix (Bio-Rad) with the primer sets listed in Supporting information Table S3. The MxP3005P real-time PCR system with MxPro qPCR software (Stratagene) was used to measure cycle threshold (Ct) values, which were subsequently corrected for primer efficiency differences before normalizing to the housekeeping gene, *rplI*. No less than five biological replicates from at least two separate days were tested for each carbohydrate condition. Transcription of SP_0061 and SP_1185 as representatives of the GlcNAc/gal and lac PTS systems respectively was determined in WT, ΔSP_0451, ΔSP_1473 during mid-exponential growth in THY as described above.

β-galactosidase assay

β-galactosidase assays were performed as previously described (Iyer *et al.*, 2005). Briefly, midexponential cultures grown in 0.5% galactose CDM conditions were collected, washed, permeabilized and assayed for β-galactosidase activity by monitoring the cleavage of ONPG at absorbance 420 nm. Data reported represent the average of six biological replicates tested on at least two separate days. For analysis of temporal induction of β-galactosidase activity, mid-exponential phase cultures grown in THY were washed once and resuspended in CDM supplemented with 0.5% glucose, 0.5% lactose or both. Samples were collected and analyzed after 1 and 2 h of incubation at 37°C.

α-galactosidase assay of transcriptional fusion strains

Mid-exponential phase THY cultures were, washed once and resuspended in CDM with no added carbohydrate or CDM supplemented with the following; 0.5% galactose or 0.5% lactose for the P_{lacA}, 0.5% GlcN or 0.5% GlcNAc for the P_{nagB}, or 0.5% fructose for the P_{fruR} transcriptional reporter strains. Following resuspension in CDM, samples were collected by centrifugation after 30, 60 and 120 min of incubation at 37°C, and resuspended in half the original volume of 100 mM sodium phosphate buffer (pH 7.5). The OD_{600} of each cell suspension was recorded, and the remaining volume of cells were permeabilized by incubating for 10 min at 37°C with Triton X-100 at a final concentration of 0.1% (w/v). The α-galactosidase activity in 10 μl of each lysate was assayed by monitoring absorbance at 405 nm as a measure of hydrolysis of *p*-nitrophenol α-D-galactopyranoside as described previously (Rosenow *et al.*, 1999). Samples were taken from the original THY cultures to determine basal activity of each strain.

Radio-labelled carbohydrate uptake assays

Glucose incorporation was assayed generally as described previously but with the following modifications (Fleming *et al.*, 2015). WT and Δ*manLMN* were grown in THY to mid-exponential phase, washed twice and resuspended in CDM containing no added carbohydrate. Cell suspensions were kept on ice until the start of the experiment. The cells were added to a chilled flask containing an equivalent volume of 800 μM unlabelled glucose CDM with 1 μCi of D-glucose [C6-H^3] (American Radiochemicals). In a final volume of 6.5 ml the final OD_{600} was 0.2 and the final unlabelled carbohydrate concentration was 400 μM (0.007% w/v). A time 0 sample was collected before the flask was put into a 37°C water bath to account for nonspecific association of radioactivity to the cells. Additional samples were collected at 1, 2.5, 5, 7.5,10, 12.5 and 15 min. Each sample was applied to a 0.45 μm nitrocellulose filter assembled on a vacuum apparatus, and washed twice with 1 ml of PBS (EMD Millipore). After drying, filters were mixed with 10 ml of Ecoscint H scintillation fluid (National Diagnostics), before measuring disintegrations per minute (DPMs) on a Beckman LS 6500 Scintillation System (Beckman). DPMs were converted to nmols of total carbohydrate incorporated per 10^9 cells using a DPM to μCi standard curve generated for D-glucose [C6-H^3] and normalizing to the number of cells based on dilution plating of cell solutions. The graphed data represent the average value of five biological replicates tested on multiple days, with error bars representing the standard error of the mean.

Selection of ΔmanLMN suppressor mutants

Approximately 10^8 cells of CDM-glucose grown Δ*manLMN* (both *manLMN::*CHESHIRE and *manLMN::aad9* [Spectinomycin-resistance gene]) were plated on a CDM plate with 0.5% final concentration of one of the following carbohydrates; galactose, lactose, GlcN, GlcNAc or fructose. Cells were plated directly or following one wash in CDM with no added carbohydrate. Each plating scheme was performed in biological duplicate such that each carbohydrate condition comprised a total of four plates per strain. The plates were incubated at 37°C in 5% CO_2 and suppressor colonies were picked after 2–5 days after plating. Colonies were picked and single colony purified on either the identical CDM carbohydrate plate condition or blood agar plates. The ability of each suppressor mutant to grow in the condition it was isolated from was confirmed by either passaging on CDM plates or liquid broth with the corresponding carbohydrate. Illumina DNA sequencing libraries were prepared for at least six suppressor isolates from each carbohydrate selection condition, using the Nextera XT DNA library prep kit (Illumina) according to the manufacturer's instructions. Whole genome sequencing was performed by the Tufts University Core Facility (http://tucf-genomics.tufts.edu/) using an Illumina HiSeq 2500. Sequence reads from each isolate were mapped to the reference TIGR4 genome (accessension number NC_003028.3), and mutations were identified using CLC Genomic Workbench software (Version 6.8 or 8; CLC Bio, Denmark)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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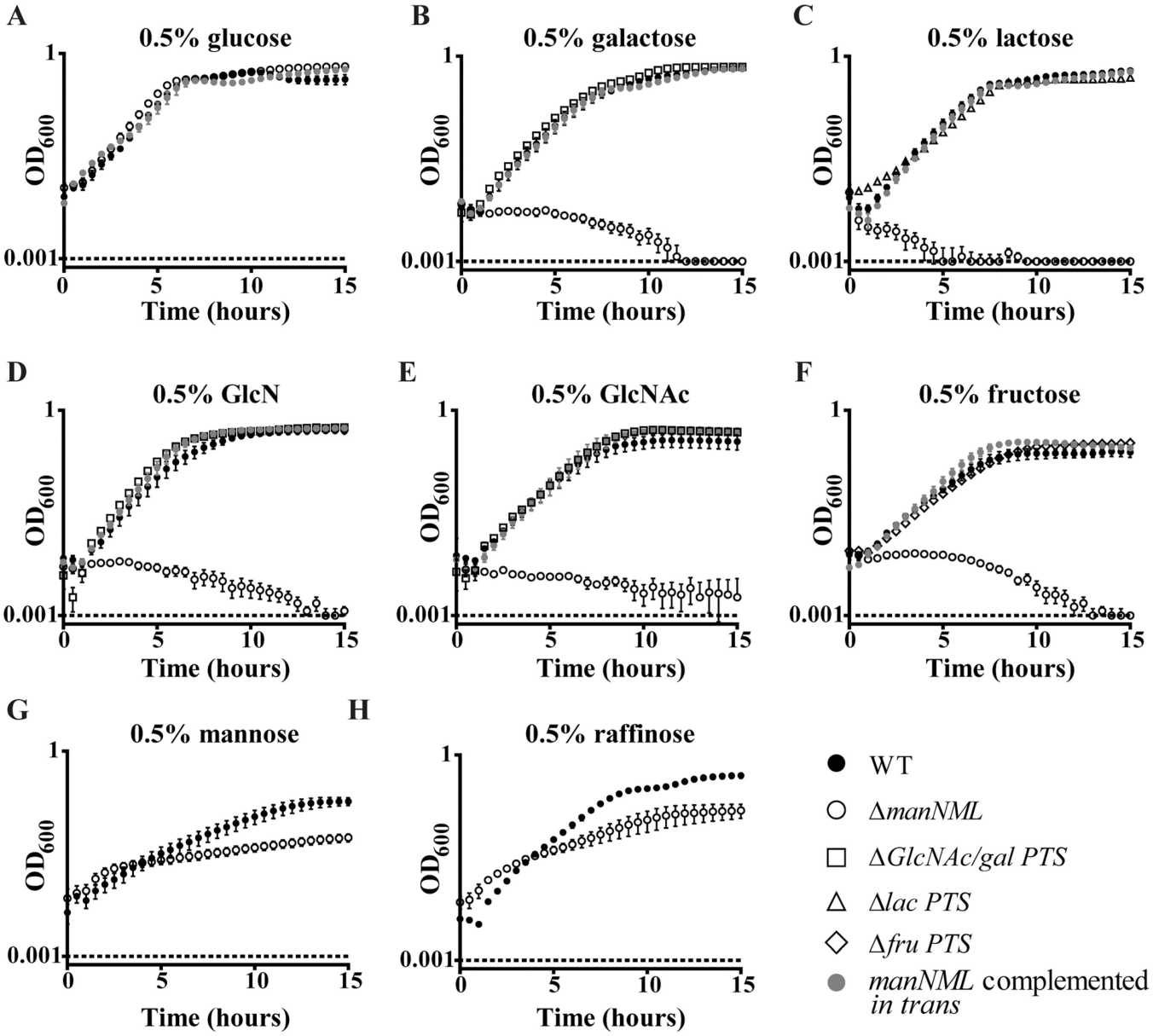

Fig. 1.

