Regulation of *ldh* expression during biotin-limited growth of *Corynebacterium glutamicum*

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Corynebacterium glutamicum is a biotin-auxotrophic bacterium and some strains efficiently produce glutamic acid under biotin-limiting conditions. In an effort to understand *C. glutamicum* metabolism under biotin limitation, growth of the type strain ATCC 13032 was investigated in batch cultures and a time-course analysis was performed. A transient excretion of organic acids was observed and we focused our attention on lactate synthesis. Lactate synthesis was due to the *ldh*-encoded L-lactate dehydrogenase (Ldh). Features of Ldh activity and *ldh* transcription were analysed. The *ldh* gene was shown to be regulated at the transcriptional level by SugR, a pleiotropic transcriptional repressor also acting on most phosphotransferase system (PTS) genes. Electrophoretic mobility shift assays (EMSAs) and site-directed mutagenesis allowed the identification of the SugR-binding site. Effector studies using EMSAs and analysis of *ldh* expression in a *ptsF* mutant revealed fructose 1-phosphate as a highly efficient negative effector of SugR. Fructose 1,6-bisphosphate also affected SugR binding.

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INTRODUCTION

Corynebacterium glutamicum is a Gram-positive organism, isolated by Kinoshita and coworkers as a biotin-auxotrophic bacterium, that excretes large amounts of L-glutamate when grown aerobically under biotin limitation with glucose as a carbon source (Kinoshita *et al.*, 1957; Kikuchi & Nakao, 1986). *C. glutamicum* is now widely used in the industrial production of amino acids such as L-glutamate and L-lysine (Abe *et al.*, 1967). Improvements of the fermentation strategies employed and of the bacterial strains have been achieved, leading to progressively increasing rates of production and/or yields of glutamate and lysine. The development of tools for genetic engineering (Kirchner & Tauch, 2003), the availability of the genome sequence (Kalinowski *et al.*, 2003), genome-wide expression analyses using microarrays (Wendisch, 2003), proteome analyses (Hermann *et al.*, 2001) and carbon-flux distribution analyses (Dominguez *et al.*, 1998; Rollin *et al.*, 1995) allowed a comprehensive knowledge of *C. glutamicum* metabolism during exponential growth and led to the rational improvement of *C. glutamicum* strains by metabolic design for the production of D-pantothenate, L-isoleucine, L-valine or L-threonine (Sahm *et al.*, 2000; Hüser *et al.*, 2005).

Despite its described habitat, soil, which is unlikely to contain excess nutrients, only few studies have attempted to understand *C. glutamicum* metabolism under nutrient limitation. To our knowledge, only iron (Wennerhold *et al.*, 2005), phosphate (Ishige *et al.*, 2003), ammonium (Silberbach *et al.*, 2005a) and nitrogen (Silberbach *et al.*, 2005b) starvation, and glucose limitation (Cocaign-Bousquet *et al.*, 1996), have been investigated. The effects of biotin limitation on *C. glutamicum* metabolism have not been studied to date, even though biotin was postulated to be essentially supplied by plant roots and then limiting in soils (Rovira & Harris, 1961).

Biotin is an important cofactor for the activity of a family of enzymes that includes pyruvate carboxylases, acyl-CoA carboxylases, oxaloacetate decarboxylases and transcarboxylases (Samols *et al.*, 1988; Toh *et al.*, 1993). In microorganisms, biotin is produced in four steps from L-alanine and pimeloyl-CoA (Eisenberg, 1987). In *C. glutamicum*, biotin auxotrophy is at least due to the lack of the *bioF*-encoded 7-keto-8-aminopelargonic acid synthetase (EC 2.3.1.47), the enzyme that catalyses the first step of biotin synthesis from L-alanine and pimeloyl-CoA. There is also no evidence of the presence of either the *bioC* and *bioH* genes, which are postulated to be involved in pimeloyl-CoA synthesis in *Escherichia coli* (Eisenberg, 1987), or *bioI* and *bioW*, involved in pimelic acid synthesis in *Bacillus subtilis* (Bower *et al.*, 1996), or *bioZ*, involved in pimeloyl-CoA synthesis in *Mesorhizobium* sp. (Sullivan *et al.*, 2001).

