microbial biotechnology

Microbial Biotechnology (2010) **4**(3), 345–356 doi:10.1111/j.1751-7915.2010.00223.x

Functional analysis of the role of CggR (central glycolytic gene regulator) in *Lactobacillus plantarum* by transcriptome analysis

Ida Rud,[1,2] Kristine Naterstad,[1] Roger S. Bongers,[3] Douwe Molenaar,[3] Michiel Kleerebezem[3,4] and Lars Axelsson[1]*

[1]*Nofima Mat, Osloveien 1, N-1430 Ås, Norway.*
[2]*Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PO Box 5003, N-1432 Ås, Norway.*
[3]*TI Food & Nutrition; NIZO food research, PO Box 20, 6710 BA Ede, the Netherlands.*
[4]*Wageningen University, Laboratory of Microbiology, Dreijenplein 10, Wageningen, the Netherlands.*

Summary

The level of the central glycolytic gene regulator (CggR) was engineered in *Lactobacillus plantarum* NC8 and WCFS1 by overexpression and in-frame mutation of the *cggR* gene in order to evaluate its regulatory role on the glycolytic *gap* operon and the glycolytic flux. The repressor role of CggR on the *gap* operon was indicated through identification of a putative CggR operator and transcriptome analysis, which coincided with decreased growth rate and glycolytic flux when *cggR* was overexpressed in NC8 and WCFS1. The mutation of *cggR* did not affect regulation of the *gap* operon, indicating a more prominent regulatory role of CggR on the *gap* operon under other conditions than tested (e.g. fermentation of other sugars than glucose or ribose) and when the level of the putative effector molecule FBP is reduced. Interestingly, the mutation of *cggR* had several effects in NC8, i.e. increased growth rate and glycolytic flux and regulation of genes with functions associated with glycerol and pyruvate metabolism; however, no effects were observed in WCFS1. The affected genes in NC8 are presumably regulated by CcpA, since putative CRE sites were identified in their upstream regions. The interconnection with CggR and CcpA-mediated control on growth and metabolism needs to be further elucidated.

Received 1 June, 2010; accepted 14 September, 2010. *For correspondence. E-mail lars.axelsson@nofima.no; Tel. (+47) 64 97 01 00; Fax (+47) 64 97 03 33.

Introduction

Lactobacillus plantarum is one of the most versatile and flexible lactic acid bacteria (LAB) and is encountered in a variety of niches (e.g. in plant material, meat, dairy products and the human gastrointestinal tract). A variety of strains of this species is used as starter cultures in the food industry, primarily aimed at preservative effects through the production of lactic acid, but also contributing to flavour and texture of the fermented food. Some strains have also shown to have probiotic effects in humans and animals (de Vries *et al*., 2006). The important role of *L. plantarum* in food fermentation and in the human gastrointestinal tract makes it an important and interesting species to investigate in terms of metabolic control, including genetic regulation mechanisms involved in carbon metabolism. In addition, the process of production of lactic acid by LAB is of general interest because of its clear biotechnological relevance, not only on basis of its use as food preservative, but also based on its use as precursor for biodegradable polymers (Singh *et al*., 2006).

Lactobacillus plantarum is a facultative heterofermentative LAB fermenting hexoses via glycolysis and pentoses via the phosphoketolase pathway that funnels into glycolysis at the central metabolite, glyceraldehyde-3-phosphate (Axelsson, 2004). Interestingly, four of the central glycolytic genes of *L. plantarum* are organized in a glycolytic operon (*gap* operon; *cggR-gap-pgk-tpi-enoA1*), encoding enzymes that catalyse steps of the central glycolysis, and the putative central glycolytic gene regulator (CggR) (Kleerebezem *et al*., 2003; Naterstad *et al*., 2007). The operon organization of the glycolytic genes facilitates efficient and concerted regulation of expression of these essential enzymes. In addition, more specific regulation of *gap* and *enoA1* transcription has been suggested by detection of their mono-cistronic expression (Naterstad *et al*., 2007).

The role of CggR has not been elucidated for *L. plantarum*. In *Bacillus subtilis*, the CggR function as repressor of the *gapA* operon (Fillinger *et al*., 2000) by binding to an operator between the promoter and the *cggR* start codon (Doan and Aymerich, 2003). *Bacillus subtilis* has a similar organization of the *gapA* operon compared with *L. plantarum*, but it is transcribed hexacistronic (*cggR-gapA-pgk-tpi-pgm-eno*) with the transcriptional start site identified

upstream *cggR* (Ludwig *et al*., 2001). Near the 3′ end of *cggR*, the transcript is processed, resulting in a stable transcript of the glycolytic genes while the *cggR* transcript is rapidly degraded (Ludwig *et al*., 2001; Meinken *et al*., 2003). Fructose-1,6-bisphosphate (FBP) has been identified as the effector molecule of CggR, acting as inhibitor of CggR DNA-binding activity when the cells are growing on carbohydrates that are metabolized into FBP (Doan and Aymerich, 2003; Zorrilla *et al*., 2007).

FBP is also a major signal for one of the global regulatory control proteins, catabolite control protein A (CcpA), involved in carbon catabolite repression (CCR) in Gram-positive bacteria (Deutscher *et al*., 1995; Stulke and Hillen, 1999; Bruckner and Titgemeyer, 2002; Titgemeyer and Hillen, 2002). CcpA activity involves binding to a conserved DNA sequence called catabolite-responsive element (CRE), thereby either activating or repressing gene expression, depending on the position of the CRE site with respect to the promoter sequence (Weickert and Chambliss, 1990). The HPr protein of the PTS systems is an important cofactor for CcpA binding when it is phosphorylated at the Ser-46 residue, and FBP and glucose-6-phosphate (G6P) have been shown to enhance HPr-Ser-P-mediated binding of CcpA to CRE (Deutscher *et al*., 1995; Gosseringer *et al*., 1997; Seidel *et al*., 2005).