ManLMN, but not substrate-specific PTS transporters, is required for growth on multiple carbohydrates. WT, Δ*manLMN*, Δ*GlcNAc/gal PTS*, Δ*lac PTS*, Δ*fru PTS* and the *manLMN* complemented strain were grown in chemically defined medium (CDM) with 0.5% final concentration of one of the following carbohydrates; glucose (A), galactose (B), lactose (C), GlcN (D), GlcNAc (E), fructose (F), mannose (G) or raffinose (H). Absorbance at 600 nm was measured every 30 min over the course of 15 h of growth at 37°C. Each data point represents the average of at least six biological replicates from at least two separate days. Error bars represent the standard error of the mean (SEM).

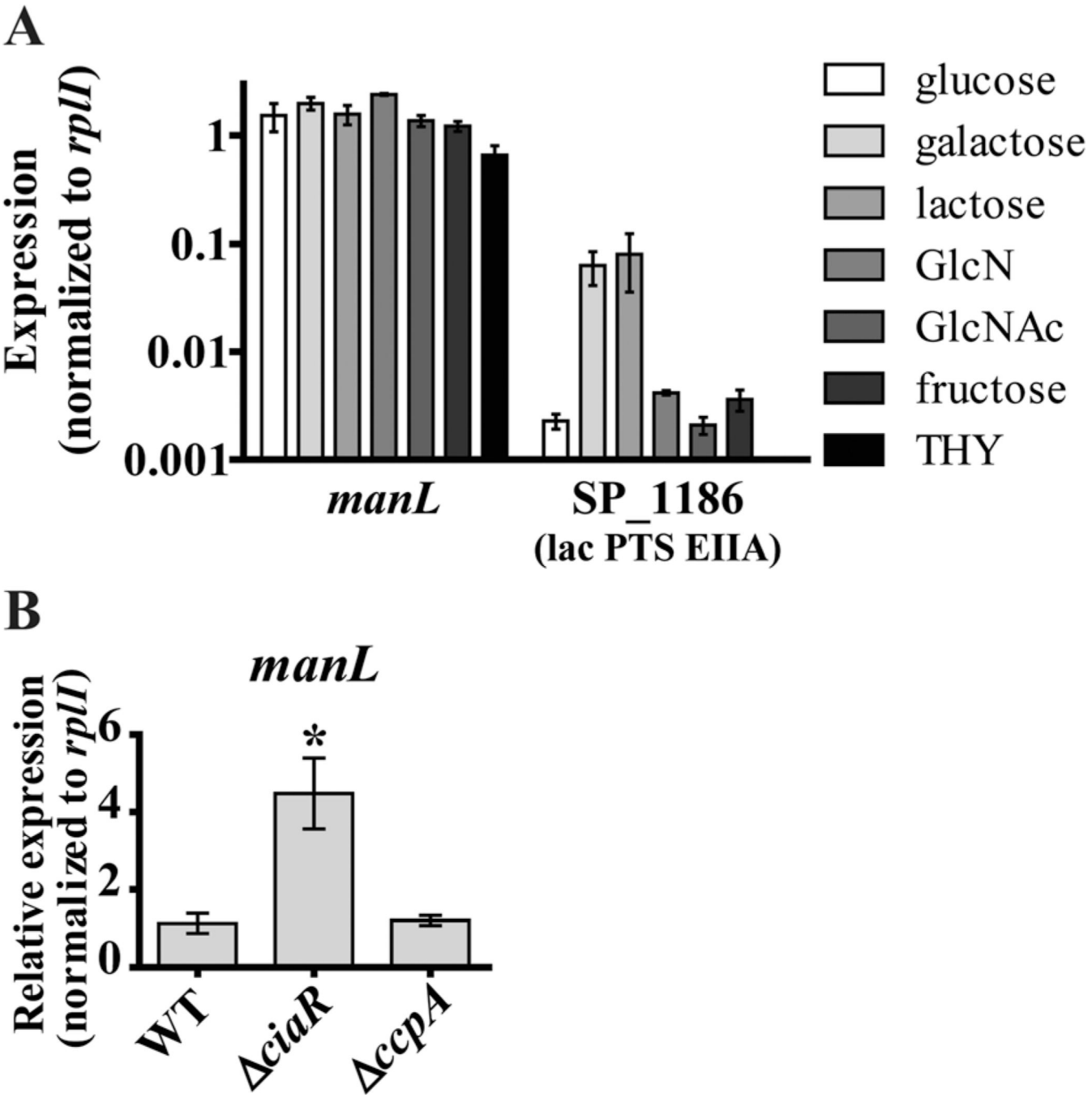

Fig. 2.

The *manLMN* operon is constitutively expressed and repressed by CiaR.

A. Transcription of *manL* as a representative of the *manLMN* operon was assessed by quantitative reverse transcriptase PCR (qRT-PCR) during mid-exponential growth of WT in THY or CDM with 0.5% final concentration of glucose, galactose, lactose, GlcN, GlcNAc or fructose. Transcription of SP_1186 encoding the lac PTS EIIA was assessed as a representative limited-substrate carbohydrate transporter gene. Each bar represents the

average of five biological replicates collected on at least two separate days, normalized to *rplI*. Error bars represent the SEM.

B. Transcription of *manL* in WT, Δ*ciaR* and Δ*ccpA* during mid-exponential growth in THY was assessed by qRT-PCR. The average of five biological replicates collected on at least two separate days, normalized to *rplI* and relative to the WT sample, is graphed for each. Error bars represent the SEM. * indicates *P* value ≤ 0.05.

