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σ^{54}-mediated control of the mannose phosphotransferase sytem in *Lactobacillus plantarum* impacts on carbohydrate metabolism

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Sigma factors direct specific binding of the bacterial RNA polymerase to the promoter. Here we present the elucidation of the σ^{54} regulon in *Lactobacillus plantarum*. A sequence-based regulon prediction of σ^{54}-dependent promoters revealed an operon encoding a mannose phosphotransferase system (PTS) as the best candidate for σ^{54}-mediated control. A σ^{54} (*rpoN*) mutant derivative did not grow on mannose, confirming this prediction. Additional mutational analyses established the presence of one functional mannose PTS in *L. plantarum*, the expression of which is controlled by σ^{54} in concert with the σ^{54}-activator ManR. Genome-wide transcription comparison of the wild-type and the *rpoN*-deletion strain revealed nine upregulated genes in the wild-type, including the genes of the mannose PTS, and 21 upregulated genes in the *rpoN* mutant. The σ^{54}-controlled mannose PTS was shown also to transport glucose in *L. plantarum* wild-type cells, and its presence causes a lag phase when cultures are transferred from glucose- to galactose-containing media. The mannose PTS appeared to drain phosphoenolpyruvate (PEP) pools in resting cells, since no PEP could be detected in resting wild-type cells, while mannose PTS mutant derivatives contained 1–3 μM PEP (mg protein)$^{-1}$. Our data provide new insight into the role of σ^{54} in *L. plantarum* and possibly other Gram-positive bacteria in the control of expression of an important glucose transporter that contributes to glucose-mediated catabolite control via modulation of the PEP pool.

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INTRODUCTION

Bacterial transcription initiation requires a sigma (σ) factor to direct the RNA polymerase core enzyme to the promoter. Most bacteria possess more than one sigma factor, each with its own specific promoter sequence, generating an important mechanism for differential gene expression in bacteria. Based on structural and functional criteria, sigma factors can be categorized into two main classes (Merrick, 1993): a class containing all the *Escherichia coli* σ^{70}-like factors and a class containing only a single member, the σ^{54} factor (Merrick & Gibbins, 1985). Although not immediately recognized as a sigma factor, σ^{54} was originally discovered in the Gram-negative bacterium *Salmonella typhimurium*, where it was found to regulate expression of the glutamine synthetase (Garcia *et al.*, 1977). The σ^{54} transcription factor (also known as σ^{N} or σ^{L}) differs from all other sigma factors by binding to a promoter with a conserved −12/−24 motif (Buck *et al.*, 1986; Morett & Buck, 1989) and by the absolute requirement for a dedicated activator protein to initiate transcription by the DNA-bound σ^{54}–RNA polymerase (RNAP) complex. This activator protein catalyses ATP-

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Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphotransferase system; RNAP, RNA polymerase.

The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) accession numbers for the array design and primary data for the microarray experiments in this study are GPL6368 and GSE11351, respectively.

Supplementary material is available with the online version of this paper.

dependent transition from a closed to an open complex of the RNAP, which enables initiation of transcription (Sasse-Dwight & Gralla, 1988). The activator protein usually binds upstream of the σ^{54} promoter site, and DNA looping is required for the activator to contact the RNAP, resembling a mechanism similar to transcriptional initiation in eukaryotes. For this reason the σ^{54} activators are also called bacterial enhancer binding proteins (EBPs) (Studholme & Dixon, 2003).

Since transcription of σ^{54}-dependent genes requires the activity of a dedicated activator protein, transcription of these genes is generally tightly controlled, with low levels of promoter leakage (Wang & Gralla, 1998). Another advantage of σ^{54}-dependent regulation is the possibility to modulate gene expression from a promoter over a wide range without requirement for additional transcription regulators, thereby allowing rapid and strict regulation of gene expression under changing physiological conditions (Buck *et al.*, 2000).

Comparison of 186 σ^{54}-dependent bacterial promoters led to a defined promoter sequence ($-12/-24$) and spacer-length consensus (Barrios *et al.*, 1999). Deletion of one or more nucleotides in the spacer region of σ^{54}-dependent promoters resulted in a dramatic decrease or complete loss of promoter activity (Buck, 1986), and although there are no data available about promoter activity after insertion of nucleotides, the spacer-length between the -12 and -24 regions is considered to be essential for promoter function (Barrios *et al.*, 1999). Similarly, a range of σ^{54}-dependent genes has been identified, and σ^{54} appears to be involved in a variety of cellular processes, including nitrogen assimilation and fixation, glutamine synthesis and transport, and sugar transport (for a review see Studholme & Buck, 2000). *In silico* analysis of bacterial genomes has led to the identification of σ^{54}-encoding genes in various bacteria, representing all bacterial phyla. Bacteria that possess a σ^{54} gene generally also have one or more predicted σ^{54}-activator genes, which are usually genetically linked to the corresponding regulated genes (Reitzer & Schneider, 2001).

In low-GC Gram-positive bacteria, σ^{54}-dependent genes have been described in several species. In *Bacillus subtilis*, σ^{54} (there designated σ^{L}) is involved in levanase and acetoin import (Ali *et al.*, 2001; Debarbouille *et al.*, 1991) and in arginine and valine degradation (Gardan *et al.*, 1997; Debarbouille *et al.*, 1999). In *Listeria monocytogenes*, *Enterococcus faecalis* and *Lactobacillus casei* the expression of the operon encoding the mannose phosphotransferase system (mannose PTS) appears to be strictly σ^{54}-dependent (Dalet *et al.*, 2001; Hechard *et al.*, 2001; Yebra *et al.*, 2004).

Here we describe the *in silico* and experimental characterization of the σ^{54} regulon of *Lactobacillus plantarum*, a lactic acid bacterium that is found in vegetable, meat and dairy fermentations. *L. plantarum* is a natural inhabitant of mammalian gastrointestinal tracts and specific strains are marketed as probiotics (Ahrne *et al.*, 1998; de Vries *et al.*, 2006). The genome of *L. plantarum* WCFS1 contains an *rpoN*-like gene, encoding a 444-residue protein with high identity to σ^{54} proteins in other bacteria, including closely related species such as *Pediococcus pentosaceus* (53 % identity), *Enterococcus faecalis* (51 %) and *Listeria monocytogenes* (40 %). The *in silico* predictions and transcriptome analysis described here show that only the mannose PTS operon is directly controlled by σ^{54} in *L. plantarum*. Furthermore, this mannose PTS also functions as a glucose-uptake system in *L. plantarum*, as exemplified by the reduced growth rate of a mannose PTS mutant on glucose-containing media. σ^{54} is thus shown not only to control the expression of the mannose PTS but also to have a marked effect on transport of other carbon sources via an indirect regulatory mechanism, mediated by the intracellular concentration of the glycolytic intermediate PEP.

METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α was used as an intermediate cloning host and was grown aerobically at 37 °C in TY medium (Sambrook *et al.*, 1989). When appropriate, chloramphenicol was added to a final concentration of 8 μg ml^{-1}. *L. plantarum* WCFS1 and its derivatives were anaerobically grown in MRS broth (De Man *et al.*, 1960) supplemented with 2 % (w/v) of selected carbon source (mannose, glucose, galactose, etc.), or in a chemically defined medium (CDM) designed for *L. plantarum* (Teusink *et al.*, 2005). Cells were grown at 30 °C. Carbon sources were obtained from Sigma-Aldrich. Growth was monitored by measurement of the OD_{600} in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech).

DNA manipulations and gene disruption. Molecular cloning and DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). Plasmids constructed in this study are listed in Table 1. Large-scale plasmid DNA isolations from *E. coli* were performed using a Jet Star Maxiprep kit (Genomed). DNA was isolated from *L. plantarum* as described previously (Ferain *et al.*, 1994). Restriction enzymes and *Pwo* polymerase were obtained from Promega. T4 ligase was obtained from Boehringer. Primers were purchased from Proligo.