In *L. plantarum*, the role of CcpA for CCR has also been established (Muscariello *et al*., 2001) and CRE sites presumed to mediate CcpA regulation of genes encoding proteins responsible for sugar uptake and cell-surface proteins have been identified (Andersson *et al*., 2005; Siezen *et al*., 2006). Besides that, knowledge on glycolytic regulation and control is limited in *L. plantarum* and in lactobacilli in general. In contrast, the regulation of glycolysis and carbon flux has been studied extensively in *Lactococcus* (*Lc.*) *lactis*, which can be regarded as the paradigm LAB. Organization of the glycolytic genes in *Lc. lactis* is very different compared with the lactobacilli, since the *cggR* gene is lacking and most of the glycolytic genes in *Lc. lactis* are not genetically linked (Bolotin *et al*., 2001). One exception is the *las* operon encoding phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH), which has shown to be transcriptionally activated by CcpA (Luesink *et al*., 1998). The PFK- and PK-encoding genes are also organized in an operon in *L. plantarum* (Kleerebezem *et al*., 2003) but without LDH. Interestingly, studies in *Lc. lactis* where the level of several of the glycolytic enzymes were engineered showed that neither PFK (Koebmann *et al*., 2005), triosephosphate isomerase (Solem *et al*., 2008), glyceraldehyde-3-phosphate dehydrogenase (Solem *et al*., 2003), phosphoglycerate enolase (Koebmann *et al*., 2006), PK (Koebmann *et al*., 2005) or LDH (Andersen *et al*., 2001) have any control on the glycolytic flux in *Lc. lactis*. Moreover, the ATP-consuming processes exert no control on the glycolytic flux in *Lc. lactis* (Koebmann *et al*., 2002), which is in contrast to *L. plantarum*, where the ATP-consuming processes to a large extent control the metabolic fluxes (i.e. of glycolysis and ribolysis) (Rud *et al*., 2008). These studies indicate a different mode of regulation of glycolysis in *Lc. lactis* and *L. plantarum*, which might also be reflected by the different organization of the glycolytic genes of the two species and could include a regulatory role of CggR in *L. plantarum*.

In this report, we aim to present a post-genomic description of the role of CggR by engineering the level of CggR through mutation and overexpression of the *cggR* gene in two different *L. plantarum* strains, NC8 and WCFS1. The repressor role of CggR on the *gap* operon was indicated through *in silico* analyses, in addition to transcriptome and physiological analyses in the *cggR*-overexpressed strains of NC8 and WCFS1. Mutation of the *cggR* gene had only effects in NC8, where the growth rate and glycolytic flux increased and genes involved in glycerol and pyruvate metabolism were affected, presumably regulated by CcpA. It was speculated that CggR also regulates other targets than the *gap* operon in NC8, and that the *gap* operon in the wild-type strains of NC8 and WCFS1 was maximally expressed under the conditions tested.

Results

In this study, the role of the central glycolytic gene regulator (CggR) in *L. plantarum* has been analysed in two different strains, NC8 and WCFS1, by engineering of the *cggR* gene expression level. Construction of the *cggR* null-mutant derivatives was successfully achieved by double-cross-over mutagenesis using the Cre-*lox*-based mutagenesis system developed for *L. plantarum* WCFS1 (Lambert *et al*., 2007) (Table S1 and S4 in *Supporting information*). In addition, strains with constitutive overexpression of *cggR* (*cggR*-P25) were constructed in the pSIP409 vector harbouring a synthetic promoter upstream the *cggR* gene (Table S1) (Rud *et al*., 2006). Physiological and genome-wide transcriptional effects (transcriptome) of the *cggR*-engineered strains were investigated during growth on glucose or ribose.

Organization and putative regulation elements of the cggR *gene/*gap *operon*

The organization of the *gap* operon, including the *cggR* gene, was compared between *L. plantarum* and *B. subtilis*, showing high similarities, although the *pgm* gene was missing in the *gap* operon of *L. plantarum* (Fig. 1A). Promoter prediction analysis of the *cggR* gene in *L. plantarum* revealed a close to perfect putative promoter (Fig. 1B) and with high similarity to that identified for the

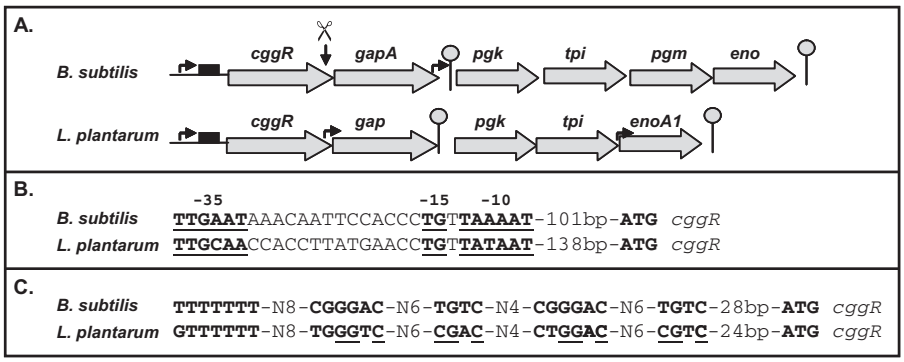

Fig. 1. Comparison between *L. plantarum* and *B. subtilis* in relation to *gap* operon and putative regulation sites upstream the *cggR* gene.
A. Organization of the *gap* operon (Ludwig *et al*., 2001; Naterstad *et al*., 2007). Promoters and rho-independent terminator structures are indicated by small arrows and loops respectively. CggR operators are shown as black boxes. Processing site of *cggR* in *B. subtilis* is indicated by a scissor.
B. Promoter prediction of the *cggR* gene in *L. plantarum* compared with *B. subtilis* (Ludwig *et al*., 2001). Consensus sequences (–35 and –10) and TG motifs are underlined. Distances to the *atg* start of *cggR* are indicated.
C. Comparison of the putative CggR operator of *L. plantarum* with the CggR operator of *B. subtilis* (Doan and Aymerich, 2003). Direct repeats in *L. plantarum* are underlined. Distances between the repeats and distances to the *atg* start of *cggR* are indicated.

cggR gene in *B. subtilis* (Ludwig *et al*., 2001). This putative promoter also contained a TG motif in position –15 previously shown to be conserved in 16S rRNA promoters of *L. plantarum* (Rud *et al*., 2006). Sequence analysis upstream of the *cggR* gene also revealed direct repeats showing homology to the similar area in *B. subtilis* (Fig. 1C).