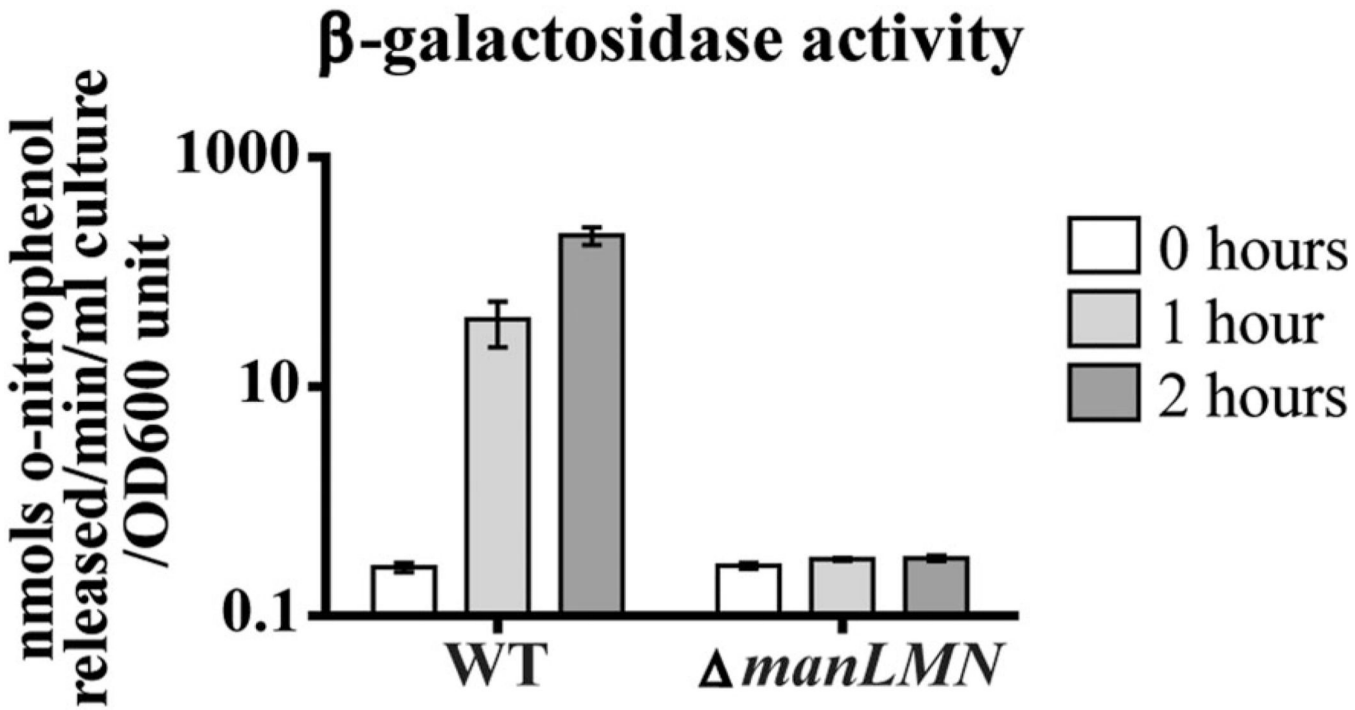

Fig. 3.

ManLMN is required for induction of β-galactosidase activity. Mid-exponential cultures of WT and Δ*manLMN* grown in 0.5% glucose CDM were washed and then resuspended in 0.5% lactose CDM. β-galactosidase activity was measured at the end of growth in glucose and 1 and 2 h after introduction to lactose CDM. Each bar represents the average of six biological replicates from two separate days. Error bars represent the SEM.

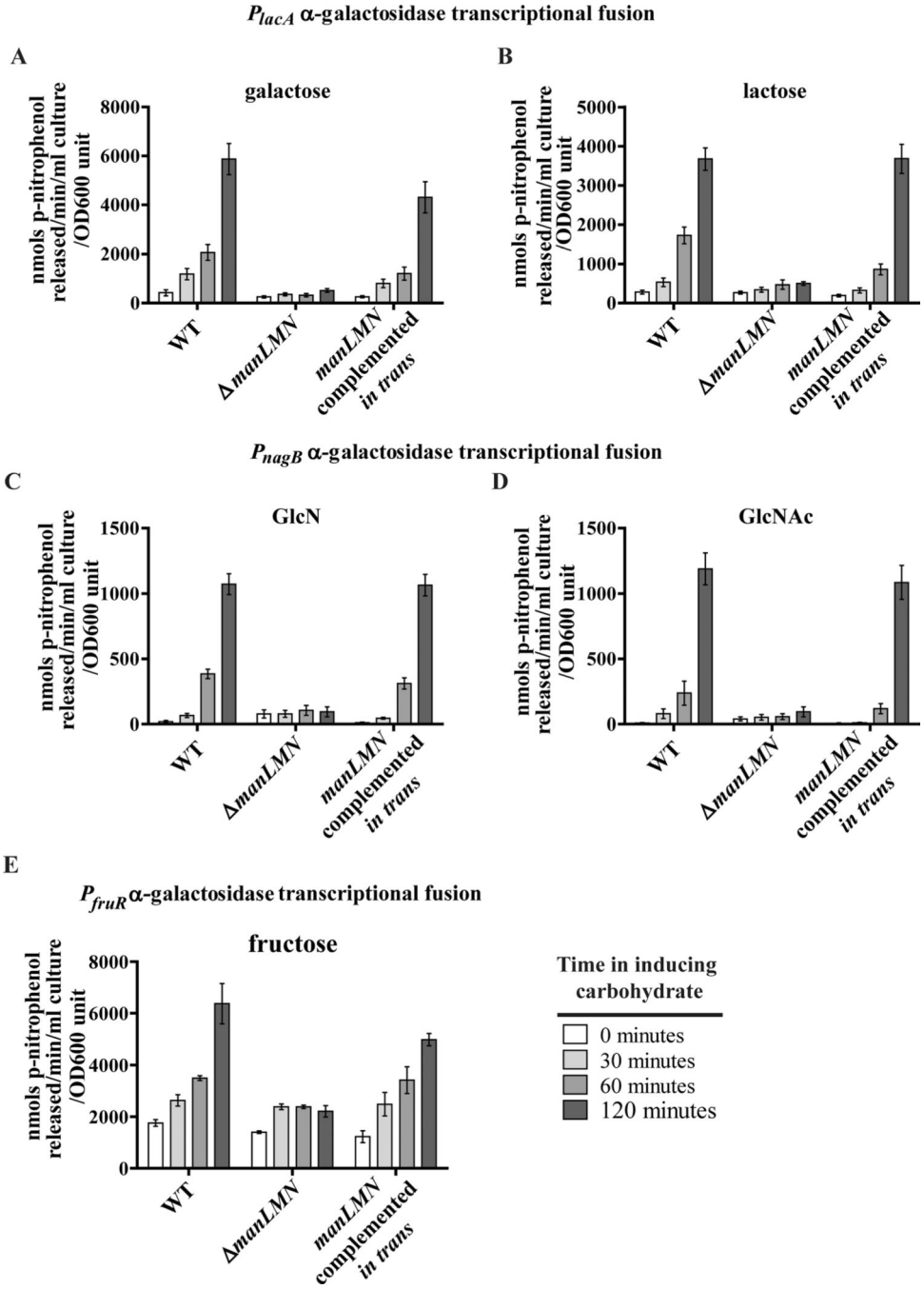

Fig. 4.

ManLMN is required for induction of galactose/lactose and GlcN/GlcNAc metabolism genes and partially required for induction of fructose metabolism genes. Carbohydrate-dependent transcriptional induction was assessed using α-galactosidase reporter strains. Strains were grown in 0.5% glucose CDM to mid-exponential phase, washed and then resuspended in CDM with 0.5% of the corresponding carbohydrate. α-galactosidase activity was measured at the end of growth in glucose and after 30, 60 and 120 min of incubation with the inducing carbohydrate. A tagatose-6-phosphate promoter reporter construct was

tested for response to galactose (A) or lactose (B). A glucosamine-6-phosphate deaminase promoter reporter construct was tested for response to GlcN (C) or GlcNAc (D). A fructose operon promoter reporter construct was tested for response to fructose (E). Each bar represents the average of six biological replicates from at least two separate days. Error bars represent the SEM.