Construction of plasmids and strains. Gene disruption was performed using a double-crossover gene replacement strategy (except for strain NZ7308, *manIIC*::*pNZ7350*; see below), which resulted in replacement of the target gene by a chloramphenicol-resistance gene cassette (P_{32}*cat*; Bron, 2004). For disruption of the *rpoN* gene a 1.2 kb fragment of the upstream region of the target locus was amplified using a proofreading DNA polymerase (*Pwo*) and the primers *rpoN-U-5′* and *rpoN-U-3′* (Table 2). Similarly, a 1.2 kb fragment of the downstream region of *rpoN* was amplified using primers *rpoN-D-5′* and *rpoN-D-3′* (Table 2). A similar strategy was used for the *manR* gene, using primers *manR-U-5′* and *manR-U-3′* and primers *manR-D-5′* and *manR-D-3′* to amplify the upstream and downstream fragments, respectively (Table 2). The up- and downstream fragments were sequentially cloned into the *Swa*I and *Ecl*136II sites, respectively, of the gene-replacement vector pNZ5318 (Lambert *et al.*, 2007), resulting in the *rpoN*-replacement vector pNZ7373 and the *manR*-replacement vector pNZ7332 (Table 1). All mutagenesis constructs aimed to replace the target gene by the chloramphenicol-resistance cassette, with the *cat* gene in the same orientation as the target gene. Correct plasmid construction was checked by restriction analyses.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features*	Source or reference
E. coli		
DH5α	Cloning host	Woodcock *et al.* (1989)
L. plantarum		
WCFS1	Wild-type strain, human saliva isolate	Kleerebezem *et al.* (2003)
NZ7306	*rpoN* gene replacement (*rpoN*::P_{32}*cat*) derivative of WCFS1	This work
NZ7307	*manR*::*cat*	This work
NZ7308	*manIIC*::*pNZ7350* (*pts9C*::*pNZ7350*)	This work
NZ7309	*manIIAB*::*cat* (*pts9AB*::*cat*)	This work
NZ7310	*manIIB*::*cat* (*pts9B*::*cat*)	This work
NZ7311	*manIIABCD*::*cat* (*pts9ABCD*::*cat*)	This work
NZ7312	*manIIAB*::*cat* (*pts10AB*::*cat*)	This work
Plasmids		
pNZ5318	Cm^R Em^R, gene replacement mutagenesis vector	Lambert *et al.* (2007)
pNZ5319	Cm^R Em^R, gene replacement mutagenesis vector	Lambert *et al.* (2007)
pNZ7373	Cm^R Em^R, pNZ5318 *rpoN*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *rpoN*	This work
pNZ7332	Cm^R Em^R, pNZ5319 *manR*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *man*	This work
pNZ7350	Cm^R Em^R, pNZ5319 derivative containing a 0.4 kb internal fragment of *manIIC*	This work
pNZ7364	Cm^R Em^R, pNZ5319 *manIIAB*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *manIIAB*	This work
pNZ7365	Cm^R Em^R, pNZ5319 *manIIB*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *manIIB*	This work
pNZ7366	Cm^R Em^R, pNZ5319 *manIIABCD*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *manIIABCD*	This work
pNZ7372	Cm^R Em^R, pNZ5319 *manIIAB*::*cat* gene replacement mutagenesis vector containing 5′- and 3′-flanking regions of *manIIA*+*B*	This work

*Cm^R, chloramphenicol-resistant; Em^R, erythromycin-resistant.

Table 2. Primers used in this study

Name	Sequence (5′–3′)	Name	Sequence (5′–3′)
rpoN-U-5′	AGTCGAGCTCTGTCCTTCACTGAATCTTGG	*manIIA+B-U-5′*	TTATGATGTTCAGGCTTTCG
rpoN-U-3′	AACCCTTGTGTTTTTATCCA	*manIIA+B-U-3′*	CAAAACTCCCATGACTGACA
rpoN-D-5′	AGTCCTGCAGCAACCACCTTATGAACCTGT	*manIIA+B-D-5′*	TTTATGGCGCCAGATAAGTA
rpoN-D-3′	AGTCAAGCTTAATGTTCTTCATATACGCGG	*manIIA+B-D-3′*	GCACACCCCTACTTCTTGAT
manR-U-5′	GTTTCAGACACGCTTGGAAC	*Con-rpoN-5′*	CTTGCCCTTAGTACCAGATG
manR-U-3′	GCACAGGCCGCCCTGTCAGC	*Con-rpoN-3′*	GAATGCTAAACGACCGATAC
manR-D-5′	CGAAGTGGTACCCACTTGTG	*Con-manR-5′*	ACGTTAGCTGTATCATTGGC
manR-D-3′	TAGCGGCATCACATCAACCC	*Con-manR-3′*	AACATATTGTCCCATGGCGG
manIIC-I-5′	CAAACGTTGGTGCCGCTGTT	*Con-manIIC*	ATGAATTTGAATGCAATTCA
manIIC-I-3′	GCGTAACCAACGGCAACAAC	*Con-manIIAB-5′*	GGCGAGGACCAGGCATTGAT
manIIAB-U-5′	AGTAGATGGTGGTGGCGTAT	*Con-manIIAB-3′*	GGTGAGGCAACGTATGGATG
manIIAB-U-3′	AGCAATGATAATGCTTACCAT	*Con-manIIPTS-3′*	ACAAATCTCCCCAATCTGCC
manIIAB-D-5′	AACATGTTAAATGAACAAAAGTAGTA	*Con-manIIB-5′*	TGCCAGCCACGTGCCGCGCA
manIIAB-D-3′	AGAATTCCAAATGACGCTTT	*Con-manIIA+B-5′*	TTCCATGGCACAGGTCGTCG
manIIB-U-5′	CGAATCCTAACACTGGCTTT	*Con-manIIA+B-3′*	CCCTGTCGCGCGTATTTCTC
manIIB-U-3′	GTTATTGTTGGTCGTTGCAG	*Con-cam-for*	GATAGGCCTAATGACTGGCT
manPTS-D-5′	ATGCATGTTCTTGGTGTAATGT	*Con-cam-rev*	CTCTTCCAATTGTCTAAATC
manPTS-D-3′	TCATTCATCTTGCCATCAAC		

A similar double-crossover strategy was used to disrupt *manIIABCD* (*pts9ABCD*), *manIIAB* (*pts9AB*), *manIIB* (*pts9B*) and *manIIA+B* (*pts10AB*), with the modification that the pNZ5318 derivative pNZ5319 (Lambert *et al.*, 2007) was used as cloning vector; this vector harbours *lox* sites up- and downstream of the P_{32}*cat* cassette, which enables *cat*-gene deletion by a resolvase, resulting in a clean gene deletion as described by Lambert *et al.* (2007). Flanking regions of the mannose operon (*manIIABCD*) were amplified using the primer sets *manIIAB-U-5′* and *manIIAB-U-3′* for the upstream region and *manPTS-D-5′* and *manPTS-D-3′* for the downstream region. Flanking regions of *manIIAB* were amplified using primers *manIIAB-U-5′* and *manIIAB-U-3′* (upstream) and *manIIAB-D-5′* and *manIIAB-D-3′* (downstream), flanking regions of *manIIB* using *manIIB-U-5′* and *manIIB-U-3′* (upstream) and *manIIAB-D-5′* and *manIIAB-D-3′* (downstream), and flanking regions of *manIIA+B* using *manIIA+B-U-5′* and *manIIA+B-U-3′* (upstream) and *manIIA+B-D-5′* and *manIIA+B-D-3′* (downstream). The upstream regions obtained were cloned into the *Swa*I site and the downstream regions into the *Ecl*136II site of the gene replacement vector pNZ5319, resulting in the *manIIABCD*-replacement vector pNZ7366, the *manIIAB*-replacement vector pNZ7364, the *manIIB*-replacement vector pNZ7365 and the *manIIA+B*-replacement vector pNZ7372 (Table 1).