Physiology of the cggR*-engineered strains*

The growth rate and metabolic fluxes of the *cggR*-engineered strains of *L. plantarum* (NC8 and WCFS1) were measured on either glucose or ribose as carbon source (Table 1). Higher growth rate was observed for all the strains when grown on glucose compared with ribose and the wild-type strain of WCFS1 grew faster than the wild-type strain of NC8. Interestingly, introduction of a *cggR* deletion in NC8 increased the growth rate and metabolic fluxes (in between 105% and 118%) compared with the wild-type strain; however no such effects were observed for WCFS1 when *cggR* was deleted (Table 1). In contrast, *cggR* overexpression (*cggR*-P25) in both NC8 and WCFS1, verified by GusA reporter activities (> 250 MU), led to a significant reduction of growth rates and metabolic fluxes compared with the parental strains (below 80%), which appeared to be independent of the carbon source used (Table 1). Notably, no other differences in growth characteristics between the strains (e.g. lag phase) were observed (data not shown).

Global transcriptome analysis

The global transcriptome responses of *cggR*-engineered *st*rains of *L. plantarum* (NC8 and WCFS1) during growth on glucose or ribose were determined using oligonucleotide-based whole-genome microarrays based on the WCFS1 genome sequence (GEO Accession No. GPL4318) (Kleerebezem *et al*., 2003) with a loop design

Table 1. Growth rate and metabolic fluxes of *cggR*-engineered strains of *L. plantarum* NC8 and WCFS1 during glucose or ribose fermentation.

		Growth rate (h^{-1}/% relative to wild type)		Glycolytic flux (mmol*h^{-1}*gdw/% relative to wild type)		Lactate flux (mmol*h^{-1}*gdw/% relative to wild type)	
Carbon source	Strain	NC8	WCFS1	NC8	WCFS1	NC8	WCFS1
Glucose	Wild type	0.48/100 ± 0	0.53/100 ± 0	9.6/100 ± 5	10.1/100	18.6/100 ± 1	19.6/100
	cggR mutant	0.52/109 ± 1	0.52/98 ± 0	11.3/118 ± 4	9.6/95	21.1/113 ± 2	18.7/95
	cggR-P25	0.36/76 ± 10	0.41/77 ± 2	6.9/72 ± 14	7.4/74	11.7/63 ± 4	14.4/73
Ribose	Wild type	0.31/100 ± 0	0.32/100 ± 0	ND	ND	ND	ND
	cggR mutant	0.32/105 ± 2	0.31/97 ± 1	ND	ND	ND	ND
	cggR-P25	0.24/79 ± 3	0.25/77 ± 1	ND	ND	ND	ND

Standard deviations of duplicate cultures are included, except for metabolic fluxes of WCFS1 where only one culture was measured on HPLC. However, the collected samples from WCFS1 were measured twice on the HPLC, showing statistically the same results.
ND, not determined.

(Fig. S1). The genes that displayed significant regulation in terms of any of the three effects: CE (carbon source effect), ME (mutation effect) or IE (interaction effect) (described in *Experimental procedures*), in NC8 are represented in Table 2. The main findings of Table 2 are illustrated in Fig. 2, which represents genes with functions related to sugar uptake, energy metabolism, fatty acid and phospholipid metabolism. The individual effects (defined in *Experimental procedures*) of the genes with significant CE, ME or IE in NC8 are listed in *Supporting information* (Table S2). In WCFS1, genes were only significantly regulated in terms of CE and OE (overexpression effect) (Table S3 in *Supporting information*).

Identification of putative CRE sites

A manual search for putative CRE sites was performed within the genes of NC8 with significant IE and with functions predicted to energy metabolism, fatty acid and phospholipid metabolism. The initial searches were performed using the WCFS1 genome sequence. The presence of putative CRE sites were identified upstream to all of the relevant genes (Fig. 3). Identical regions were subsequently identified upstream corresponding genes in NC8 through the use of a partial genome sequence that is currently available for this strain.

Discussion

Putative regulation mechanism of the gap *operon in* L. plantarum

The similar organization of the *gap* operon in *L. plantarum* in comparison with several other Gram-positive bacteria, such as *B. subtilis* (Fig. 1A), could reflect a similar regulation of the operon. In contrast to *B. subtilis*, no transcriptional start site of *cggR* has been revealed for *L. plantarum*. This was suggested to be due to a similar processing event as in *B. subtilis*, causing rapid degradation of the *cggR* transcript and thus too small amount of the transcript to be detected (Naterstad *et al*., 2007). In our study, the almost perfect putative *cggR* promoter sequence (Fig. 1B) might thus initiate *cggR* transcription or perhaps penta-cistronic transcription of the entire *gap* operon. In *B. subtilis*, CggR acts as repressor of the *gapA* operon by binding to an operator localized upstream *cggR*, a process shown to be modulated by the level of FBP (Doan and Aymerich, 2003). It seems likely that *L. plantarum* utilizes a similar mechanism to modulate *gap* operon expression, since a putative operator upstream *cggR* of *L. plantarum* was identified with significant similarity to the CggR operator in *B. subtilis* (Fig. 1C). Searches in the genome sequence of *L. plantarum* WCFS1 with the putative operator sequence (searches were performed using sequence motifs that lack the T-stretch) for the occurrence of other target sequences revealed no significant hits, suggesting a *cggR*-dependent regulation mechanism specific for the *gap* operon (data not shown).

Physiological effects of the cggR*-engineered strains*

Glucose and ribose was selected as carbon sources since these two sugars are taken up into the cell by two different uptake systems (PTS and permease) and because they are catabolized through different metabolic pathways, i.e. glycolytic and phosphoketolase pathways respectively (Axelsson, 2004). In addition, they have shown to induce the *cggR* promoter in *B. subtilis* differently (Ludwig *et al*., 2001).

The higher growth rate of both NC8 and WCFS1 when grown on glucose as carbon source compared with the growth rate on ribose (Table 1) confirms that glucose is the preferred carbon source. The effects with increased growth rate and metabolic fluxes in the *cggR* deletion derivative of NC8, and the reduced growth rates and metabolic fluxes of the *cggR*-overexpressed strains of NC8 and WCFS1, indicate a connection between CggR and a mechanism leading to growth impairment.