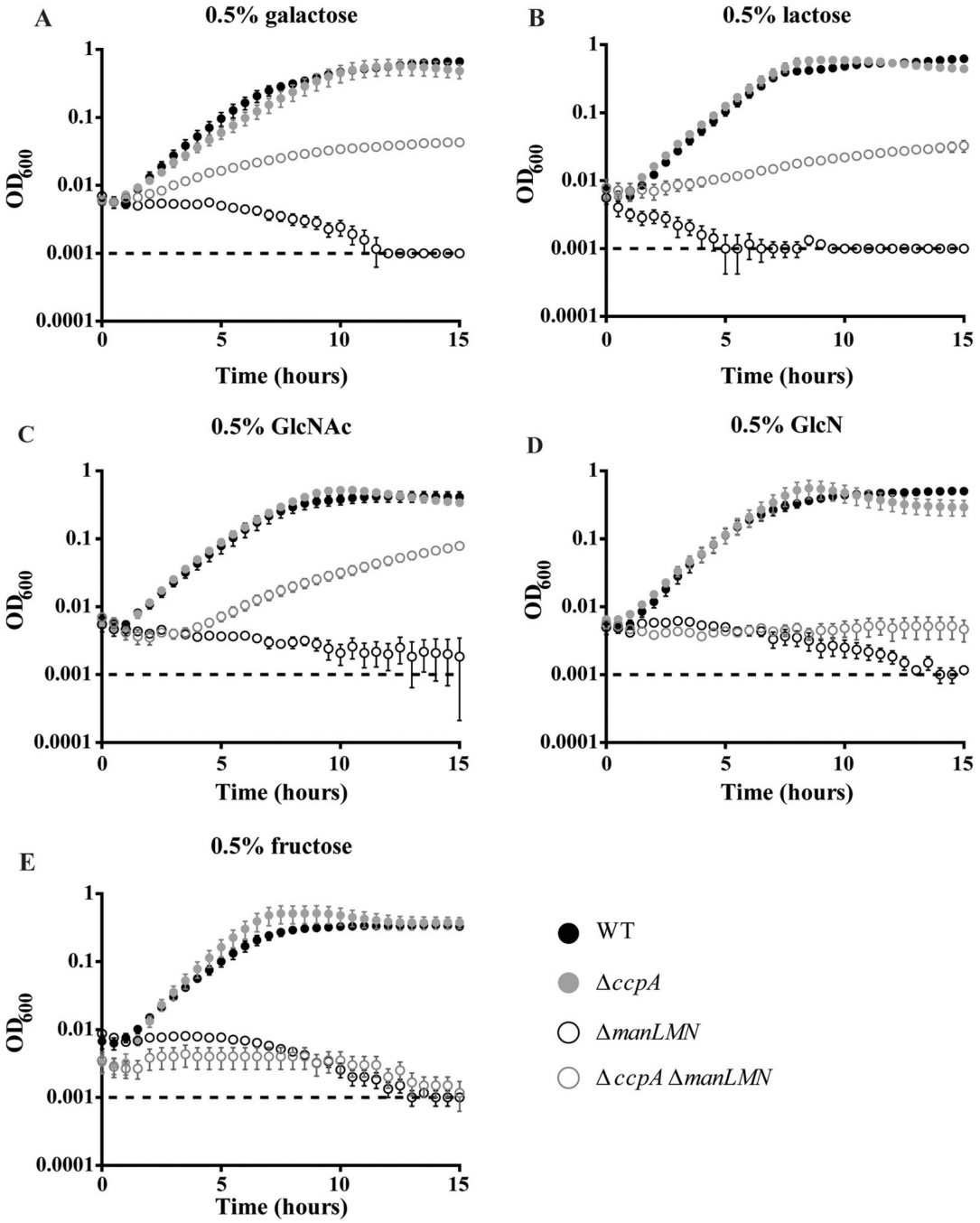

Fig. 5.

Deletion of *ccpA* rescues growth of Δ*manLMN* in select conditions. WT, Δ*manLMN*, Δ*ccpA* and Δ*manLMN* Δ*ccpA* were grown in CDM with 0.5% final concentration of one of the following carbohydrates; galactose (A), lactose (B), GlcN (C), GlcNAc (D), fructose (E). Absorbance at 600 nm was measured every 30 min over the course of 15 h of growth at 37°C. Each data point represents the average of at least six biological replicates from two separate days. Error bars represent the SEM.

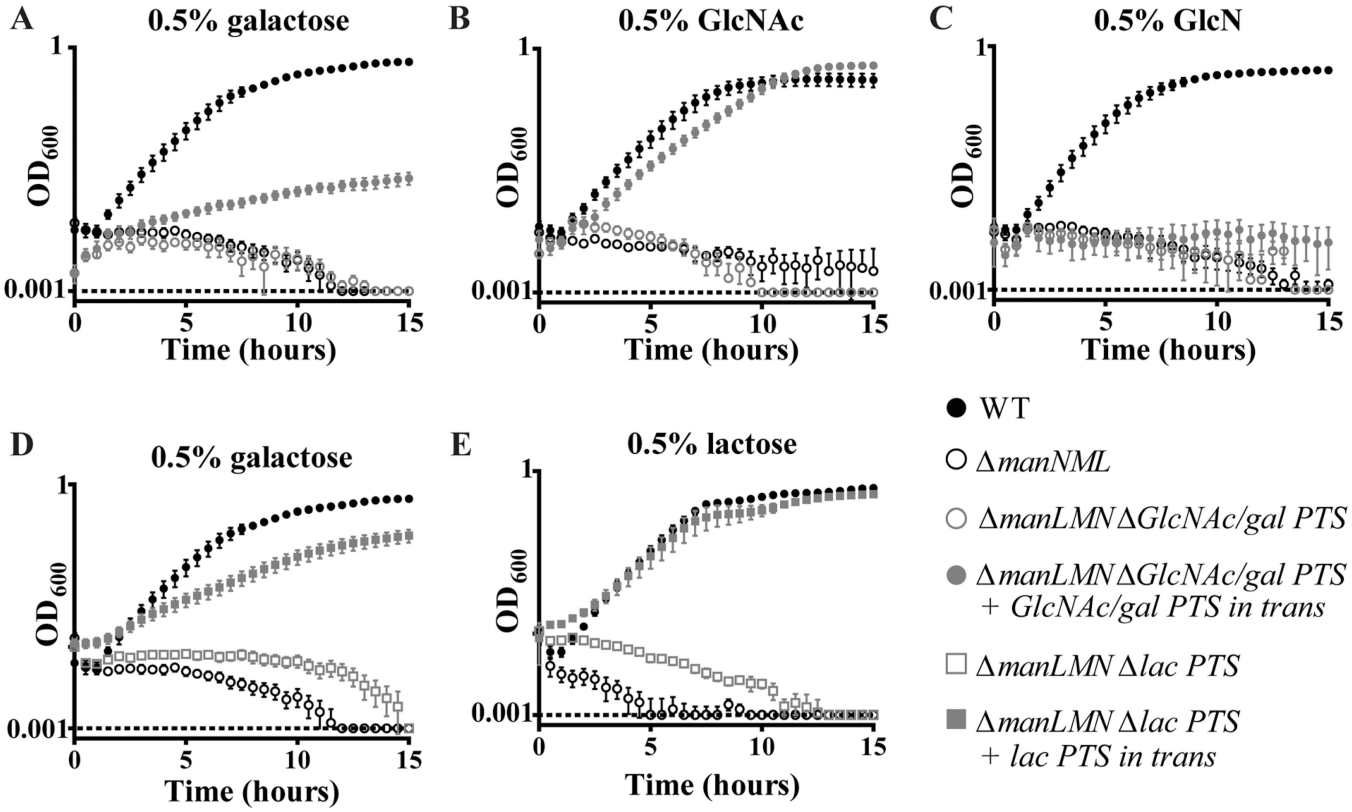

Fig. 6.

Constitutive expression of substrate-specific PTS systems rescues growth of Δ*manLMN* in select conditions. Growth of the ManLMN-GlcNAc/gal PTS double deletion strain (Δ*manLMN* Δ*GlcNAc/gal PTS*) and its corresponding complemented strain (Δ*manLMN* Δ*GlcNAc/gal PTS* + *GlcNAc/gal PTS* complemented *in trans*) were compared to growth of WT and Δ*manLMN* in galactose (A), GlcNAc (B) and GlcN (C). Growth of the ManLMN-lac PTS double deletion strain (Δ*manLMN* Δ*lac PTS*) and its corresponding complemented strain (Δ*manLMN* Δ*lac PTS* + *lac PTS* complemented *in trans*) was compared to growth of WT and Δ*manLMN* in galactose (D) and lactose (E). For all strains, optical density readings were taken every 30 min over the course of 15 h of growth at 37°C. Each data point represents the average of at least six biological replicates from at least two separate days. Error bars represent the SEM.