The resulting plasmids were transformed into *L. plantarum* as described previously (Josson *et al.*, 1989), and primary plasmid integrants were selected on MRS plates with 8 μg chloramphenicol ml^{-1} and 1 % glycerol at 30 °C. To check for erythromycin sensitivity, colonies were picked and transferred to MRS plates with 20, 40, 60, and 80 μg erythromycin ml^{-1} and grown overnight at 30 °C. The anticipated genetic organization after correct gene replacement leads to chloramphenicol resistance and erythromycin sensitivity; integrants with this phenotype were checked by PCR using universal primers (*Con-cam-for* and *Con-cam-rev*) annealing in the *cat* gene and a site-specific primer (*Con*-primers in Table 2) annealing outside of the chromosomal region used for homologous recombination. The 5′-primers were combined with primer *Con-cam-rev* and the 3′-primers were combined with *Con-cam-for*.

For the disruption of *manIIC* (*pts9C*) a single-crossover strategy was used. A 400 bp internal fragment was amplified using primers *manIIC-I-5′* and *manIIC-I-3′* (Table 2) and cloned into the *Swa*I site of pNZ5319, resulting in the *manIIC*-disruption vector pNZ7350. The resulting plasmid was transformed to *L. plantarum*, and primary plasmid integrants were selected on MRS plates with 8 μg chloramphenicol ml^{-1} and 1 % glycerol at 30 °C. Correct integration of the disruption plasmid was checked by PCR using primer *Con-manIIC*, annealing upstream of the integration site, and the *Con-cam-rev* primer.

RNA extraction and quality control. RNA was isolated as described previously (Stevens *et al.*, 2008), using a phenol/chloroform extraction followed by further purification with the High Pure RNA isolation kit (Roche). The yield and purity of the RNA were determined by measurement of A_{260} and A_{280} (Ultrospec 3000, Pharmacia Biotech). The RNA quality was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies), following the manufacturer's instructions. Only RNA samples displaying 16S : 23S rRNA ratios of 1.6 or higher were labelled and used for microarray experiments.

cDNA synthesis, labelling and hybridization. The Cyscribe Post-labelling kit (Amersham Biosciences) was used to synthesize and to label cDNA. Labelled cDNAs were hybridized on amplicon-based microarrays containing fragments of approximately 97 % of the genes of *L. plantarum* WCFS1 as described previously (Stevens *et al.*, 2008). Array design was submitted to the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under GEO accession no. GPL6368.

Scanning, data extraction and analyses. The slides were scanned with a ScanArray Express 4000 scanner (Perkin Elmer) and the images were analysed with Imagene software 4.2 (BioDiscovery). Primary data were submitted to GEO under accession number GSE11351.

Statistical analyses were performed with R (http://www.r-project.org/) using the linear models for microarray database limma (Smyth, 2005). Background-corrected spot intensities in both channels ($I1$ and $I2$) were converted to M-A coordinates, where $M=\log_2(I1/I2)$ and $A=\log_2(I1/I2)/2$ and subsequently normalized using a LOESS fit, assuming that, on average, M is independent of A and centred around 0 (Smyth, 2005). Normalized intensities were used for further analysis. Log odds for differential expression (B-value) higher than 1 were taken as a cut-off. As amplicons were spotted in duplicate, two measurements per gene were performed, and only genes of which both measurements matched the criteria mentioned above were taken into account.

Physiological characterization. The rate of lactate production was estimated by measuring the acidification rate in acidification buffer (0.5 mM potassium phosphate, 70 mM KCl and 1 mM $MgSO_4$), pH 6.4, containing 0.5 % (w/v) of a specified carbon source, as described previously (Poolman *et al.*, 1987). Cells were grown to OD_{600} 1.0, washed twice in acidification buffer to remove all residual carbon sources and suspended to a density of OD_{600} 1.0. After calibration of the cell suspension, a carbon source was added (t_0) and pH change was followed over time. Acidification rates were calculated for each suspension within the linear range using Microsoft Excel XP. The pH change was converted to H^+ production by calibration of the cell suspension with 2 μl aliquots of 50 mM HCl.

Transcription analysis of the mannose operon. RNA was isolated from exponentially growing *L. plantarum* WCFS1 cells cultured in CDM (Teusink *et al.*, 2005) supplemented with specific carbon sources as described above. Total RNA (5 μg) was blotted on a Gene Screen filter (New England Nuclear) as recommended by the manufacturer. An internal fragment of the *manIIC* gene was synthesized using the primers *manIIC-I-5′* and *manIIC-I-3′* (Table 2). The fragment was labelled with [α-^{32}P]ATP (Amersham Biosciences) by nick translation (Sambrook *et al.*, 1989) and used for hybridization at 65 °C in 6× SSC/0.2 % BSA for 2 h. Blots were washed 10 min with 6× SSC followed by 30 min washing with 1× SSC and finally with 0.1× SSC at 65 °C for 30 min prior to autoradiography.

Bioinformatic methodology. To identify putative σ^{54} promoters, the algorithm for fitting a mixture model by expectation maximization, MEME (Bailey & Elkan, 1994), was used on 17 experimentally verified σ^{54}-dependent promoters from *E. coli* (Reitzer & Schneider, 2001). MEME parameters were set as follows: one motif per sequence should be found (−mod oops) and only the given strand should be searched. The motif in the form of a position-specific scoring matrix (PSSM) was used to search the complete genome of *L. plantarum* WCFS1, using the search tool MAST (Bailey & Gribskov, 1998). The E-value cut-off used in MAST was 1.00×10^{-6}. Only motifs lying between the start codon and 300 bp upstream of the start codon were selected, and overlap with other genes was allowed.

Analyses of glycolytic intermediates. *L. plantarum* cells grown in CDM (Teusink *et al.*, 2005) containing 1 % glucose to an OD_{600} of 1.0 were harvested by centrifugation (3360 ***g***, 30 °C, 5 min), washed twice with CDM without carbon source and resuspended in CDM without carbon source at an OD_{600} of 5.0. Samples (10 ml) taken after 0, 15, 30, 60, 120 and 180 min were quenched in −40 °C 60 %

methanol/HEPES buffer as described by Pieterse *et al.* (2005). Quenched cells were harvested by centrifugation at 3500 ***g*** at −20 °C using a Sorvall RC5B Plus centrifuge and, to remove medium components, then washed once with quenching buffer (−40 °C) and once with ice-cold water. Subsequently, cells were stored overnight at −80 °C before lyophilization. The lyophilizate was resuspended in 500 µl water, and cell debris was removed by centrifugation (20 800 ***g***, 4 °C, 5 min) to obtain a cell-free extract. The extract was passed through a 0.22 µm filter before HPLC analysis. HPLC analyses were performed as described previously (Bhattacharya *et al.*, 1995; Boogaard, 2002), using an anion-exchange DX-300 column and a conductivity detector in combination with an anion self-regenerating suppressor (Dionex) to enhance signal-to-noise ratio. The flow-rate of the elution buffer was adjusted to 1 ml min^{-1} (half-speed) leading to doubled runtime (from 45 to 90 min), improving separation of the individual peaks.