Transcriptional regulation of the gap *operon*

Intriguingly, there was no change in expression of the *gap* operon when wild-type strains of *L. plantarum* NC8 or WCFS1 were grown on ribose compared with glucose (no CE observed for these genes), which is in contrast to what has been reported for *B. subtilis* (Ludwig *et al*., 2001). Doan and Aymerich (2003) have shown that low levels of FBP lead to stronger CggR inhibition of the *gap* operon in *B. subtilis*. The fermentation of ribose compared with glucose in *L. plantarum* would theoretically lead to lower levels of FBP since the ribose fermentation first coincides with glycolysis at the level of glyceraldehyde-3-phosphate. However, the level of FBP was shown to be more or less equal (~30 mM, data not shown) in *L. plantarum* NC8 and WCFS1 when grown on either of the two carbon sources, which could be a consequence of the high induction of transketolase and transaldolase during ribose growth shown as CE (Table 2 and Table S3 in *Supporting information*). Transketolase and transaldolase are involved in the conversion of ribose-5-phosphate and xylulose-5-phosphate into glyceraldehyde-3-phosphate and fructose-6-phosphate, and glyceraldehyde-3-phosphate into fructose-6-phosphate respectively. In that way, they are important for the synthesis of essential six-carbon compounds for biosynthetic pathways during pentose fermentation. Overall, these observations could be in good agreement with a role of FBP as the effector molecule that inhibits CggR-mediated repression of the *gap* operon expression in *L. plantarum*.

Table 2. Genes with significant CE, ME or IE in *L. plantarum* NC8.

Gene locus	Gene	Product	CE	ME	IE
Amino acid biosynthesis					
lp_1375	*metE*	5-Methyltetrahydropteroyltriglutamate – homocysteine *S*-methyltransferase			0.5
lp_2685	*dapA2*	Dihydrodipicolinate synthase			0.8[a]
Biosynthesis of cofactors, prosthetic groups and carriers					
lp_2612		Pyrazinamidase/nicotinamidase			–0.6
Cell envelope					
lp_1070		Lipoprotein precursor			0.7
lp_3679		Extracellular protein			0.5[a]
Cellular processes					
lp_0409	*plnM*	Immunity protein PlnM	2.6[a]		
lp_0412	*plnP*	Immunity protein PlnP, membrane-bound protease CAAX family	2.2		
lp_2544	*npr2*	NADH peroxidase			0.6
lp_2906	*endA*	DNA-entry nuclease		–0.9	–1.0
lp_3128		Stress induced DNA-binding protein			–0.6
Central intermediary metabolism					
lp_0193	*agl3*	Alpha-glucosidase	2.6[a]		1.0[a]
DNA metabolism					
lp_0432		DNA helicase (putative)		–0.6	
lp_0772	*uvrB*	Excinuclease ABC, subunit B		–0.7	–0.6
lp_0773	*uvrA1*	Excinuclease ABC, subunit A			–0.8
lp_2280	*dinP*	DNA-damage-inducible protein P		–0.9	–1.2
lp_2301	*recA*	Recombinase A		–0.8	–0.7
lp_2693	*rexA*	ATP-dependent nuclease, subunit A		–0.8	–0.7
lp_2694	*rexB*	ATP-dependent nuclease, subunit B			–0.7
lp_3023	*umuC*	UV-damage repair protein		–1.5	–1.6
Energy metabolism					
lp_0329	*acdH*	Acetaldehyde dehydrogenase	–3.3[a]		
lp_0852	*pox2*	Pyruvate oxidase	2.3		
lp_1112	*fum*	Fumarate hydratase		–0.8	
lp_2151	*pdhD*	Pyruvate dehydrogenase complex, E3 component	2.8		
lp_2152	*pdhC*	Pyruvate dehydrogenase complex, E2 component	3.0		
lp_2153	*pdhB*	Pyruvate dehydrogenase complex, E1 component, beta subunit	3.7		1.0
lp_2154	*pdhA*	Pyruvate dehydrogenase complex, E1 component, alpha subunit	4.1	–0.9	1.1
lp_2629	*pox3*	Pyruvate oxidase	2.5		1.5
lp_3045		Short-chain dehydrogenase/oxidoreductase			–0.5
lp_3313	*pflB2*	Formate C-acetyltransferase	3.1		1.2
lp_3314	*pflA2*	Formate acetyltransferase-activating enzyme	2.7		0.9
lp_3418	*pck*	Phosphoenolpyruvate carboxykinase (ATP)	2.5		0.9
lp_3420	*gadB*	Glutamate decarboxylase			–0.5
lp_3483	*lacL*	Beta-galactosidase, large subunit	2.3		
lp_3484	*lacM*	Beta-galactosidase, small subunit	2.0		
lp_3487	*galM3*	Aldose 1-epimerase	2.8		0.6
lp_3525	*pbg9*	6-Phospho-beta-glucosidase	2.1		
lp_3538	*tkt4*	Transketolase	6.5		0.8
lp_3539	*tal2*	Transaldolase	6.5		
lp_3589	*pox5*	Pyruvate oxidase	2.3		0.7
Fatty acid and phospholipid metabolism					
lp_0168	*dak1B*	Dihydroxyacetone kinase			0.7
lp_0169	*dak2*	Dihydroxyacetone phosphotransferase, dihydroxyacetone binding subunit			0.8
lp_0371	*glpD*	Glycerol-3-phosphate dehydrogenase	4.0		2.5
Purines, pyrimidines, nucleosides and nucleotides					
lp_0242	*ndk*	Nucleoside-diphosphate kinase	3.9		1.2
lp_0692	*nrdF*	Ribonucleoside-diphosphate reductase, beta chain		–0.6	–0.5
lp_0693	*nrdE*	Ribonucleoside-diphosphate reductase, alpha chain		–0.6	
lp_2697	*pyrE*	Orotate phosphoribosyltransferase			–1.0
lp_2702	*pyrC*	Dihydroorotase			–0.5
lp_2931	*nrdG*	Anaerobic ribonucleotide reductase activator protein		–1.1	–1.1
lp_2932	*nrdD*	Anaerobic ribonucleoside-triphosphate reductase		–1.0	–0.8
lp_3271	*guaC*	GMP reductase			–0.5
Regulatory functions					
lp_0788	*cggR*	Central glycolytic gene regulator		2.8[b]	
lp_0889		Transcription regulator		0.6	0.5
lp_2964		Transcription regulator (putative)			–0.5
lp_3345	*spx4*	Regulatory protein Spx			0.8[a]
lp_3655	*srlM2*	Sorbitol operon activator			0.8[a]