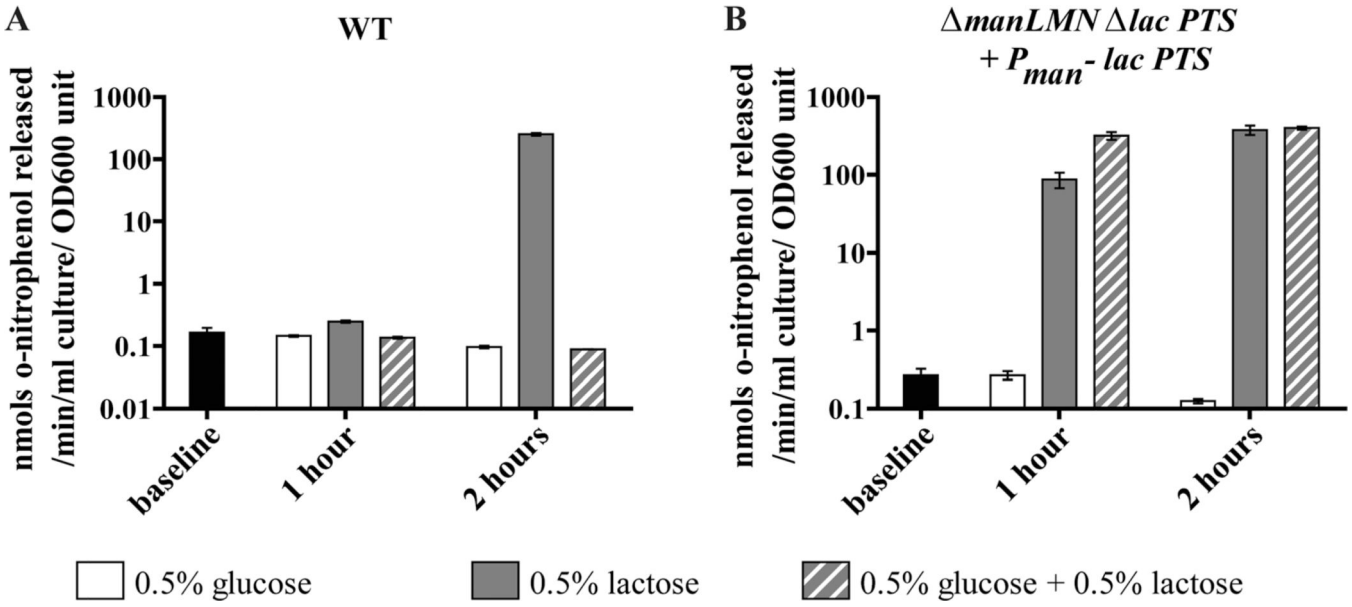

Fig. 7.

A strain constitutively expressing the lac PTS and lacking *manLMN* is unable to repress β-galactosidase activity. Mid-exponential THY cultures of WT (A), and Δ*manLMN* Δ*lac PTS* + *lac PTS* complemented *in trans* (B) were washed and switched to CDM containing 0.5% glucose, 0.5% lactose or 0.5% glucose + 0.5% lactose. Samples were collected from the THY cultures (‘baseline’) as well as one and 2 h after switching to the CDM conditions. Each bar represents the average of six biological replicates collected on at least two separate days, except the 2 h glucose only condition which was only assayed three times. Error bars represent the SEM.

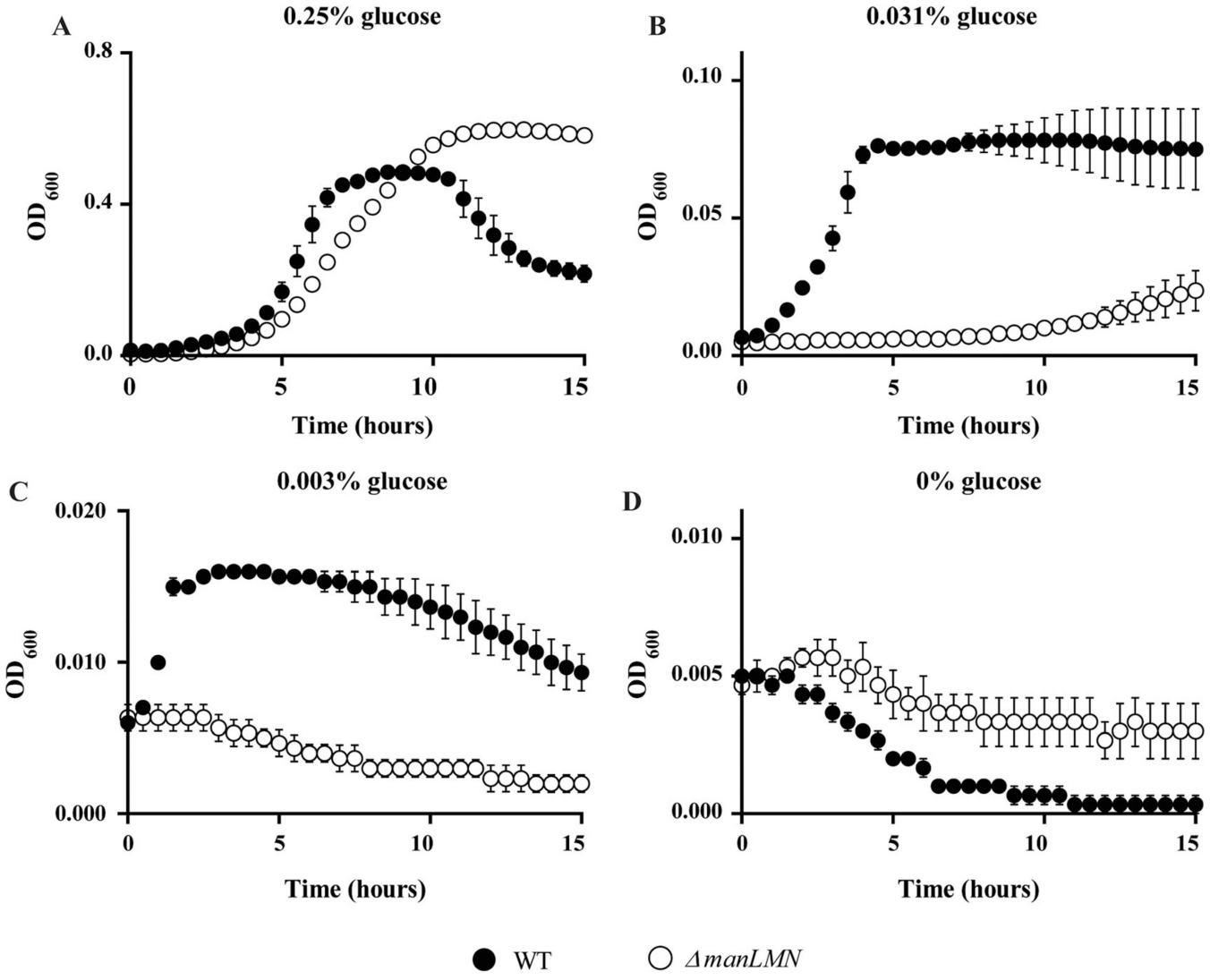

Fig. 8.

The Δ*manLMN* strain has a reduced ability to grow in limiting glucose. Growth of WT and Δ*manLMN* was compared in CDM containing the following growth limiting concentrations of glucose; 0.25% (A), 0.031% (B), 0.003% (C) and 0% (D). Both strains were tested at least six times over the course of multiple days. Each data point represents the average of three biological replicates from a representative day and error bars represent the SEM from a representative experiment.