RESULTS

In silico σ^{54} regulon prediction in *L. plantarum* WCFS1

To elucidate the role of σ^{54} in *L. plantarum*, we initially set out to predict the σ^{54} regulon in this species. To obtain a reliable prediction of the σ^{54} regulon, experimentally verified σ^{54}-dependent promoters from *E. coli* (Reitzer & Schneider, 2001) were used to construct a position-specific scoring matrix (PSSM; see Supplementary material, Table S1) using MEME (Bailey & Elkan, 1994). This PSSM was used to search the *L. plantarum* WCFS1 genome using MAST (Bailey & Gribskov, 1998). A limited set of significant hits was obtained (Table 3) and the best hit (significance score 5.6×10^{-9}) was a sequence encountered 123 bp upstream of an operon encoding genes of a putative mannose PTS (PTS9). The next-best hit had a drastically poorer score, but still remained within the significance cut-off employed here (Table 3).

RNAP containing σ^{54} requires an activator protein to initiate transcription (Sasse-Dwight & Gralla, 1988). These activators possess a unique σ^{54}-interaction domain, whose Pfam signature (accession no. PF00158) (Finn *et al.*, 2006; Studholme & Dixon, 2003) was used to search in bacterial genomes for σ^{54}-activator genes. Such a search revealed a single candidate σ^{54}-activator-encoding gene (probability $E=9 \times 10^{-64}$; Supplementary material, Fig. S1) in the *L. plantarum* genome (lp_0585), which was designated *manR*. Genes encoding σ^{54} activators are commonly genetically linked to the genes regulated by the activator. Notably, the σ^{54}-activator gene in the *L. plantarum* genome is located immediately upstream of a *manIIAB* gene cluster (lp_0586-0587, PTS10) that is predicted to encode components of a mannose PTS, but appears to be incomplete since it lacks a permease-encoding gene. Importantly, the predicted *L. plantarum* σ^{54}-activator is located downstream of the mannose PTS operon (PTS9), which is preceded by the best-scoring σ^{54}-dependent promoter sequence (Table 3,

Table 3. Predicted σ^{54}-dependent promoters in *L. plantarum* WCFS1

The position of the promoter (−12 region) in relation to the start codon is listed in the last column.

ORF	Gene	Product	Sequence	Score	Position
lp_0575	*pts9AB*	Mannose PTS, IIAB	TATTT **TGGC**ACGGAAT**TTGC** ATATA	5.6E–09	−123
lp_0562	*nagA*	*N*-Acetylglucosamine-6-phosphate deacetylase	CCTGG **TGGC**ACGACGG**TTGC** TGGAT	1.2E–07	−169
lp_2712		Transport protein	CGACT **TGGC**ATAAAAC**TTGC** CCATG	1.8E–07	−154
lp_1048	*rpsN*	Ribosomal protein S14	CAATT **TGGC**ATGCCAT**TTGC** TAAAT	3.6E–07	−27
lp_3070		Hypothetical protein	GCAAG **TGGC**ACAGATT**CTGC** ACCTG	4.0E–07	−271
lp_0220	*gpo*	Glutathione peroxidase	CGTGA **TGGC**ACCCCAG**TTGC** TATGA	6.6E–07	−149
lp_0586	*pts10A*	Mannose PTS, IIA	TAACT **TGGC**ATGCTTT**TTGC** ATGTA	1.5E–06	−45
lp_3688a	*rpmH*	Ribosomal protein L34	ACGAT **TGGC**ATACGAG**TTGC** GGGTC	1.5E–06	−170
lp_1541	*gnd*	Phosphogluconate dehydrogenase (decarboxylating)	TTTTT **TGGT**ACGATAG**TTGC** GTATT	1.7E–06	−28
lp_3331	*ISP2_5 N*	Transposase, N-terminal fragment	ATCTA **TGGC**ACAATAG**TTGA** TAAGT	1.9E–06	−273
lp_2613		ABC transporter, permease protein	GAACA **TGGC**ATGGAAC**TTGA** AGATT	2.1E–06	−38
lp_1110		Hypothetical protein	CGCGA **TGGC**ACGATTA**TTGA** TACGA	2.3E–06	−224
lp_1510	*coaE*	Dephospho-CoA kinase	CGGTG **TGGC**ACAATTA**TTGA** GAAAA	2.6E–06	−58
lp_2271	*mutS*	DNA mismatch repair protein Muts2	GATCA **TTGG**CACGGCA**TCTG** ACCGT	3.2E–06	−237
lp_2954		Integral membrane protein (putative)	GGTTA **TGGC**ATTGATT**TTGC** GCTAC	5.0E–06	−257
lp_3054		Aryl-alcohol dehydrogenase	GCGGT **TTAC**ATACTAT**TTGA** AACGA	5.0E–06	−286
lp_1395		Hypothetical protein	ATTGG **TGGA**ACGCAGG**TTGC** TAGTG	5.6E–06	−274
lp_1596	*efp*	Elongation factor P	ACGAC **TGGC**ACCTTAG**TTGC** AAACA	5.6E–06	−206
lp_2925		Cell surface protein precursor	GTTGC **TGGC**ACGTCTA**TTGA** CAGTC	5.6E–06	−154
lp_1319	*rsuA*	Pseudouridylate synthase	ATGAT **TGGA**ACGAAAG**CTGC** TGGGT	6.2E–06	−38
lp_1488	*hpk4*	Histidine protein kinase; sensor protein (putative)	CGTAG **TGGC**ATCGGCA**TTGC** CGGGC	6.2E–06	−264

Fig. 1). However, these genetic loci are separated in the *L. plantarum* WCFS1 genome by a 15 kb insertion encoding a non-ribosomal-peptide synthesis machinery (Fig. 1). This insertion is absent in other *L. plantarum* strains, as was concluded on the basis of genome-wide, array-based comparative genome hybridization (CGH) analysis of this species (Molenaar *et al.*, 2005).

In conclusion, the *in silico* analyses suggested a role for σ^{54} in transcriptional control of the mannose-PTS-encoding operon(s) found up- and downstream of the σ^{54}-activator-encoding gene. Nevertheless, these findings cannot exclude the involvement of σ^{54} in regulation of other target genes that are preceded by a promoter resembling the σ^{54}-dependent consensus sequence.

Mannose utilization in *L. plantarum* WCFS1

To study the σ^{54} regulon in *L. plantarum,* an *rpoN* deletion strain was constructed using a double crossover gene-replacement strategy, resulting in strain NZ7306 (*rpoN*::P_{32}*cat*, Table 1). Since in our *in silico* analysis the best-scoring σ^{54} promoter sequence was predicted upstream of the mannose PTS operon, and a σ^{54}-activator gene appeared to be located downstream of that same operon, we evaluated the ability of the *rpoN* deletion strain to grow on mannose. The wild-type strain had comparable growth rates in MRS medium supplemented with mannose or glucose as carbon source (Table 4). The *rpoN* deletion strain NZ7306 displayed growth characteristics similar to its parental strain when grown on MRS containing glucose. In contrast, when cells were grown on mannose the growth rate was reduced dramatically (Table 4), supporting a role for σ^{54} in the regulation of genes involved in mannose utilization in *L. plantarum*. Furthermore, biomass formation on MRS supplemented with mannose was at least two times lower by the mutant strain compared to the wild-type (Table 4).

There are two putative mannose PTS operons in *L. plantarum* WCFS1, of which one is complete (*manIIABCD* or *pts9ABCD*), while the second locus (*manIIAB* or *pts10AB*) appears to be truncated (Fig. 1). Both loci are preceded by a σ^{54} promoter sequence (Table 3) and genetically linked to the σ^{54}-activator gene. To characterize the role of these genes in mannose uptake and their postulated σ^{54} dependency, gene-deletion mutant strains were constructed of the putative regulator (*manR*), both mannose operons, and of the separate *manIIAB* locus, the *manIIB* gene and the permease-encoding *manIIC* gene present in the *manIIABCD* (PTS9) operon (Table 2, Fig. 1). Glucose- and mannose-dependent growth characteristics of all strains were determined (Table 4). All strains appeared to grow approximately equally well in MRS medium supplemented with glucose. In contrast, strains mutated in the *manIIABCD* (PTS9) operon or in the σ^{54}-activator gene *manR* displayed impaired growth and lower biomass yields on MRS supplemented with mannose (Table 4). The strain in which the *manIIAB* locus of PTS10 was deleted appeared to be unaffected in its capacity to grow on mannose-containing media. These experiments clearly establish that *L. plantarum* contains a single functional mannose PTS system, encoded by the genes lp_0575–0577 (*manIIABCD* or *pts9ABCD*), which is under control of σ^{54} in concert with the σ^{54}-activator, or mannose-operon regulator, ManR (lp_0585).