Table 2. *cont.*

Gene locus	Gene	Product	CE	ME	IE
Transport and binding protein					
lp_0171	*dhaP*	Dihydroxyacetone transport protein (putative)			0.7
lp_0349	*amtB*	Ammonium transport protein	–2.8		
lp_0372	*glpF3*	Glycerol uptake facilitator protein	3.1		1.8
lp_0436	*pts7C*	Cellobiose PTS, EIIC			0.6
lp_0439	*pts8C*	Cellobiose PTS, EIIC			0.9
lp_0575	*pts9AB*	Mannose PTS, EIIAB	–2.4		0.6
lp_0576	*pts9C*	Mannose PTS, EIIC	–2.6	0.6	0.6
lp_0749	*pstB*	Phosphate ABC transporter, ATP-binding protein	–2.1[a]		
lp_0770		Multidrug transport protein			–0.7
lp_1120		Amino acid transport protein	–2.0		
lp_1945		ABC transporter, ATP-binding protein	2.8		
lp_2509		Transport protein	–2.2		
lp_2780	*pts20A*	Cellobiose PTS, EIIA	2.9	–0.6	0.6
lp_3008	*pts23A*	Cellobiose PTS, EIIA	2.1		
lp_3278		Amino acid transport protein	–2.1		
lp_3279	*kup2*	Potassium uptake protein		–0.6	–0.5
lp_3303		Multidrug transport protein		0.6	
lp_3540		Transport protein	6.5[a]		0.6[a]
lp_3541	*pts34B*	PTS, EIIB	6.5		
lp_3547	*pts35B*	Galactitol PTS, EIIB			0.6
lp_3658	*rbsU*	Ribose transport protein	6.7		
lp_3659	*rbsD*	Ribose transport protein, membrane-associated protein	7.1		
Hypothetical proteins					
lp_0058		Unknown	2.7		
lp_0063		Unknown	2.2		
lp_0089		Unknown			–0.6
lp_0137		Oxidoreductase			–0.7
lp_0170	*dak3*	Dihydroxyacetone phosphotransferase, phosphoryl donor protein			0.9
lp_0214		Integral membrane protein	–2.2		
lp_0240		Unknown	3.6	–0.5	1.0
lp_0402		Unknown			–0.5
lp_0691		Unknown		–0.8	–0.6
lp_0960		Unknown		–1.4	–1.2
lp_1068		Unknown			0.5
lp_1611		Unknown		–1.2	–1.1
lp_1908		Integral membrane protein			–0.8
lp_2732		Oxidoreductase			0.5
lp_2813		Unknown	2.2		
lp_2948		Unknown			0.8
lp_3022		Unknown		–1.4	–1.6
lp_3078		Hydrolase, HAD superfamily	2.3		0.5
lp_3142		Unknown		–1.1	–1.1
lp_3318		Oxidoreductase	2.5		
lp_3537		Hydrolase, HAD superfamily, Cof family	6.3		
Other categories					
lp_0655		Prophage P1 protein 32		–0.7[a]	–0.6[a]
lp_2442		Prophage P2a protein 15	2.6[a]		

a. Log_2-value based on spot intensity of one probe.
b. Log_2-value based on spot intensities of the two *cggR* probes that were not in the deleted region of *cggR* (FDR < 0.001).
CE (carbon source effect), log_2 of > 2.0 or < –2.0.
ME (mutation effect), log_2 of > 0.5 or < –0.5.
IE (interaction effect), log_2 of > 0.5 or < –0.5.

The highest and, for WCFS1 the only, affected gene in the *cggR* mutant strains was seen for the *cggR* gene itself in terms of ME (Table 2 and Table S3), which was based on the signals of the *cggR*-specific probes that are localized outside the deleted region. As expected, the single *cggR* probe that corresponds to the deletion region of *cggR* displayed a significantly lower signal (data not shown). One reason for the upregulated probes outside the deletion region could be due to release of CggR repression on the *cggR* transcript; however, no release of repression of the remaining *gap* operon was observed.

Another reason could be that the native *cggR* transcript is highly unstable, analogous to what has been reported for *cggR* in *B. subtilis* (Ludwig *et al*., 2001), but has gained considerable stability characteristics as a consequence of the truncation of the *cggR* transcript (600 bp of *cggR* has been deleted in the *cggR* mutant strains).