Abbreviations: EMSA, electrophoretic mobility shift assay; β -Gal, β -galactosidase; Ldh, lactate dehydrogenase; PTS, phosphotransferase system.

On the other hand, the *bioA*, *bioD* and *bioB* genes are present and encode functional enzymes that are able to catalyse the last three steps (Hatakeyama *et al.*, 1993a, b).

In *C. glutamicum*, pyruvate carboxylase (EC 6.4.1.1), the major anaplerotic enzyme, and acetyl-CoA carboxylase (EC 6.4.1.2), which catalyses the committed step in fatty acid synthesis, have been shown to be biotinylated *in vivo* (Peters-Wendisch *et al.*, 1998; Jäger *et al.*, 1996). As the reactions catalysed by these two enzymes are closely linked to pyruvic acid, a rigid branch point of central metabolism (Vallino & Stephanopoulos, 1994) and the precursor of many important metabolites, biotin limitation is expected to lead to an important disturbance of central metabolism. In addition, this disturbance could be magnified by the expected biotin dependency of propionyl-CoA carboxylase β -chain (EC 6.4.1.3), the *pccB*-encoded carboxylase involved in mycolic acid synthesis (Portevin *et al.*, 2005). Based on these assumptions of a possible metabolic disturbance at the pyruvate node during biotin-limited growth of *C. glutamicum*, we followed the growth features of *C. glutamicum* during biotin-limited batch cultures and focused our attention on the effect of biotin limitation on pyruvate redirection under these conditions. An unexpected

mixed-acid fermentation occurred under aerobiosis without stress. We restricted our investigation to L-lactate synthesis and the expression and regulation of the L-lactate dehydrogenase (Ldh)-encoding *ldh* gene. In-depth analysis of *ldh* expression allowed us to identify SugR, the product of the *cg2115* gene, as the transcriptional regulator of *ldh*. During the preparation of this manuscript, Engels & Wendisch (2007), Gaigalat *et al.* (2007) and Tanaka *et al.* (2008a) reported the identification of SugR as the transcriptional repressor of sugar PTS (phosphotransferase system) genes in *C. glutamicum*. None of these papers reported the role of SugR in *ldh* transcriptional regulation.

METHODS

Bacterial strains, growth conditions, metabolite analysis, plasmids and oligonucleotides. The bacterial strains and plasmids used in this study are listed in Table 1. The sequences of oligonucleotides are listed in Table 2. *C. glutamicum* strains were grown aerobically (250 r.p.m.) at 34 °C in Brain Heart Infusion (BHI) rich medium (Difco) or in GCXII chemically defined medium (Keilhauer *et al.*, 1993) containing 0.02 mg biotin l⁻¹ and either 2 % (w/v) glucose, 2 % (w/v) fructose, 4 % (w/v) sucrose, 2 % (w/v) acetate or a mixture of glucose and fructose (2 % each). Growth was