Comparative transcriptome analysis of wild-type *L. plantarum* and its *rpoN* derivative

To evaluate the accuracy of the predictions of the σ^{54} target sites, transcriptome profiles of exponentially growing wild-type cells were compared to those of the *rpoN*-mutant cells, both grown on MRS medium supplemented with glucose, using genome-wide, amplicon-based DNA-microarrays.

Microarray analysis identified nine genes that were expressed at a higher level in the wild-type compared to the *rpoN* mutant (Table 5). The *rpoN* gene showed higher expression in the wild-type, which is in agreement with deletion of the gene in the mutant strain (Table 5). The set of genes with highest relative expression in the wild-type was the *manIIABCD* genes of PTS9, confirming the involvement of σ^{54} in their transcriptional control. The other genes were expressed at a slightly higher level,

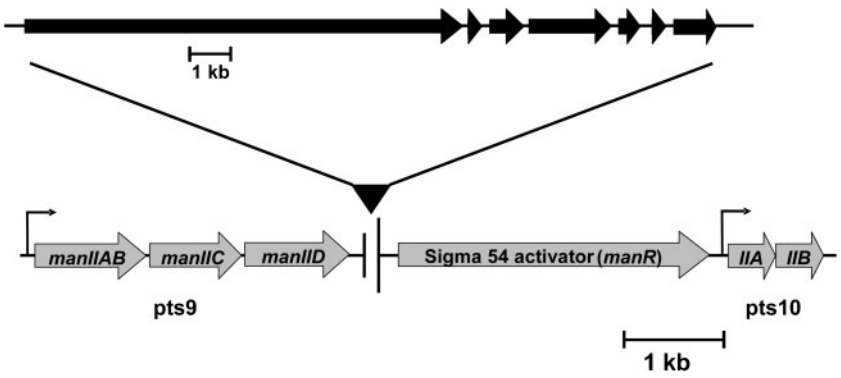

Fig. 1. Schematic representation of the mannose gene cluster in *L. plantarum* WCFS1. The 15 kb insertion encoding the non-ribosomal-peptide machinery is printed at a smaller scale. Predicted σ^{54}-dependent promoters are indicated.

Table 4. Growth rates of *L. plantarum* WCFS1 and its derivatives on different carbon sources

The maximal growth rates (μ_{max}) and optical densities after 24 h of culture are given for strains grown in MRS medium or in a chemically defined medium (CDM), both supplemented with 2 % (w/v) of the specific carbohydrate.

Strain	Glucose		Mannose	
	μ_{max}	OD_{600} 24 h	μ_{max}	OD_{600} 24 h
MRS				
WCFS1 (wild-type)	0.87 ± 0.02	6.84 ± 0.4	0.83 ± 0.03	6.25 ± 0.1
NZ7306 (*rpoN*)	0.80 ± 0.04	6.43 ± 0.4	0.14 ± 0.02	2.64 ± 0.3
NZ7307 (*manR*)	0.79 ± 0.01	6.08 ± 0.3	0.11 ± 0.01	2.38 ± 0.2
NZ7308 (*pts9C*	0.68 ± 0.00	6.03 ± 0.3	0.11 ± 0.00	2.12 ± 0.1
NZ7309 (*pts9AB*)	0.79 ± 0.02	6.15 ± 0.3	0.11 ± 0.01	2.53 ± 0.1
NZ7310 (*pts9B*)	0.77 ± 0.02	5.94 ± 0.1	0.11 ± 0.01	2.30 ± 0.2
NZ7311 (*pts9ABCD*)	0.77 ± 0.01	6.51 ± 0.3	0.11 ± 0.01	2.40 ± 0.1
NZ7312 (*pts10AB*)	0.88 ± 0.02	6.74 ± 0.1	0.64 ± 0.02	5.57 ± 0.1
CDM				
WCFS1 (wild-type)	0.74 ± 0.01	4.20 ± 0.3	0.65 ± 0.02	2.02 ± 0.1
NZ7306 (*rpoN*)	0.61 ± 0.02	2.72 ± 0.2	0.11 ± 0.01	1.02 ± 0.1
NZ7307 (*manR*)	0.63 ± 0.00	2.68 ± 0.0	0.11 ± 0.03	1.18 ± 0.1
NZ7308 (*pts9C*	0.61 ± 0.01	2.70 ± 0.1	0.16 ± 0.02	1.15 ± 0.1
NZ7309 (*pts9AB*)	0.64 ± 0.02	2.77 ± 0.3	0.10 ± 0.03	1.18 ± 0.1
NZ7310 (*pts9B*)	0.62 ± 0.01	2.66 ± 0.2	0.11 ± 0.02	1.06 ± 0.1
NZ7311 (*pts9ABCD*)	0.63 ± 0.03	2.90 ± 0.2	0.12 ± 0.01	1.07 ± 0.1
NZ7312 (*pts10AB*)	0.64 ± 0.02	2.91 ± 0.3	0.58 ± 0.06	2.45 ± 0.6

indicating that they were less affected by *rpoN* deletion. Except for the mannose operon, the genes with higher expression levels in the wild-type compared to its *rpoN* derivative were not preceded by σ^{54}-dependent promoters (Table 3), suggesting that their regulation is not directly dependent on σ^{54}.

In the *rpoN* mutant derivative, 23 genes showed higher expression compared to the wild-type. However, since sigma factors are not known to block transcription of genes, higher expression of these genes is most likely the result of secondary effects of the *rpoN* mutation.

The global transcription analyses strongly suggest that the σ^{54} regulon of *L. plantarum* consists of a single locus, the mannose operon *manIIABCD* encoding PTS9. This is in agreement with the particular significance of the predicted σ^{54} promoter sequence upstream of this locus and its genetic linkage to the cognate, activator-encoding gene *manR*. As a consequence the consensus sequence for σ^{54}-binding sites in *L. plantarum* is identical to the sequence upstream of the mannose operon encoded by lp_0575–0577 (see Table 3).

Characterization of the mannose PTS in *L. plantarum* WCFS1

The mannose operon under control of σ^{54} belongs to a separate family of PTSs, the mannose family, characterized by a unique IID enzyme and a fused IIAB enzyme (Saier & Reizer, 1992; Zuniga *et al.*, 2005). Although it is annotated as a mannose transporter, a similar PTS is also known to transport glucose in *E. coli* (Grenier *et al.*, 1985), and additional homologues are presumably the major glucose-uptake systems in lactic acid bacteria (Chaillou *et al.*, 2001). A similar role in *L. plantarum* would require expression of the mannose operon in cells grown on glucose, and therefore transcriptional analyses of the mannose PTS genes was performed in cells grown on different carbon sources. The mannose PTS appeared to be transcribed only in cells growing on glucose or mannose, and not in cells growing on lactose, maltose, fructose, cellobiose or sucrose (Fig. 2), supporting a role for the mannose PTS in mannose and glucose transport.

Involvement of the mannose PTS in glucose transport could lead to decreased glucose uptake in strains lacking this PTS, as already described in *Listeria monocytogenes* and *Lactobacillus pentosus* (Chaillou *et al.*, 2001; Vadyvaloo *et al.*, 2004). When cells were grown on glucose-containing MRS, only a very minor, but consistent, reduction of the growth rate was observed in any of the mutants affected in mannose PTS expression (*rpoN*, *manR*, *manIIABCD*) compared to the wild-type (Table 4). However, this difference in growth rates was more pronounced when cells were grown in chemically defined medium (CDM), again supporting a role for the mannose PTS in glucose import (Table 4).