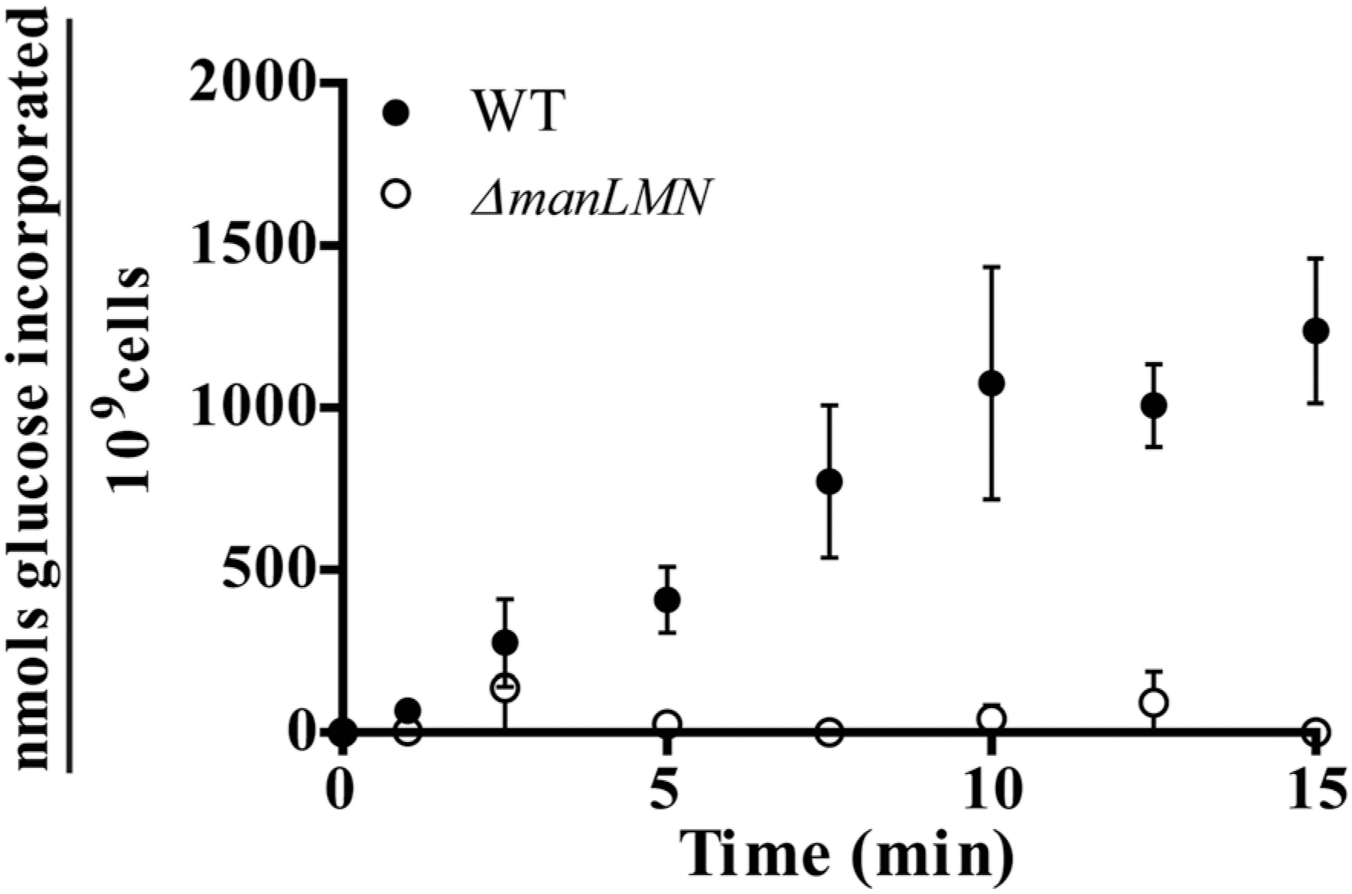

Fig. 9.

The Δ*manLMN* strain is deficient at glucose transport in a glucose limiting condition. Glucose incorporation by WT (closed circles) and Δ*manLMN* (open circles) was measured using a radiolabelled-carbohydrate incorporation assay. Mid-exponential phase glucose-grown cells were washed before exposure to a mixture of 400 μM unlabelled glucose plus H^3-labelled glucose at 37°C. Accumulation of the H^3-labelled glucose was determined in filtered samples over time according by standard liquid scintillation method. Values are reported as total nmols of carbohydrate accumulated/10^9 cells by accounting for the concentration of unlabelled carbohydrate and the colony forming units in each reaction. Each point is the average of five biological replicates collected on multiple days, and error bars are the standard error of the mean.

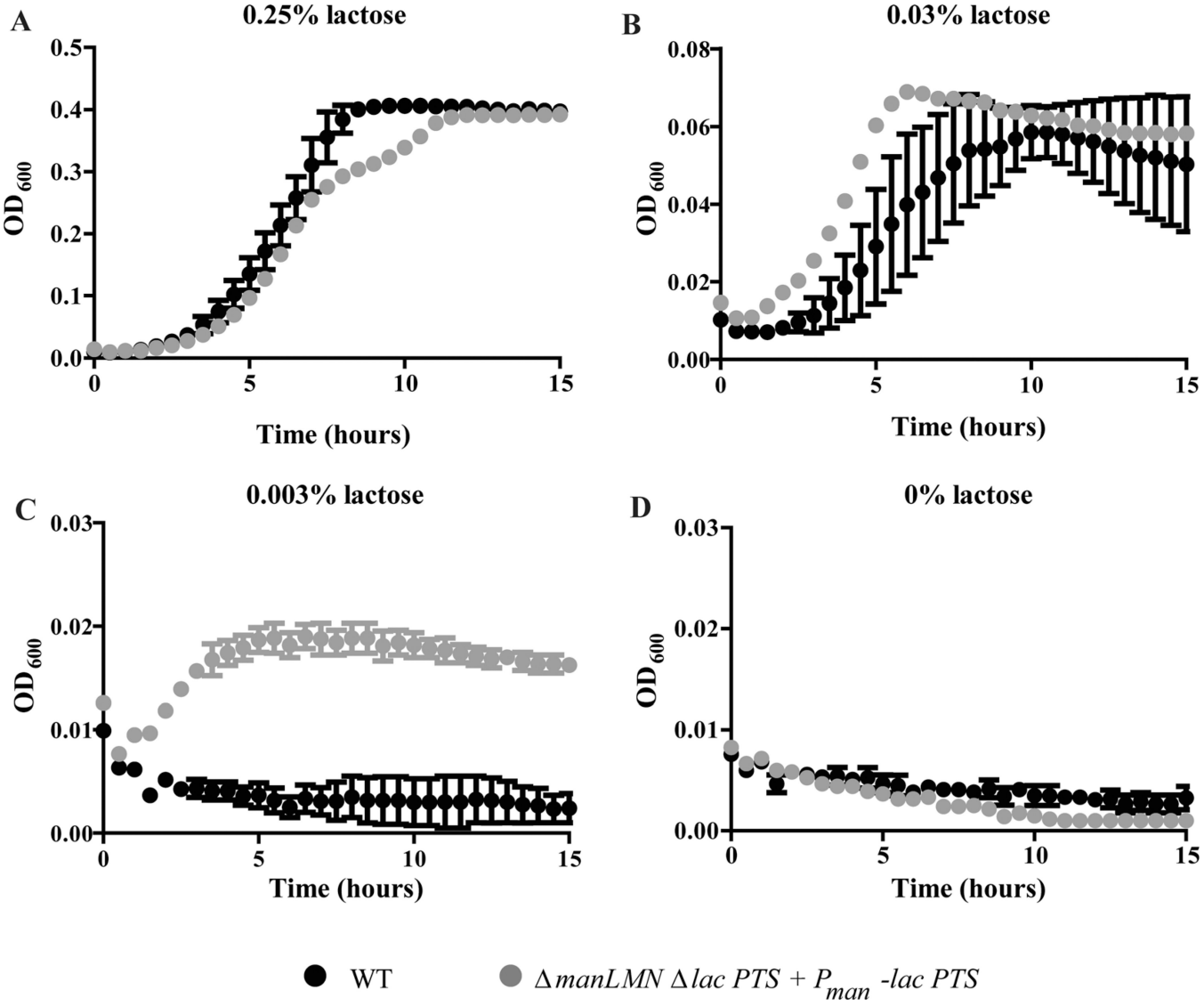

Fig. 10.

The Δ*manLMN* strain constitutively expressing the lac PTS has a heightened ability to grow in limiting lactose. Growth of WT and the lac PTS constitutive expressing strain (Δ*manLMN* Δ*lac PTS* + *lac PTS* complemented *in trans*) was compared in CDM containing the following growth-limiting concentrations of lactose; 0.25% (A), 0.031% (B), 0.003% (C) and 0% (D). Both strains were tested at least six times over the course of multiple days. Each data point represents the average of three biological replicates from a representative day and error bars represent the SEM from a representative experiment.