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
DH5 α	F ⁻ <i>thi endA1 hsdR17(r⁻ m⁻) supE44 ΔlacU169 (ϕ80lacZΔM15) <i>recA1 gyrA96 relA1</i></i>	BRL
BL21(DE3)pLysS	<i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) pLysS	Novagen
<i>C. glutamicum</i>		
RES167	Wild-type, restriction-deficient mutant of ATCC 13032	Dusch <i>et al.</i> (1999)
RES167::pMM29	<i>ldh</i> insertion mutant, Km ^r	This study
RES167::pAG1022	Integrated <i>Pldh-lacZYA</i> fusion, Km ^r	This study
RES167::pAG1030	Integrated <i>PsugR-lacZYA</i> fusion, Km ^r	This study
RES167::pAG1032	<i>ptsF</i> insertion mutant, Km ^r	This study
RES167::pAG1033	Integrated mutated <i>Pldh-lacZYA</i> fusion, Km ^r	This study
RES167::pAG1034	SugR ^{His} expression in <i>C. glutamicum</i> , Km ^r	This study
RES167::pAG1035	<i>fruR</i> insertion mutant, Km ^r	This study
Δ <i>sugR</i>	In-frame deletion of <i>sugR</i>	This study
Δ <i>cg0146</i>	In-frame deletion of <i>cg0146</i>	This study
Δ <i>cg3224</i>	In-frame deletion of <i>cg3224</i>	This study
Δ <i>cg3315</i>	In-frame deletion of <i>cg3315</i>	This study
Plasmids		
pCR2.1-TOPO	Cloning vector for PCR products, Ap ^r Km ^r	Invitrogen
pMF2	Promoter-probe vector, Ap ^r Km ^r	Soual-Hoebeke <i>et al.</i> (1999)
pK18 <i>mobsacB</i>	Vector for the construction of insertion and deletion mutants in <i>C. glutamicum</i> , Km ^r	Schäfer <i>et al.</i> (1994)
pET9-sn1	Vector for the overexpression of His-tagged proteins, Ap ^r Km ^r	Trésaugues <i>et al.</i> (2004)
pMM29	pCR2.1-TOPO with a 355 bp <i>ldh</i> fragment, Km ^r	This study
pAG1022	pMF2 with the full-length promoter of <i>ldh</i> , Km ^r	This study
pAG1033	Derivative of pAG1022, mutated SugR-binding site, Km ^r	This study
pAG1025	Overproduction of SugR with a C-terminal hexahistidine tag, Km ^r	This study
pAG1034	Expression of SugR ^{His} in <i>C. glutamicum</i> , Km ^r	This study
pAG1030	pMF2 with the full-length promoter of <i>sugR</i> , Km ^r	This study
pAG1032	pCR2.1-TOPO with a 500 bp <i>ptsF</i> fragment, Km ^r	This study
pAG1035	pCR2.1-TOPO with a 501 bp <i>fruR</i> fragment, Km ^r	This study

Table 2. Oligonucleotides used in this study

Name	Sequence (modifications in bold)	5' nt	Modifications
Ldh-1	GTCAAGATTATGAAATCC	3086400	
Ldh-2	GTGTCTTCGAAAATTTTC	3086045	
Ldh-10	7-GAGTCATCTTGGCAGAGCAT	3086922	5' biotin label
Ldh-11	TTCCCTAACCGGACAACCCA	3086718	
Ldh-20	GAAT TCGCGAACTAGCTCTGCAATG	3087050	<i>EcoRI</i>
Ldh-21	GGATCC ATTTTCGATCCCACTTCCTG	3086695	<i>BamHI</i>
Ldh-24	ATTTTCGGACATAATCGGGCATAATTAAA	3086820	
Ldh-26	CAAAAATAACACTTGGTCTGACCACATTTT	3086846	
Ldh-27	TAAAGGTGTAACAAAGGAATCCGGGCACAA	3086795	
Ldh-30	CAATCTTGTTACCGACGGTT	3086670	
Ldh-31	ATCTCCTGCGCCAATGAGGA	3086650	
Ldh-32	TATGCGTATGCAACTCCAAC	3086630	
Ldh-37	ACCACATT TAGCCTGTAT ATCGGGCATAATTAAAGGTGTA	3086825	SugR-binding site
Ldh-38	ATTATGCCCGAT ATACAGGCTAAAT GTGGTCAGACCAAGT	3086796	SugR-binding site
Regg1-1	GGTACCA AGGATTCATCTGGCATCTG	2007449	<i>KpnI</i>
Regg1-2	CCATGGC ACTCCTTAAAGCAAAAAGC	2007861	<i>NcoI</i>
Regg1-3	CCATGGT TCTTACAGTCACTGCAAGT	2008644	<i>NcoI</i>
Regg1-4	GCATGCA ATTGCCACCCAACAACACC	2009048	<i>NheI</i>
Regg1-5	GGAAC ATATGT ACGCAGAGGAGCGCCGT	2007864	<i>NdeI</i>
Regg1-6	TTTT GCGGCCGCTTAATGGTGATGGTGATGGT TTCTGCAATCACAACCTCTAC	2008640	<i>NotI</i> and 6 × His
Regg1-8	TGCCGTTAATGAGGCAATCT	2007909	
Regg1-9	ACATTTACACGTCCCTCAAC	2007928	
Regg1-10	CGTCTCTGCAGTGACATCGA	2007968	
Regg1-11	AAACCACAGAGTTGAGCTTG	2008051	
Regg1-12	ATAGCTACCTTCACATGAGG	2009130	
Regg1-13	GAAT TCAAGCTTTAAGTCGCTTTTC	2007550	<i>EcoRI</i>
Regg1-14	GGATCC ATGTCACCTCCTTAAAGCAA	2007866	<i>BamHI</i>
Rega1-1	GGTACCG GCAACACCGTAAACAGTCA	123651	<i>KpnI</i>
Rega1-2	CCATGGG AAAAAGTTACCACGGAAT	124119	<i>NcoI</i>
Rega1-3	CCATGGT GATGAAATTTTGGATACCC	124931	<i>NcoI</i>
Rega1-4	GCATGCATTTTCTGGTTCATGATCAC	125382	<i>NheI</i>
Regg2-1	GGTACCA TGTGGAATTTGAACTCATC	3089486	<i>KpnI</i>
Regg2-2	CCATGGG TATTTAGAGCCTTAATCG	3089944	<i>NcoI</i>
Regg2-3	CCATGGC CCGCACTCTGTGGGCGGTT	3090647	<i>NcoI</i>
Regg2-4	GCATGC AGGCGGGGATGTCTACGTAG	3091105	<i>NheI</i>
Mar-1	GGTACCA GAAATCAGCTGGCCGTCGCG	3162596	<i>KpnI</i>
Mar-2	GCCTGCCACCATGGCGGTAT	3163195	<i>NcoI</i>
Mar-3	CTCCGGAATT CCATGGA ACA	3163676	<i>NcoI</i>
Mar-4	GCATGC ATTGGTGAAAGCGTAGTTGA	3164215	<i>NheI</i>
Fruca-1	AGCCTCCAAGAACCCAAACG	2014381	
Fruca-2	TGAGACCAGCCAAGATACCG	2014880	
FruR-1	GTGTCTGTAGGAGATTTAGC	2011837	
FruR-2	GATTGAATCGCATCAACCTC	2012336	