Reduced glucose uptake rates in *L. plantarum* should be reflected in decreased rates of lactate formation rates, which can be evaluated by measuring the acidification rate

Table 5. Genes significantly up- and downregulated in the wild-type compared to the mutant NZ7306

Putative operons are indicated in bold.

ORF	Gene name	Product	Ratio (log_2)	*P*-value (FDR)	*B*-value
Upregulated					
lp_0179	*amy2*	α-Amylase	−1.73	1.85E–02	1.02
lp_0180	*msmK1*	Multiple sugar ABC transporter, ATP-binding protein	−1.47	7.43E–03	2.63
lp_0181	*map2*	Maltose phosphorylase	−2.22	9.11E–03	2.23
lp_0575	*pts9AB*	Mannose PTS, EIIAB	−4.45	1.35E–03	5.18
lp_0576	*pts9C*	Mannose PTS, EIIC	−3.81	7.55E–04	8.00
lp_0577	*pts9D*	Mannose PTS, EIID	−4.68	1.29E–03	5.53
lp_0693	*nrdE*	Ribonucleoside-diphosphate reductase, α chain	−1.52	1.67E–03	4.81
lp_0787	*rpoN*	DNA-directed RNA polymerase, σ^{54}	−1.86	1.80E–02	1.06
lp_1730	*map3*	Maltose phosphorylase	−1.34	7.43E–03	2.64
Downregulated					
lp_0199		Unknown	1.02	9.53E–03	2.11
lp_0291		Oxidoreductase	1.47	3.98E–03	3.48
lp_0788	*cggR*	Central glycolytic genes regulator	1.20	6.48E–03	2.88
lp_1670	*fabZ1*	(3*R*)-Hydroxymyristoyl-(acyl carrier protein) dehydratase	2.68	1.22E–03	7.12
lp_1671	*fabH2*	3-Oxoacyl-(acyl-carrier protein) synthase III	2.36	8.23E–03	2.47
lp_1672	*acpA2*	Acyl carrier protein	2.85	1.67E–03	4.70
lp_1673	*fabD*	(Acyl-carrier protein) *S*-malonyltransferase	2.46	8.23E–03	2.42
lp_1674	*fabG1*	3-Oxoacyl-(acyl-carrier protein) reductase	2.14	1.48E–02	1.41
lp_1675	*fabF*	3-Oxoacyl-(acyl-carrier protein) synthase II	2.56	1.34E–03	5.31
lp_1676	*accB2*	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	2.30	1.29E–03	5.45
lp_1677	*fabZ2*	(3*R*)-Hydroxymyristoyl-(acyl carrier protein) dehydratase	2.17	7.18E–03	2.75
lp_1678	*accC2*	Acetyl-CoA carboxylase, biotin carboxylase subunit	2.16	2.90E–03	3.95
lp_1679	*accD2*	Acetyl-CoA carboxylase, carboxyl transferase subunit β	1.94	1.49E–02	1.38
lp_1680	*accA2*	Acetyl-CoA carboxylase, carboxyl transferase subunit α	1.81	9.53E–03	2.14
lp_1681	*fabI*	Enoyl-(acyl-carrier protein) reductase (NADH)	2.12	1.49E–02	1.31
lp_1682		Phosphopantetheinyltransferase	2.28	1.22E–03	5.78
lp_1684		Integral membrane protein	2.05	1.57E–03	4.99
lp_1685		Transcription regulator	2.09	1.22E–03	6.44
lp_1686		Acyl-CoA thioester hydrolase (putative)	1.81	1.67E–03	4.67
lp_1695		Integral membrane protein	1.70	1.40E–02	1.59
lp_1708		Unknown	2.49	3.42E–03	3.69
lp_3256		DegV family protein	1.89	1.22E–03	5.83
lp_3487	*galM3*	Aldose 1-epimerase	1.05	8.23E–03	2.39

in a weakly buffered cell suspension (Poolman *et al.*, 1987). Wild-type cells were grown on either glucose or mannose, harvested and resuspended in the acidification assay buffer. Both suspensions acidified when glucose was added [acidification rate of 238 ± 12.4 and 168 ± 2.8 nmol protons (mg protein)$^{-1}$ s^{-1}, in glucose and mannose pre-grown cells, respectively]. This result primarily indicates that cells grown on mannose contain a complete glucose transport

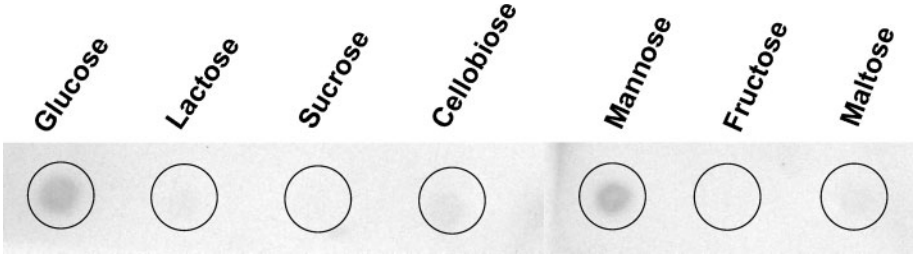

Fig. 2. Transcription analysis of the mannose operon on different carbohydrates. Total RNA from exponentially growing cells was spotted and an internal 300 bp fragment of the *manIIC* gene was used as probe. The carbohydrates are indicated above the spots.

and utilization machinery, which also supports a role for the mannose PTS in glucose import in *L. plantarum* WCFS1.

The acidification rate observed in glucose-containing assay buffer for glucose-grown NZ7306 (Δ*rpoN*) cells was significantly reduced compared to that of glucose-grown wild-type cells [acidification rate 160 ± 8.5 nmol protons (mg protein)$^{-1}$ s^{-1}, versus 238 ± 12.4 nmol protons (mg protein)$^{-1}$ s^{-1} observed for the wild-type], indicating a reduced glycolysis rate in the *rpoN* mutant. In contrast to wild-type cells, the glucose-pre-grown NZ7306 cells were not able to convert mannose to lactate, confirming the crucial role of *rpoN* in regulation of mannose utilization in *L. plantarum*.

Overall, these experiments support an important role for the mannose PTS (PTS9) of *L. plantarum* in glucose uptake.

Pleiotropic effects of the mannose PTS on overall carbohydrate metabolism

Apart from mannose and glucose, other carbohydrates have also been reported to be transported by homologues of the mannose PTS family (Vadeboncoeur & Pelletier, 1997). The availability of a mutant (*manIIABCD*::P_{32}*cat*) provided the opportunity to evaluate the substrate spectrum of the *L. plantarum* mannose PTS system extensively. To this end, growth characteristics of the mutant on a range of carbon sources were monitored and compared to those of the parental strain. The mutant and wild-type strains were pre-cultured on glucose, washed twice to remove all traces of glucose and inoculated in fresh media at an OD_{600} of approximately 0.05. To minimize the effects of undefined medium composition, cells were grown in CDM (Teusink *et al.*, 2005). The maximal growth rate of the wild-type and NZ7306 were similar for a range of carbon sources, including cellobiose, maltose, *N*-acetylglucosamine and sucrose (data not shown). However, significant differences were observed between the wild-type and mutant strain when they were transferred to medium containing galactose as the sole carbon and energy source. The wild-type strain WCFS1 displayed a growth arrest that lasted for 20–30 h (Fig. 3), irrespective of the pre-culture growth phase at the time of transfer [stationary phase of growth (overnight culture), or exponential growth phase (OD_{600} 1.0), data not shown]. Notably, the growth initiation delay of the wild-type cells when transferred to galactose was not observed when a trace amount (0.001 %, w/v) of glucose was added to the galactose-containing medium (Fig. 3). In contrast, all mutant strains that lack mannose PTS expression (NZ7306, NZ7307, NZ7308, NZ7309, NZ7310 and NZ7311; see Table 1) were able to initiate growth immediately after inoculation (illustrated for strain NZ7311 in Fig. 3), suggesting involvement of the mannose PTS in repression of galactose utilization. The direct involvement of mannose PTS expression in the delayed growth initiation in the wild-type was further supported by the observation that wild-type cells pre-cultured on CDM with maltose (a carbon source that does not lead to mannose PTS expression; see above) started to grow on galactose immediately after the medium transfer (Fig. 3).