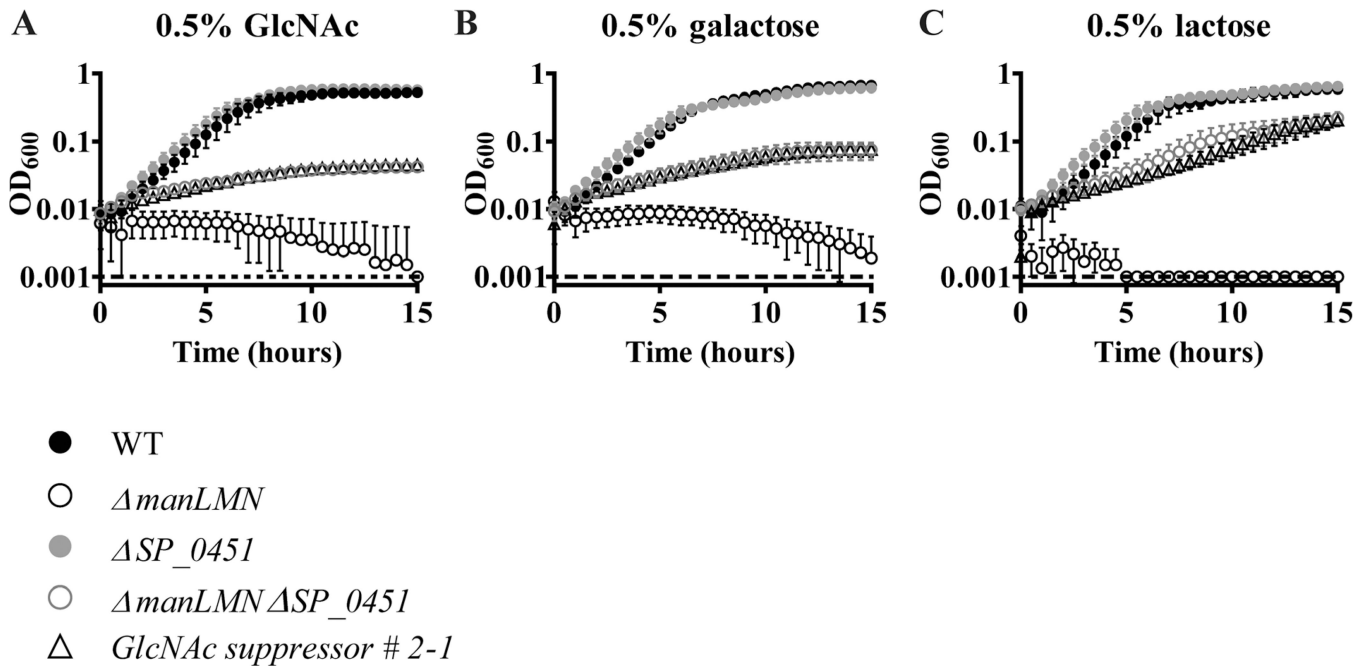

Fig. 11.

Inactivation of SP_0451 rescues growth of Δ*manLMN*. Growth of Δ*manLMN* Δ*SP_0451* was assessed in CDM containing 0.5% galactose (A), GlcNAc (B) or lactose (C). WT and Δ*manLMN* controls were included in all conditions. Each data point represents the average of at least four biological replicates collected on multiple days and error bars represent the SEM.

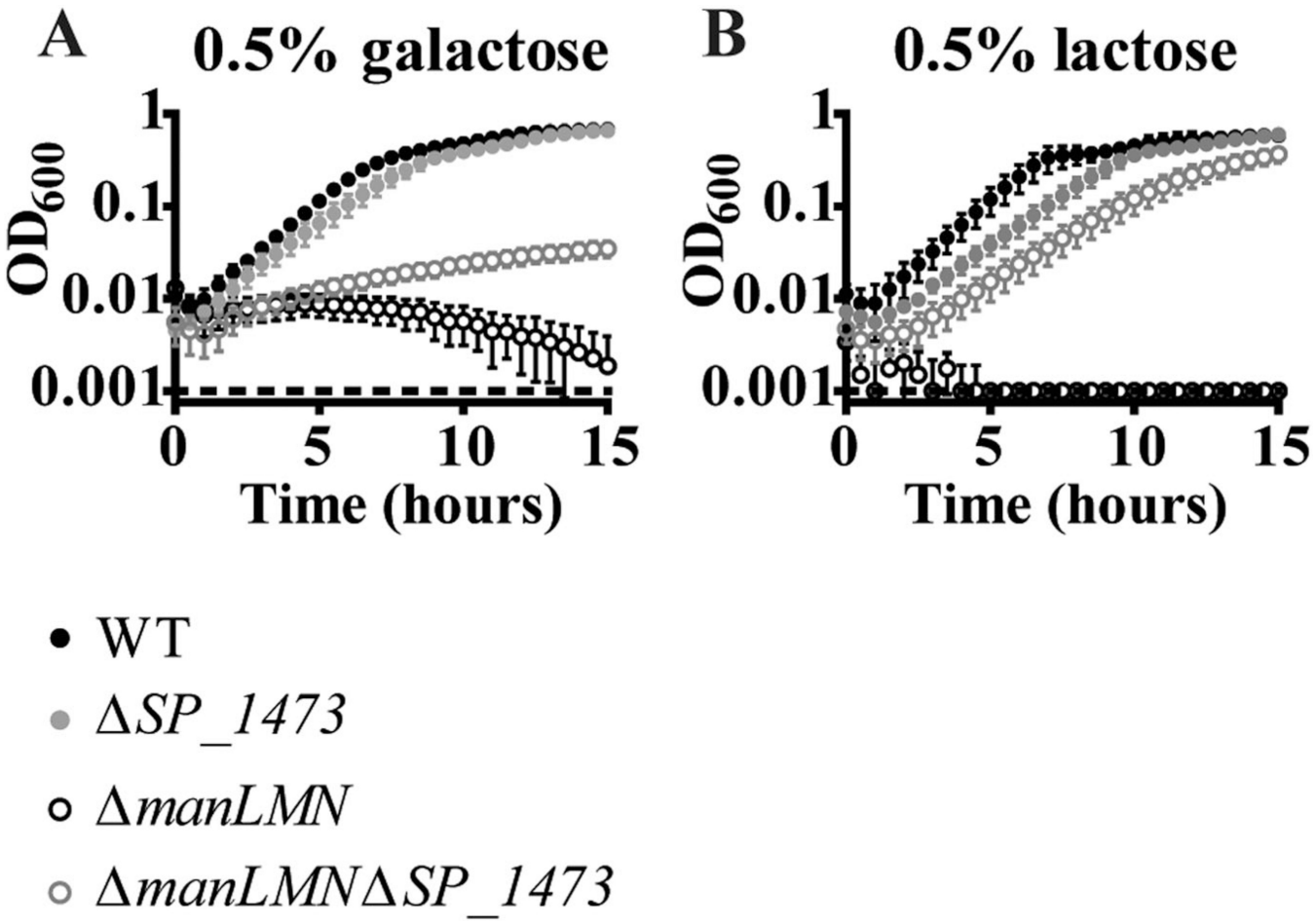

Fig. 12.

Inactivation of SP_1473 rescues growth of Δ*manLMN*. Growth of Δ*manLMN* Δ*SP_1473* was assessed in CDM containing 0.5% galactose (A) or lactose (B). WT and Δ*manLMN* controls were included in all conditions. Each data point represents the average of at least four biological replicates collected on multiple days and error bars represent the SEM.

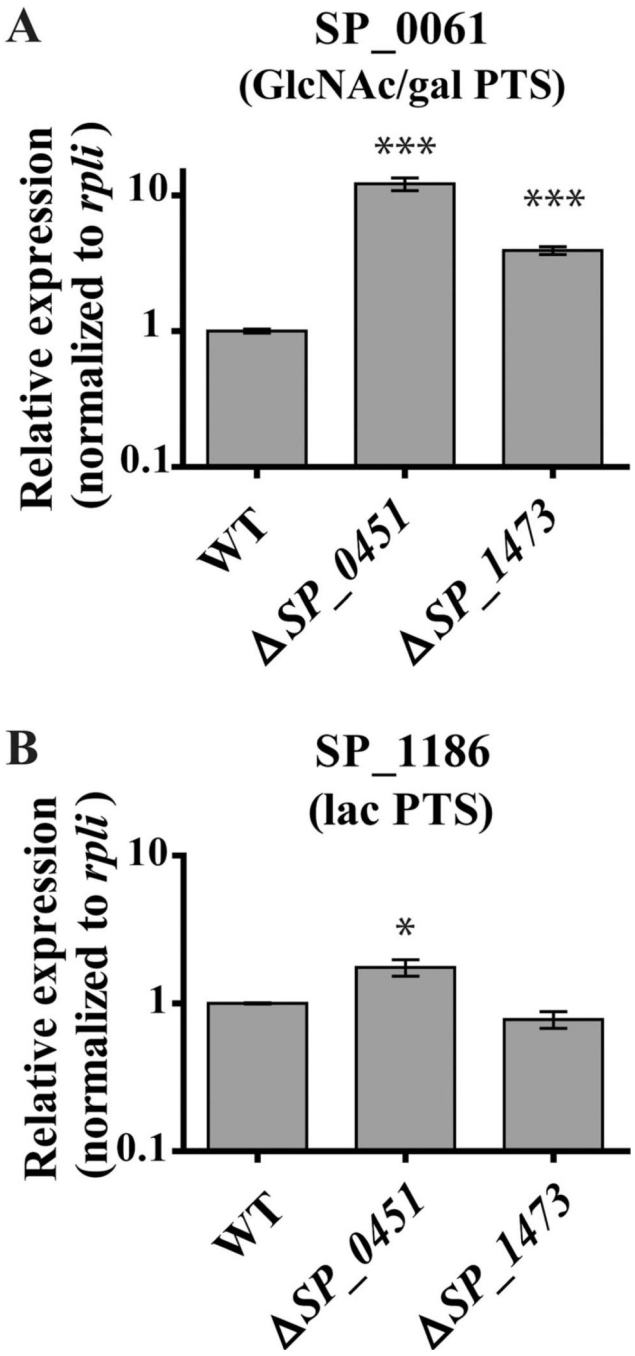

Fig. 13.