followed by measuring the OD₆₀₀ with a Beckman DU 7400 spectrophotometer. Time-course experiments were performed as follows. *C. glutamicum* strains were grown overnight in BHI medium. Cells were concentrated by centrifugation and resuspended in CGXII medium at an initial OD₆₀₀ of 2. Samples were taken every 1.5 h. Experiments were done in triplicate and all the analyses were done in triplicate for each sampling time. *Escherichia coli* strains (DH5 α and BL21(DE3)pLysS) were grown aerobically at 37 °C in Luria–Bertani medium (Difco). Antibiotics used were kanamycin (25 μ g ml⁻¹), chloramphenicol (10 μ g ml⁻¹) and ampicillin (100 μ g ml⁻¹). IPTG was used at 0.1 mM final concentration. All chemicals were purchased from Sigma-Aldrich. The dry mass was estimated by measuring the OD₆₀₀ and assuming that an optical density equal to 1

corresponds to 0.34 mg dry mass ml⁻¹ (Ruffert *et al.*, 1997). The elemental formula of *C. glutamicum* biomass was taken to be C_{4.5}H₈O_{2.2}N with a molecular mass of 121 Da when ash content (9 % total cell dry mass) was allowed for (Dominguez *et al.*, 1998). Extracellular metabolites were measured with a Beckman System Gold HPLC. L-Lactate concentrations were determined enzymically with the lactate reagent kit from Sigma-Aldrich. D-Lactate was determined spectrophotometrically at 340 nm by combining 100 μ l of the solution to be tested and 900 μ l of 0.1 M sodium pyrophosphate buffer pH 8.5 containing 10 mM NAD⁺ and 2 U D-lactate dehydrogenase from *Leuconostoc mesenteroides* (Sigma-Aldrich). Glutamate concentration was determined enzymically (Sigma-Aldrich). Intracellular fructose 1,6-bisphosphate was measured as

described by Dominguez *et al.* (1998). Emission was measured at 460 nm after excitation at 350 nm with an SFM 25A fluorescence spectrophotometer (BIO-TEK Instruments). Intracellular concentrations were expressed as molar concentrations using the intracellular water content value of 1.8 ml (mg dry mass)⁻¹ as determined by Hoischen & Krämer (1989).

DNA isolation, manipulation and transfer. All the molecular biology procedures used in this study were as described by Sambrook *et al.* (1989), Ausubel *et al.* (1987) and Merkamm & Guyonvarch (2001). *C. glutamicum* and *E. coli* cells were transformed by electroporation as described by Bonamy *et al.* (1990). Unless otherwise stated, enzymes were purchased from Promega.