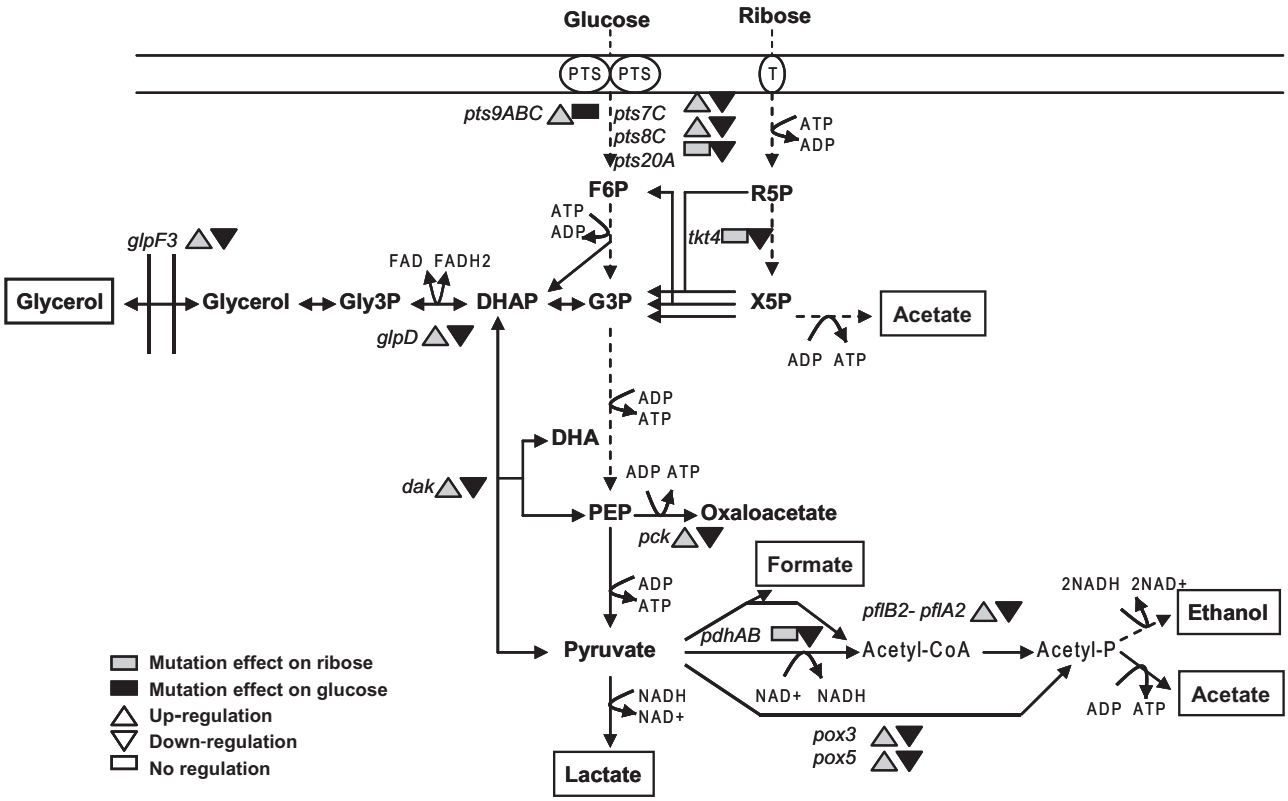

Fig. 2. Schematic representation of the metabolic pathways for glucose and ribose fermentation in *L. plantarum*, including significant IE genes in NC8. The IE genes are divided into the individual MEs on ribose (grey symbols) and glucose (black symbols) respectively. Upward-pointing triangles indicate upregulated genes, downward-pointing triangles indicate downregulated genes and boxes indicate none-regulated genes. The functions of the genes are described in Table 2.

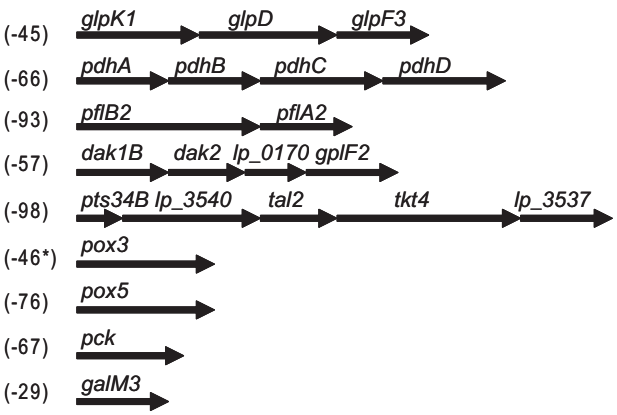

Fig. 3. Genes and operons identified with a putative CRE site upstream to the (first) gene. Only genes of NC8 with significant IE, and with functions predicted to energy metabolism, fatty acid and phospholipid metabolism are included. The identification is based on the genome sequence of WCFS1. Position of the CRE boxes relative to the start of the gene is indicated. Asterisk indicates a previous identified CRE box (Lorquet *et al.*, 2004) with three base mismatches. The functions of the genes are described in Table 2.

The *cggR* overexpression was verified in *cggR*-P25 strains of WCFS1 (and NC8, data not shown) where *cggR* was the strongest upregulated gene in terms of OE (Table S3 in *Supporting information*). The main OEs observed were downregulation of the glycolytic genes of the *gap* operon in WCFS1 (and NC8). This supports the repressor role of CggR on *gap* operon expression in WCFS1 and NC8, and is in good agreement with the conclusions drawn from the observed physiological effects upon *cggR* overexpression (i.e. decreased growth rate and glycolytic flux).

Since no regulation of the *gap* operon was observed in the *cggR* mutant derivatives of NC8 and WCFS1, it seems that CggR does not, or only to a very limited extend, repress the *gap* operon in the wild-type strains growing on either glucose or ribose, which probably reflects the already maximum induction of the operon by the high FBP levels in these cells. In *B. subtilis*, it has been shown that maximum level of FBP activation is at 10 mM (Doan and Aymerich, 2003), which is far below the intracellular FBP levels measured in this study. This potentially indicates a role of CggR on the *gap* operon under conditions when

the level of FBP is lower, e.g. during growth on other sugars or combinations of other carbon and nitrogen sources. It could also be speculated that CggR is involved in regulation of the *gap* operon in other growth phases or during transitions between different growth phases, as only the exponential phase was evaluated in our study.

It should be mentioned that no redundancy of the glycolytic genes of the *gap* operon has been identified in the annotated genome of *L. plantarum* WCFS1 (Kleerebezem *et al*., 2003), except for the *enoA1* gene. Thus, expression of these genes is also essential during gluconeogenesis, for instance during starvation when low levels of FBP are expected. The identified promoter of the *gap* gene in *L. plantarum*, based on primer extension analysis (Naterstad *et al*., 2007), could thus provide a constant basal expression of the glycolytic genes of the *gap* operon (*gap-pgk-tpi-enoA1*); however, it cannot be ruled out that the transcriptional start site identified was a result of a processing event. Previously observed difficulties in detection of a *cggR* transcript in *L. plantarum* using Northern blotting techniques (Naterstad *et al*., 2007) prohibit any straightforward experimental approaches to investigate the possibility of post-transcriptional processing of the *cggR* messenger or its eventual transcript stability.