PTS system expression is de-repressed in Δ*SP_0451* and Δ*SP_1473*. Transcription of SP_0061 as a representative of the GlcNAc/gal PTS (A) and SP_1186 as a representative of the lac PTS (B) was assessed by qRT-PCR during mid-exponential growth of WT, Δ*SP_0451* and Δ*SP_1473* in THY. The average of at least three biological replicates normalized to *rplI* and relative to the WT sample, is graphed for each. Error bars represent the SEM. * indicates *P* value ≤ 0.05, and *** indicates *P* value ≤ 0.001.

Table 1

Reoccurring suppressor mutations.

Gene and/or operon	Genes hit	Encoded protein	Consequence of mutation	Selection carbohydrate(s)
SP_0058-SP_0063 *	SP_0058	SP_0058, repressor of GlcNAc/gal PTS operon	Ile9Arg	GlcNAc
	Upstream of SP_0060	Upstream of GlcNAc/gal PTS system	Disruption of regulation site?	GlcNAc
SP_0148-0152	SP_0151	ABC transporter ATP-binding domain	Val323fs	Galactose
			Gln97His	Fructose
	SP_0152	ABC transporter permease	Trp145 *	Fructose
SP_0451	SP_0451	Hypothetical small protein	−35 mutation (TTGACA–>TTGAAA)	GlcNAc
			−35 mutation (TTGACA–>TTGATA)	GlcNAc
	promoter		Start codon (ATG–> ATT)	GlcNAc
			Glu63 *	GlcNAc
SP_0576-0578 *	SP_0576	LicT transcriptional anti-terminator	Pro117Leu	GlcN
			Tyr266Asp	GlcN
	SP_0577	β-Glucoside specific PTS system EIIABC	Met107Ile	GlcNAc
			His534Tyr	GlcN (2)
			His513Asn	GlcN
			Ser471 *	Fructose
SP_0875-0877 *	SP_0877	fru PTS system EIIABC	Phe44Ser	Fructose
			Gly73Cys	Fructose
			Gly177Val	Fructose
			Ala223Asp	Fructose
			Ala484Asp	Fructose
			Ser442 *	Fructose
SP_0927-0939 *	SP_0927	SmrC; LysR family transcriptional regulator	Glu20Lys	GlcNAc, lactose (2)
			Pro103Leu	GlcN
			Ser124Leu	Lactose (2)
			Ser138Ile	GlcN
			Thr202Ile	Lactose
			Thr245Ile	Galactose, lactose, fructose
SP_1184-1187	Upstream	Upstream of lac PTS system operon	Disruption of regulation site?	Galactose
	SP_1185	lac PTS system EIIBC	Val466Gly	Galactose
	SP_1186	lac PTS system EIIA	Ala31Asp	Galactose
			Ala53Asp	Galactose
SP_1895-1898 *	SP_1895	Raffinose ABC transporter permease	Gly48Val	Fructose
	SP_1895		5’ 10 bp deletion -	Fructose

Gene and/or operon	Genes hit	Encoded protein	Consequence of mutation	Selection carbohydrate(s)
			frameshift	
SP_2173-2176	SP_2173	Lipoteichoic acid synthesis enzyme DltD	C–>T bp change 2bp upstream of start	Galactose (2)
	SP_2176	Lipoteichoic acid synthesis enzyme DltB	Glu67*	Galactose

This chart shows all genes and/or operons that had a suppressor mutation in at least two independent isolates. The gene number, encoded protein, the consequence of the mutation and the carbohydrate selection condition are all listed. References for gene functions are listed in Supporting information Table S2 which provides the complete list of all suppressor mutations identified.

* Signifies genes and/or operons that had suppressor mutations in both Δ*manLMN* backgrounds; *manLMN::CHESHIRE* and *manLMN::spec*.

Table 2

Strains used in the study.

***Streptococcus pneumoniae* strain**	**Genotype**	**Reference**
Wild type	TIGR4	Laboratory strain
Δ*manLMN*	*manLMN::spec*	This study
Δ*manLMN*	*manLMN::CHESHIRE*	This study
Δ*manLMN* complemented in trans	*manLMN::CHESHIRE* with *manLMN* promoter and coding region cloned into the SP_1773 locus	This study
Δ*manLMN, manL* complemented in trans	*manLMN::CHESHIRE* with *manL* promoter and coding region cloned into the SP_1773 locus	This study
Δ*GlcNAc/gal* PTS	*SP_0061-3::cat*	This study
Δ*lac* PTS	*SP_1185-6::cat*	This study
Δ*fru* PTS	*SP_0877::cat*	This study
Δ*ciaR*	*ciaR::spec*	This study
Δ*ccpA*	*ccpA::cat*	Laboratory strain
P_{lacA} - *agaA*	TIGR4, *lacA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
P_{nagB} - *agaA*	TIGR4, *nagB* promoter region inserted upstream of *agaA* start codon, cat^R	This study
P_{fruR} - *agaA*	TIGR4, *fruRCA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* P_{lacA} - *agaA*	Δ*manLMN, lacA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* P_{nagB} - *agaA*	Δ*manLMN, nagB* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* P_{fruR} - *agaA*	Δ*manLMN, fruRCA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* complemented in trans P_{lacA} - *agaA*	Δ*manLMN* complemented in trans, *lacA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* complemented in trans P_{nagB} - *agaA*	Δ*manLMN* complemented in trans, *nagB* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN* complemented in trans P_{fruR} - *agaA*	Δ*manLMN* complemented in trans, *fruRCA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN, manL* complemented in trans P_{lacA} - *agaA*	Δ*manLMN, manL* complemented in trans, *lacA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN, manL* complemented in trans P_{nagB} - *agaA*	Δ*manLMN, manL* complemented in trans, *nagB* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN, manL* complemented in trans P_{fruR} - *agaA*	Δ*manLMN, manL* complemented in trans, *fruRCA* promoter region inserted upstream of *agaA* start codon, cat^R	This study
Δ*manLMN*Δ*GlcNAc/gal* PTS	*manLMN::spec, SP_0061-3::cat*	This study
Δ*manLMN*Δ*lac* PTS	*manLMN::spec, SP_1185-6::cat*	This study
Δ*manLMN*Δ*fru* PTS	*manLMN::spec, fruA::cat*	This study
Δ*manLMN*Δ*GlcNAc/gal* PTS, *GlcNAc/gal PTS* complemented in trans	*manLMN::spec, SP_0061-3::cat* Pman-SP_0061-3 cloned into the SP_1773 locus	This study
Δ*manLMN*Δ*lac* PTS, *lac* PTS complemented in trans	*manLMN::spec, SP_1185-6::cat* Pman-SP_1185-5 cloned into the SP_1773 locus	This study
Δ*manLMN*Δ*fru* PTS, *fru* PTS complemented in	*manLMN::spec, fruA::cat*	This study

***Streptococcus pneumoniae* strain**	**Genotype**	**Reference**
trans	Pman-fruA cloned into the SP_1773 locus	
Δ*SP_0451*	*SP_0451::spec*	This study
Δ*SP_1473*	*SP_1473::spec*	This study
Δ*manLMN*Δ*SP_0451*	*manLMN::CHESHIRE, SP_0451::spec*	This study
Δ*manLMN*Δ*SP_1473*	*manLMN::CHESHIRE, SP_1473::spec*	This study