Construction of mutants. PCR experiments were carried out with either *Taq* DNA Polymerase (Promega) or High Fidelity PCR enzyme mix (Fermentas) and a GeneAmp PCR System 2700 thermocycler (Applied Biosystems). Chromosomal *C. glutamicum* DNA was used as template. PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics). All oligonucleotides were purchased from Sigma-Genosys. The genome sequence of *C. glutamicum* deposited in GenBank with the accession number NC_006958 was used throughout this work as the reference.

To disrupt the chromosomal *ldh* gene (*cg3219*), an internal *ldh* fragment was amplified by PCR using primers Ldh-1 and Ldh-2. The purified 356 bp PCR fragment was cloned into the pCR2.1-TOPO cloning vector (Invitrogen) to create pMM29. Plasmid pMM29 was introduced into *C. glutamicum* by electroporation. Kanamycin-resistant transformants were the result of chromosomal integration by single cross-over events, as verified by Southern blotting. The same procedure was followed to disrupt the chromosomal *ptsF* gene (*cg2120*), with primers Fruca-1 and Fruca-2 (plasmid pAG1032) and the chromosomal *fruR* gene (*cg2118*) with primers FruR-1 and FruR-2 (plasmid pAG1035). As *fruR* is the first cistron of the *cg2118-cg2119-cg2120* operon (Gaigalat *et al.*, 2007), the orientation of the *fruR* fragment in pAG1035 was chosen in order to allow the expression of the *cg2119-cg2120* genes under the control of *Plac*, then allowing the recombinant strain to grow on minimal media containing glucose or fructose as carbon sources.

Defined chromosomal deletions of the four putative transcriptional-regulator-encoding genes were constructed with the pK18 *mobsacB* vector system (Schäfer *et al.*, 1994) as follows. The DNA region upstream of the *cg2115* gene encoding YP_226173 was amplified by PCR with oligonucleotides Regg1-1 and Regg1-2 (Table 2). The DNA region downstream of *cg2115* was amplified with oligonucleotides Regg1-3 and Regg1-4 (Table 2). The PCR fragments were cloned independently into the pCR2.1-TOPO cloning vector. The upstream region was isolated as a *KpnI/NcoI* fragment and the downstream region as a *NcoI/NheI* fragment. These fragments were cloned into *KpnI/NheI*-digested vector pK18 *mobsacB*. The construction was transferred into *C. glutamicum* RES167 by electroporation. Allelic exchanges were performed as described by Schäfer *et al.* (1994). PCR experiments were performed to verify the chromosomal deletions. The same procedure was followed to delete the YP_227153-encoding *cg3224* gene (oligonucleotides Regg2-1, Regg2-2 and Regg2-3, Regg2-4), the YP_224408-encoding *cg0146* gene (Rega1-1, Rega1-2 and Rega1-3, Rega1-4) and the YP_227238-encoding *cg3315* gene (Mar-1, Mar-2 and Mar-3, Mar-4).

Construction of β -galactosidase (β -Gal) fusions. The promoter-probe vector pMF2 (Soual-Hoebeke *et al.*, 1999) was used for construction of transcriptional fusions of the *ldh* promoter region or of the *sugR-cg2116* promoter region and the promoterless *lacZ* gene. Plasmid pMF2 was first digested with *EcoRI* and *BamHI* and purified. The native promoter fragments were generated by PCR using primers Ldh-20 and Ldh-21 or Regg1-13 and Regg1-14 respectively, and

cloned into pCR2.1-TOPO. The resulting plasmids were digested with *EcoRI* and *BamHI*, and the purified fragments were cloned in front of the *lacZ* gene in pMF2. Successful cloning was verified by sequencing (Genome Express). The resulting plasmids pAG1022 and pAG1030 allowed the expression of *lacZ* under the control of the *ldh* promoter and of the *sugR-cg2116* promoter respectively and were introduced into *C. glutamicum* by electroporation. Kanamycin-resistant transformants were the result of chromosomal integration at the *icd* locus (*cg0766*) by single cross-over events, as verified by Southern blotting.