Ribose-dependent regulation

The highest number of significantly regulated genes were identified as CEs of both NC8 and WCFS1, therefore only genes with a high $\log_2$-change (CE > 2.0 or CE < –2.0) were listed in Table 2 and Table S3 (*Supporting information*) respectively. The seven genes with the highest level of CE (> 6.0) were the same in both NC8 and WCFS1, and are allocated to two operons: the *rbs* operon encoding genes involved in ribose transport, and an operon including genes encoding transketolase (*tkt4*) and transaldolase (*tal2*). The high regulation of these genes confirms their major role during ribose fermentation. The *rbs* operon of *L. plantarum* is similar to that of *Lactobacillus sakei*. In the latter, the PTS system has been suggested to be involved in the negative regulation of ribose utilization, since transport and phosphorylation of ribose were shown to increase in a *ptsI* mutant derivate (Stentz and Zagorec, 1999). As was anticipated, the genes encoding the mannose PTS (*pts9ABC*), which is known to be the main glucose PTS in LAB (Chaillou *et al*., 2001), were downregulated in both strains during ribose fermentation.

Regulation of genes involved in metabolism and transport

Although the *gap* operon and other glycolytic genes appeared unaffected by deletion of *cggR* in both NC8 and WCFS1, a total of 73 genes appeared to be significantly affected by the *cggR* mutation in NC8 (Table 2), when sorted by ME and IE ($\log_2 > 0.5$ and $\log_2 < -0.5$). In contrast, no significant transcriptional changes could be detected in WCFS1 upon mutation of the *cggR* gene in terms of ME or IE (data not shown).

Interestingly, genes with predicted functions associated with energy metabolism, fatty acid and phospholipid metabolism, and sugar transport were predominant among the significantly regulated genes in term of IE (and also CE) in NC8 (Table 2). A significant IE means that the genes are regulated in the *cggR* mutant strain of NC8; however, they are regulated differently when the strain is growing on ribose compared with glucose. In fact almost all of the genes were oppositely regulated on the two carbon sources when dividing the IE into the individual effects: $ME_{(ribose)}$ and $ME_{(glucose)}$ (Table S2 in *Supporting information*). This is illustrated in a pathway map of glucose and ribose fermentation, containing most of the metabolic genes with a significant IE in NC8 (Fig. 2). The metabolic function that was most strongly affected in terms of IE in NC8 belonged to glycerol metabolism and was encoded by the *glp* operon, containing *glpK1* (glycerol kinase, not on the array), *glpD* (glycerol-3-phosphate dehydrogenase) and *glpF3* (glycerol uptake facilitator protein). However, no fermentation of glycerol was detected using an API carbohydrate fermentation test in either the wild-type strains or the *cggR* mutant derivatives of NC8 and WCFS1 (data not shown), and no production of glycerol was detected (data not shown) that could explain this high regulation. Dihydroxyacetone phosphate (DHAP) is a metabolite linked to glycerol metabolism, and an operon encoding components of the dihydroxyacetone phosphotransferase 2 (*dak1B-dak2-dak3-dhaP*), which are involved in the phosphorylation of dihydroxyacetone into DHAP (Fig. 2), was apparently also affected. This process is known in *Escherichia coli*, where the phosphorylation occurs via a phosphotransfer mechanism involving components of the PTS (Gutknecht *et al*., 2001).

Other metabolic genes with a significant IE were dominated by genes involved in pyruvate metabolism, including genes encoding components of the pyruvate dehydrogenase complex (*pdh* operon), the pyruvate formate lyase (*pfl* operon) and pyruvate oxidase (*pox3* and *pox5*). The two *pox* genes have shown to encode the two major pyruvate oxidases in *L. plantarum* (Lorquet *et al*., 2004; Goffin *et al*., 2006). All these enzymes can be involved in converting pyruvate into other end-products than lactate, such as acetate, formate or ethanol. However, no production of acetate, formate or ethanol was detected in the different engineered NC8 strains [except for constant level of acetate production during growth on ribose (data not shown)], which could indicate that the affinity constants of these enzymes for their substrates are insufficient to

compete with LDH or that there are no or minor activities of these enzymes under the conditions tested. The latter is partly supported by previous studies, which have suggested that PDH activity is lacking in *L. plantarum* (Dirar and Collins, 1973; Hickey *et al*., 1983; Murphy and Condon, 1984), and that POX activity is dependent on the availability of molecular oxygen (Murphy and Condon, 1984; Sedewitz *et al*., 1984; Murphy *et al*., 1985; Lorquet *et al*., 2004). Interestingly, transcriptional regulation of these genes has also previously been reported in *L. plantarum* through microarray analysis (Saulnier *et al*., 2007). Another metabolic gene with significant IE was *pck* encoding phosphoenolpyruvate carboxykinase responsible for the conversion of PEP to oxaloacetate, which subsequently can be converted to malate by malate dehydrogenase leading to NAD^+ regeneration. In addition, the *tkt4* gene encoding transketolase also showed a significant IE, which was one of the most highly upregulated genes during ribose fermentation.

Genes encoding PTS systems (e.g. mannose PTS, cellobiose PTS, galactitol PTS) were among the IE genes. Interestingly, expression of the mannose PTS system in *L. plantarum*, as well as in other Gram-positive bacteria, has been shown to be dependent on the σ^{54} transcriptional factor, encoded by *rpoN* (Dalet *et al*., 2001; Hechard *et al*., 2001; Stevens *et al*., 2010). Notably, the *rpoN* gene is localized upstream of the *cggR* gene in the genome sequence of *L. plantarum* WCFS1, but it was not significantly regulated in the *cggR* mutant derivatives.

Most of the genes with a significant ME in NC8 were negatively affected, and the calculated effects were almost equal in terms of IE (Table 2). That indicates a response in the NC8 *cggR* mutant growing on ribose, which was confirmed when ME was divided into the individual effects based on carbon source as previously described [$ME_{(ribose)}$ and $ME_{(glucose)}$; Table S2 (*Supporting information*)]. The affected genes were mainly involved in DNA, nucleoside and nucleotide metabolism.