The mannose PTS drains the PEP pool

Since no differential expression of galactose utilization genes was found in the comparative transcriptome analysis of the wild-type and its *rpoN* derivative, the regulation of galactose catabolism could be due to post-transcriptional regulation. This could possibly involve phosphorylation of the *L. plantarum* LacS-type galactose transporter by PEP, as has been described in *Streptococcus thermophilus*. In this bacterium galactose is taken up by a lactose/galactose transport protein (LacS), which catalyses two modes of transport: solute-H^+ symport and lactose/galactose antiport (Foucaud & Poolman, 1992). The streptococcal LacS possesses a PTS-IIA-like domain, and phosphorylation of this domain led to an increased antiporter transport rate, while it did not affect its symporter-mode transport rate

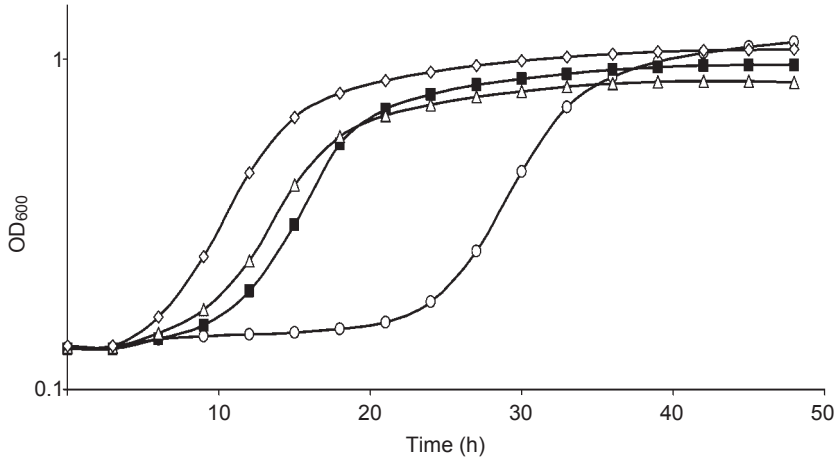

Fig. 3. Growth of *L. plantarum* WCFSI and its derivatives on galactose. The wild-type strain WCFS1 (○) shows a prolonged growth arrest compared to the mannose PTS deletion mutant NZ7311 (◇) after transfer from glucose-containing to galactose-containing CDM. Strain WCFS1 grown on maltose (■) or activated with glucose (△) initiates growth immediately after inoculation. For details see text.

(Poolman *et al.*, 1995). *L. plantarum* has two putative galactose transporters (*lasS1* and *lacS2*) that share significant similarity with each other (38 % identity) and with the galactose/lactose transporter of *S. thermophilus* (37 % and 56 % identity, respectively). Furthermore, both transporters have a phosphate-accepting IIA domain, suggesting that a similar regulatory mechanism as has been observed in *S. thermophilus* might affect transport modes of the *L. plantarum* homologues.

To investigate a potential role of LacS phosphorylation, the intracellular concentration of PEP was monitored in *L. plantarum* wild-type and the ManPTS mutant strain when grown in glucose-containing CDM followed by carbon starvation. To exclude the possibility of mannose PTS expression due to any leakage of the *rpoN*-dependent promoter, these measurements were performed using the *manIIABCD* deletion strain NZ7311 and the permease (*manIIC*) deletion strain NZ7308 (Table 1). In samples taken from wild-type and NZ7311 cultures growing on CDM containing glucose, no PEP could be detected, indicating that intracellular PEP concentrations during growth are low (data not shown). To monitor PEP pool development upon carbon starvation, cells were grown to mid-exponential growth phase, harvested, washed, and resuspended in medium without a carbon source. Samples were taken from these suspensions at different time points after the medium transfer, and intracellular PEP concentrations were determined. In the wild-type strain, no PEP could be detected in any of the samples, indicating that this strain fails to accumulate PEP upon carbon starvation (Fig. 4). In contrast, in the mutant strains NZ7308 and NZ7311, PEP was detected immediately after transfer to the starvation medium, and appeared to be maintained at a concentration of 1–3 μM up to several hours after carbon starvation (Fig. 4). These results suggest that the expression of the mannose PTS significantly affects the capacity of the wild-type cells to accumulate PEP during starvation, which may hamper initiation of growth on galactose due to a lack of phosphate-donor molecules required for activation of the galactose transporter by LacS-IIA domain phosphorylation. In contrast, the relatively high PEP pools in resting cells of the mutant strain would support LacS activation, allowing immediate growth initiation upon transfer to galactose-containing media. The observation that trace amounts of glucose facilitated growth initiation of the wild-type strain on galactose can be explained by the production of PEP from glucose and the corresponding LacS activation under these conditions.

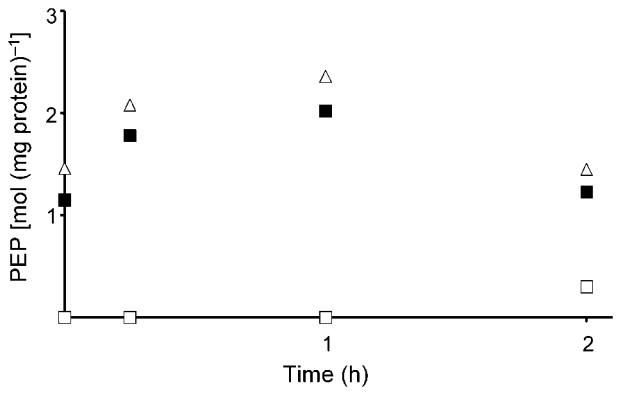

Fig. 4. Effect of mannose PTS deficiency on intracellular PEP pools: concentration of intracellular PEP in carbon-starved resting cells of *L. plantarum* wild-type (□) and its mannose PTS-mutant derivatives NZ7311 (■) and NZ7308 (△). Mutants NZ7311 and NZ7308 differ significantly from the wild-type ($P=3.66\times10^{-5}$ and 1.0×10^{-4} respectively), but not from each other ($P=0.19$).

Overall, these results indicate that the mannose PTS system of *L. plantarum* plays a pivotal role in carbon metabolism control. This role clearly exceeds the 'simple' role that is predicted on the basis of its annotated function, e.g. transport of mannose (and glucose), and includes control of central carbon metabolism by affecting the relative levels of important glycolytic intermediates such as PEP, which in their turn control the capacity to initiate growth on certain other carbon sources.

DISCUSSION

Regulation of gene expression by alternative sigma factors is a well-established mechanism of adaptation to environmental conditions in bacteria. Here we describe the prediction and validation of the σ^{54} regulon in *L. plantarum* WCFS1. The conserved binding sequence of this sigma factor enabled a prediction of the specific regulon using the pattern recognition algorithms MEME and MAST (Bailey & Elkan, 1994; Bailey & Gribskov, 1998). The prediction was verified by comparative whole-genome transcriptome analysis of the *L. plantarum* wild-type and its σ^{54}-mutant derivative. The regulon was concluded to be restricted to the mannose PTS operon, which is in agreement with the genetic linkage between the gene encoding the σ^{54}-activator protein and the target locus of the mannose PTS.