The *in vivo* binding of SugR on *Pldh* was tested with the following construct. The 5' part of *Pldh* was amplified by PCR with oligonucleotides Ldh-37 and Ldh-21. The 3' part of *Pldh* was amplified by PCR with oligonucleotides Ldh-38 and Ldh-20. The PCR fragments were combined and used as templates for PCR amplification with oligonucleotides Ldh-20 and Ldh-21. The resulting fragment was digested with *EcoRI* and *BamHI*, and cloned in front of the *lacZ* gene in pMF2 as before. The resulting transcriptional fusion (pAG1033) was introduced into *C. glutamicum* by electroporation and the integrations events were confirmed as before.

Enzyme assays and Ldh purification. Bacterial cells were harvested and resuspended in 50 mM sodium phosphate buffer (pH 6.6), 10 mM MgCl₂ to 50 OD₆₀₀ units. RNase A (1 mg ml⁻¹) and DNase I (1 mg ml⁻¹) were added when appropriate. Cells were disrupted in a French press (Spectronic Instruments) at 110 400 kPa. Cell debris was removed by centrifuging at 10 000 g for 15 min at 4 °C. The supernatant was ultracentrifuged at 130 000 g for 2 h at 4 °C. The resulting supernatant was used as the cytosoluble fraction. The protein concentration was measured by the Lowry method using bovine immunoglobulin G as a standard.

Lactate dehydrogenase (EC 1.1.1.27) activity was assayed photometrically at 30 °C as described by Bergmeyer & Bernt (1974) in a slightly modified form. The reaction was performed in 50 mM sodium phosphate buffer pH 6.6, 5 mM MgCl₂, 20 mM sodium pyruvate, 0.3 mM disodium NADH, 2 mM fructose 1,6-bisphosphate with a final concentration of 10 µg protein ml⁻¹. Activity staining for lactate dehydrogenase after non-denaturing gel electrophoresis was performed according to Selander *et al.* (1986). β -Galactosidase (EC 3.2.1.23) activity was assayed as described by Miller (1972). The glutamate dehydrogenase (EC 1.4.1.4) and isocitrate dehydrogenase (EC 1.1.1.42) activities were assayed spectrophotometrically as described by Meers *et al.* (1970) and Nachlas *et al.* (1963) respectively. All the activities were measured in triplicate. Mean values are given with confidence interval ($P=0.05$).

L-Lactate dehydrogenase purification was performed essentially according to Dartois *et al.* (1995). The ÄKTA FPLC device (Amersham Pharmacia) was used for chromatography. The cytosoluble fraction was fractionated with (NH₄)₂SO₄. The enzyme precipitated between 45 and 60% saturation. The precipitate was dialysed overnight against buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM β -mercaptoethanol). The solution was loaded on a DEAE-Sephadex A-50 column pre-equilibrated with buffer A. Ldh was eluted using increasing concentrations from 0.1 to 1 M KCl in buffer A. Fractions displaying Ldh activity were pooled and concentrated with an ultrafiltration cell PM30 (Amicon). The concentrate was then loaded onto a Cibacron Blue column pre-equilibrated with 20 mM phosphate buffer pH 6.6. The column was washed and the enzyme was eluted by addition of 1 mM NADH in phosphate buffer pH 6.6. Coomassie blue staining after SDS-PAGE electrophoresis revealed a single band.

DNA-affinity purification and identification of regulatory proteins. The purification of DNA-binding proteins was performed essentially as described by Gerstmeir *et al.* (2004). The *ldh* promoter/operator probe was generated by PCR using *C. glutamicum*

chromosomal DNA and oligonucleotides Ldh-10 and Ldh-11, the former being tagged with biotin. Cytosoluble proteins from *C. glutamicum* cells grown on minimal medium with either acetate or glucose as a carbon source were prepared as described above and dialysed against binding buffer (Gerstmeir *et al.*, 2004). For each condition, 10 mg samples of proteins were incubated with 1 mg coupled beads for 2 h at 25 °C. Unbound proteins were washed and DNA-bound proteins were eluted. Eluted proteins were concentrated and dialysed against 10 mM Tris/HCl pH 8.0, 1 mM EDTA buffer by ultrafiltration of the samples with Vivaspin 500 concentrators (Sartorius). SDS-PAGE was performed according to the method of Laemmli (1970) followed by Coomassie brilliant blue R-250-staining.