CcpA regulation of genes involved in metabolism and transport

The opposite regulation of genes involved in metabolism and transport when the *cggR* mutant of NC8 was growing on ribose compared with glucose indicates a common regulatory factor which is dependent on the carbon source the strains are catabolizing. The lower growth rate on ribose compared with glucose clearly shows that ribose is not a preferential carbon source in *L. plantarum*, and a regulation with connection to CCR could thus be involved for the genes showing significant CE. The global regulatory control protein (CcpA) involved in CCR is the plausible common factor affecting many of the mutually regulated genes in terms of CE and IE. In fact, putative target sites of CcpA (CRE sites) were identified upstream of the genes/operons with functions associated with energy metabolism, fatty acid and phospholipid metabolism (Fig. 3). The role of CcpA in CCR in *L. plantarum* has previously been established (Muscariello *et al*., 2001), and CRE sites presumed to mediate CcpA control were identified in direct proximity to genes coding for proteins responsible for sugar uptake (Andersson *et al*., 2005). CcpA-mediated regulation of some of the genes/operons represented in Fig. 3 has also previously been shown/indicated, e.g. four of the *pox* genes in *L. plantarum* (Lorquet *et al*., 2004; Goffin *et al*., 2006), and a putative gene encoding glycerol dehydrogenase and dihydroxyacetone kinase in *Enterococcus faecalis* (Leboeuf *et al*., 2000). Genes encoding important components of CcpA-mediated regulation (i.e. *ccpA*, *ptsH* and *hprK*) were not affected in NC8 in terms of CE, ME or IE, suggesting that the regulatory cofactors, such as the phosphorylated state of HPr-Ser46-P or the level of FBP/G6P, which are involved in CcpA-mediated regulation were affected rather than the core components involved. This notion is further exemplified by the preliminary finding that a slightly higher level of FBP is present in the *cggR* mutant strain of NC8 compared with the wild-type strain during growth on glucose (data not shown). The transcriptome analysis in terms of ME and OE shows that the *cggR* mutation and *cggR* overexpression affects genes both positively and negatively (Table 2 and Table S3). Although CggR is generally believed to have a repressor function, its direct or indirect interaction with other regulators, such as CcpA, which is known to act both as repressor and activator, potentially explains the bidirectional transcription control exerted by CggR.

Concluding remarks and future perspectives

The identification of the putative CggR operator sequence combined with the observed downregulation of the *gap* operon when the level of CggR was sufficiently high indicates that CggR functions as repressor on the *gap* operon in both *L. plantarum* WCFS1 and NC8, i.e. in a similar manner as in *B. subtilis*. However, our results also indicate that CggR might have a more prominent regulatory role in *gap* operon control under conditions that differ from those tested here. For example, growth conditions that lead to reduced FBP levels are bound to generate more pronounced *cggR* mediated *gap* operon control. Such conditions could include the growth on alternative carbon and/or nitrogen sources, in other phases of growth than tested here, in the transition between two growth phases, or in the transition from one carbon source to another. Thereby, it could very well be that CggR-mediated regulation is of greater importance in more natural environ-

ments where the nutritional state is more fluctuating, as compared with the rich-laboratory conditions employed here.

The fact that the *cggR* mutation in *L. plantarum* NC8 caused significant physiological and transcriptional effects even though the remaining *gap* operon was unaffected indicates that CggR also regulates another target in NC8. It could be speculated that there are no other target genes for CggR than the *gap* operon in WCFS1, since no hits with the putative operator sequence were revealed in the genome sequence of WCFS1, and thus explaining why no transcriptional regulation was observed in the *cggR* mutated strain. The answer could be divergence in evolution of genes involved in sugar transport and catabolism which has shown to be highly variable between *L. plantarum* strains (Molenaar *et al*., 2005), also including WCFS1 and NC8. The variations between the two strains are perhaps not that surprising, since they originally were isolated from two very different niches, silage (NC8) and human saliva (WCFS1) (Aukrust and Blom, 1992; Kleerebezem *et al*., 2003), and might have experienced markedly different evolutionary pressures over time.

In NC8, the growth rate and the glycolytic flux increased in the *cggR* mutated strain, but the regulation of the *gap* operon was not significantly affected, indicating that the glycolytic enzymes are in excess and that glycolytic flux is controlled by CggR by another mechanism than through transcriptional regulation of the glycolytic genes. One suggestion is that glycolytic enzymes are regulated at the protein level rather than the transcriptional level. The physiological effects observed could also be a consequence of relieved regulation by CggR on growth controlling genes or on factors interconnected with increased CcpA-mediated control on genes especially involved in glycerol and pyruvate metabolism, thus providing an even more efficient and stricter homolactic fermentation profile of this strain. However, one cannot exclude that a slight increase of *gap* operon transcription, too weak to be detected by the microarray technology, could be responsible for the effect. Increased rate of lactic acid production is interesting biotechnologically (Singh *et al*., 2006) and further research on the CggR-CcpA regulation of the central carbon metabolism and its flux could provide further insights in the control of this pathway.

To conclude, CggR has an important regulatory role on growth and metabolism in *L. plantarum* that certainly deserves further elucidation.

Experimental procedures

The description of the experimental procedures can be found in Appendix S1 in *Supporting information*.

Acknowledgements

This work was supported by The Fund for the Research Levy on Agricultural Products. We thank Inga Marie Aasen for the HPLC analysis and Birgitta Baardsen for excellent technical assistance.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Loop designed hybridization schemes of *L. plantarum* NC8 (A) and WCFS1 (B). Wild-type strains, *cggR* mutant strains and *cggR*-overexpressed strains are represented as Wt, ΔcggR and cggR-P25, respectively, and biological duplicates are indicated with A and B, and are represented in a circle. Strains grown on glucose are indicated by dark-grey boxes, whereas ribose-grown strains are indicated by grey boxes. The loop designs allow for the evaluation of putative dye effects.
Table S1. Bacterial strains and plasmids.
Table S2. Individual effects of the genes with significant CE, ME or IE in *L. plantarum* NC8.
Table S3. Genes with significant CE and OE in *L. plantarum* WCFS1.
Table S4. Cloning and sequencing primers used in the construction of *cggR*-engineered strains of *L. plantarum*.
Appendix S1. Experimental procedures.

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