The relatively higher expression in the wild-type strain of genes involved in maltose utilization (Table 5) indicates involvement of σ^{54} in control of utilization of other carbohydrates. However, the predicted regulation of the maltose genes by a LacI-transcription regulator (Francke *et al.*, 2008), and the virtually identical growth characteristics of the σ^{54} mutant and the wild-type strain on CDM supplemented with maltose (data not shown), contradict such a role for σ^{54}. The higher expression in the wild-type of a ribonucleotide reductase, an enzyme that catalyses the reduction of ribonucleosides, thereby providing the building blocks required for DNA replication, is possibly caused by the higher growth rate of the wild-type. Careful manual inspection of upstream regions of the genes showing higher expression failed to identify candidate σ^{54}-dependent promoters (Table 3), and their regulation is

therefore likely to be due to unidentified secondary effects and not directly dependent on σ^{54}.

The gene located immediately downstream of *rpoN*, the central glycolytic gene regulator (lp_0788; *cggR*), is expressed at a higher level in the mutant, which is probably due to readthrough of the P_{32} promoter upstream of the *cat* gene that replaces *rpoN* in the mutant. CggR probably regulates the expression of four glycolytic genes in *L. plantarum* (*gap*, *pgk*, *tpi*, *eno*) that lie immediately downstream of *cggR*. However, no differential expression of these glycolytic genes was observed in the transcriptome analyses, indicating that the effect of promoter readthrough is minimal. Furthermore, preliminary results in our laboratory suggest that overexpression of *cggR* does not affect the expression of glycolytic genes in strain WCFS1 (I. Rud and others, unpublished results). However, to exclude that any polar effects of the *rpoN* mutation were confounding the PEP measurements, the experiments to elucidate the role of the mannose PTS in modulation of the intracellular PEP pools were performed with mannose-PTS-deficient mutants.

The operon encoding the fatty acid biosynthesis machinery (lp_1670–1681) showed higher expression in the σ^{54} mutant, probably due to the decreased glycolytic rates observed in this strain, which may lead to an altered flux to malonyl-CoA, an essential intermediate in fatty acid biosynthesis which relieves repression of the *fab* genes in *B. subtilis* (Schujman *et al.*, 2006). The apparently higher expression in the mutant of an oxidoreductase encoded by ORF lp_0291 is an artefact due to the presence of a 189 bp fragment of this gene on the plasmid used for cloning, which is located directly downstream of the *cat* gene (Lambert *et al.*, 2007). Overall, the increased expression of these genes in the *rpoN* mutant is most likely due to secondary effects, which is in agreement with the assumption that sigma factors are generally not involved in the repression of genes.

The inability of the σ^{54}-mutant strain NZ7306 to grow on mannose as sole carbon source showed that transcription of the mannose operon is strictly σ^{54}-dependent, which is in agreement with the observation that σ^{54}-regulated genes are generally not influenced by other transcription factors (Buck *et al.*, 2000). Growth analyses of strains mutated in the mannose PTS showed that ManIIABCD is the only functional mannose transporter in *L. plantarum*.

Regulation of the mannose operon by σ^{54} has already been described in the Gram-positive bacteria *Enterococcus faecalis*, *Listeria monocytogenes* and *Lactobacillus casei* (Dalet *et al.*, 2001; Hechard *et al.*, 2001; Yebra *et al.*, 2004), indicating that this mode of regulation is conserved among some Gram-positive bacteria. In *Ent. faecalis* and *Lis. monocytogenes* mutation of the σ^{54}-encoding gene led to resistance to mesentericin Y105, a class II bacteriocin produced by *Leuconostoc mesenteroides*, which was shown to be due to impaired *manIIC* expression (Dalet *et al.*, 2000; Ramnath *et al.*, 2004; Robichon *et al.*, 1997). Additional studies proposed a conserved mechanism for bacteriocin sensitivity in which ManIIC acts as a docking protein for antimicrobial peptides (Diep *et al.*, 2007). However, *L. plantarum* appeared to be resistant to mesentericin Y105 (M. J. A. Stevens, unpublished data), suggesting that the *L. plantarum* mannose PTS does not act as a docking protein for this bacteriocin.

Homologues of the mannose PTS studied in this paper are found throughout the bacterial kingdom. It has been shown that this PTS also transports glucose and it is additionally thought to be important as a glucose PTS in several lactic acid bacteria (Chaillou *et al.*, 2001). The observation that mannose PTS deletion leads to a decreased growth rate on glucose has already been described for *Lis. monocytogenes* and *Lactobacillus pentosus* (Chaillou *et al.*, 2001; Vadyvaloo *et al.*, 2004). Our data provide further evidence for glucose transport by the mannose PTS in *L. plantarum*. Notably, the codon adaptation index of the mannose-PTS-encoding genes was high, suggesting that this transport system can be produced at a high level (Kleerebezem *et al.*, 2003); this is in agreement with its importance in uptake of favourable sugars such as glucose.

The role of the mannose PTS in glucose transport suggests a role of this PTS in canonical systems for control of carbon utilization such as carbon catabolite repression (Chaillou *et al.*, 2001). Indeed, this has been shown in the close relative of *L. plantarum*, *L. casei*, in which the mannose PTS regulates lactose operon expression via terminator modulation; only strains lacking CcpA and mannose PTS activity are able to express the lactose operon (Chaillou *et al.*, 2001; Gosalbes *et al.*, 1997). However, the expression of both *lacS1* and *lacS2* in a strain lacking a functional CcpA but still harbouring a mannose PTS indicates a different mechanism in *L. plantarum* (Stevens, 2008). Since we did not observe an effect in the transcription analysis, the regulatory role of the mannose PTS seems to be exerted at another level, possibly involving metabolic control of transport.

Phosphorylation of a IIA-like domain of the *S. thermophilus* antiporter/symporter LacS leads to an increased antiporter transport rate, whereas symporter transport rate is not affected (Poolman *et al.*, 1995). The antiporter mode (galactose/lactose exchange) is the most relevant transport mode as it is much faster than the proton-motive force (PMF)-driven symporter (lactose/H^+) mode (Knol *et al.*, 1996). The wild-type cells in our experiment were depleted of metabolic energy, as no PEP could be detected in these cells. Consequently, the prolonged growth arrest could be due to inability to build up the PMF needed for galactose transport initiation. The addition of a trace amount of glucose to resting cells (as in our experiments) has been shown to lead to changes in the concentrations of glycolytic intermediates (Neves *et al.*, 2002) and to the generation of a PMF (Poolman *et al.*, 1995), enabling galactose/H^+ transport and eventually growth initiation.

Cells mutated in the mannose PTS maintain a high intracellular PEP pool. Phosphorylation of LacS is

(His~P)-HPr and PEP-dependent (Gunnewijk & Poolman, 2000); hence it is likely that the high PEP pool in the mutant strain leads to a permanent LacS-(IIA-P) state, allowing efficient galactose import immediate after inoculation.

At first sight, it may seem that σ^{54} has only a minor role in *L. plantarum* since only the mannose operon is transcribed from a σ^{54}-dependent promoter. However, the mannose PTS operon encodes a glucose-uptake system in *L. plantarum* and σ^{54}-dependent transcription allows expression of the operon at high level without the involvement of additional transcriptional regulators in glucose-containing media. Thereby, σ^{54} exerts metabolic control via the strict control of mannose PTS expression, which affects the concentration of PEP in carbon-starved cells, thereby influencing the energy state of these cells and modulating their capacity to initiate growth on other carbon sources, such as galactose. This metabolic control is apparently directly linked to glucose transport, and the conservation of mannose PTS regulation by σ^{54} in other Gram-positive bacteria suggests similar control in other lactic acid bacteria and *Listeria* spp.

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