Spots of interest were excised and analysed by MALDI-TOF mass spectrometry as described by Gerstmeir *et al.* (2004). Spectra were acquired on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a 337 nm nitrogen laser, in positive-ion reflector mode with delayed extraction. External calibration was applied, based on a mixture of six reference peptides covering the m/z 900–3700 Da range. Proteins were identified using the search programs PeptIdent (<http://us.expasy.org>) and Mascot (<http://www.matrixscience.com>), reducing the Swiss-Prot/TrEMBL and NCBI databases to the *Bacteria* species.

Construction and purification of a His-tagged SugR protein (SugR^{His}). A PCR product fusing the coding region of the *C. glutamicum* *sugR* gene (*cg2115*) with a nucleotide sequence encoding a C-terminal His-tag was obtained by using primers Regg1-5 and Regg1-6. The PCR product was digested with *NdeI* and *NotI* and cloned into the expression vector pET9sn1 (Trésaugues *et al.*, 2004). The resulting plasmid pAG1025 was transferred to *E. coli* BL21(DE3)pLysS (Novagen) by electroporation. The His-tagged SugR protein was purified with Ni-NTA spin columns (Qiagen) according to the manufacturer's protocol. SDS-PAGE was performed to control purity of the His-tagged SugR protein (SugR^{His}).

To control the *in vivo* functionality of SugR^{His}, pAG1025 was digested by *Sall* and the digested plasmid was self-ligated to give pAG1034. This led to the deletion of both the T7 promoter and the first 20 nucleotides of *sugR* in pAG1025. The plasmid pAG1034 was then introduced into *C. glutamicum* by electroporation. In the recombinant strain, the *sugR*^{His} gene was expressed as a single copy under the control of the native *sugR* promoter.

Identification of transcriptional starts by the RACE method. Total RNA from glucose-grown cells was prepared as described by Merkamm & Guyonvarch (2001) and incubated in 0.1 M sodium acetate pH 3.0, 5 mM MgSO₄ and 0.3 U DNase I ml⁻¹ (Roche Diagnostics) for 30 min at 37 °C. After heat inactivation for 5 min at 75 °C and phenol/chloroform extraction, the RNA was precipitated with LiCl as described by Sambrook *et al.* (1989). Primers Ldh-32 and Regg1-10) binding downstream of the putative translational starts of the *ldh* and *sugR* genes respectively along with 2 µg total RNA were used for cDNA synthesis. The cDNAs were then modified and amplified by two further PCRs with nested primers (Ldh-31 and Ldh-30 or Regg1-9 and Regg1-8) using the 5'/3' RACE kit second generation (Roche Diagnostics) according to the manufacturer's protocol. The PCR products were purified and cloned into the pCR2.1-TOPO vector. Three different clones from each experiment were selected for plasmid preparation and DNA sequencing (Genome Express).

Transcriptional organization of the *sugR-cg2116* locus. cDNAs were obtained as described before with primer Regg1-12. The cDNAs were further amplified by PCR with oligonucleotides Regg1-12 and Regg1-3 or Regg1-12 and Regg1-11. PCR products were analysed by agarose gel electrophoresis.

Electrophoretic mobility shift assays (EMSAs). Standard EMSA studies were performed essentially as described by Rey *et al.* (2005), with appropriate PCR products and purified His-tagged SugR protein (SugR^{His}). The samples were loaded onto a 2% non-denaturing agarose gel prepared in gel buffer (10 mM Tris/HCl pH 7.8, 10 mM sodium acetate, 1 mM EDTA) and separated at 50 V for 2 h. DNA and DNA-protein complexes were visualized under UV light after ethidium bromide staining of the gels. Effector screening studies were performed as described by Gaigalat *et al.* (2007). After electrophoresis and ethidium bromide staining, the gels were scanned with a Typhoon TRIO Variable Imager (GE Healthcare).

Bioinformatic tools for the interpretation of the *C. glutamicum* genomic sequences. Orthologues of Ldh and SugR proteins in three other *Corynebacterium* species, namely *C. efficiens* YS-314 [GenBank accession NC_004369 (Nishio *et al.*, 2003)], *C. diphtheriae* NCTC 13129 [GenBank accession NC_002935 (Cerdeño-Tárraga *et al.*, 2003)] and *C. jeikeium* K411 [GenBank accession NC_007164 (Tauch *et al.*, 2005)], were searched for using the protein-protein BLAST program (Altschul *et al.*, 1990) with Best Reciprocal Hit method.

A search in the whole *C. glutamicum* genome for DNA motifs similar to the SugR-binding motif found in the *ldh* promoter was performed using the Scan For Matches software (Dsouza *et al.*, 1997) and specific Perl scripts to select motifs located in intergenic regions and oriented like the one in the *ldh* promoter.

Promoter regions of putative target genes of SugR in *C. glutamicum* (*ptsG*, *sugR-cg2116*, *ptsI*, *cg2118-ptsF*, *ptsH*, *ptsS*, *scrB*, *ldh*), *C. efficiens* (CE1824, CE1827, *ldh*) and *C. diphtheriae* (DIP1427, DIP1429, *ldh*) were compared with the MEME software (Bailey & Elkan, 1994) in order to determine a consensus motif for the SugR-binding site.

RESULTS AND DISCUSSION

Batch-culture characteristics during biotin-limited growth on glucose

The growth of *C. glutamicum* RES167 (Dusch *et al.*, 1999) on glucose was investigated in biotin-limited batch cultures. A typical batch fermentation profile is shown in Fig. 1. The fermentation profile was very different from that observed during growth with excess biotin, and showed three distinct phases. In all these phases, growth appeared to be arithmetic rather than exponential, as the increase in biomass concentration was a linear function of time. During the initial phase of growth, glucose was consumed at a specific rate (q_{glc}) of 1.53 ± 0.05 mmol (g dry cells)⁻¹ h⁻¹ and a growth rate of 0.558 ± 0.03 g dry cells h⁻¹ l⁻¹ was maintained. This corresponded to a rate of carbon assimilation into biomass ($Y_{X/C}$) of 12.485 ± 0.96 g dry cells (mol carbon consumed)⁻¹. Acetic acid [$\gamma_{\text{ace}} = 0.678 \pm 0.04$ mmol (g dry cells)⁻¹ h⁻¹], L-lactic acid [$\gamma_{\text{lac}} = 0.382 \pm 0.01$ mmol (g dry cells)⁻¹ h⁻¹] and trace amounts of pyruvic acid were produced during this first phase. No glutamate was detected in the culture medium. This was not surprising because the culture conditions used in this study were significantly different from those used for glutamate production under biotin limitation (Choi *et al.*, 2004). The most noticeable phenomenon observed was the overflow of organic acids

Fig. 1. Features of *C. glutamicum* RES::pAG1022 growth during biotin-limited batch cultures on glucose. (a) Biomass (□, g dry cells), glucose concentration (■, % w/v), lactate concentration (▲, g l⁻¹), acetate concentration (●, g l⁻¹). (b) L-Lactate dehydrogenase specific activity [△, mmol min⁻¹ (mg protein)⁻¹], β-Galactosidase activity [○, 10 U (mg protein)⁻¹], lactate concentration (▲, g l⁻¹), intracellular fructose 1,6-bisphosphate concentration (◇, mM). Each point corresponds to the mean value from nine measurements. Standard deviations are indicated by error bars.

during the growing phase without oxygen limitation and no heat or nutritional stress. To date, organic acid production by *C. glutamicum* has been mainly observed under oxygen deprivation, at the onset of the stationary phase or after heat shock during continuous cultivation (Dominguez *et al.*, 1993; Okino *et al.*, 2005; Inui *et al.*, 2004; Uy *et al.*, 2003; Stansen *et al.*, 2005). During growth on glucose with excess biotin, q_{glc} was 4.44 mmol (g dry cells)⁻¹ h⁻¹ and $Y_{X/C}$ was 11.02 g dry cells (mol carbon consumed)⁻¹, as recalculated from data published by Gerstmeir *et al.* (2003). In biotin excess, the specific rate of glucose consumption was significantly higher than that in biotin limitation. As the specific activity of the glucose-specific PTS was shown to be constant under various conditions with glucose as a carbon source (Cocaign-Bousquet *et al.*, 1996), and no inhibition of glucose PTS

enzymes was reported in *C. glutamicum*, the low q_{glc} value in our study was attributed to a low glycolytic flux due to the bottleneck created by biotin limitation. Acetate and lactate excretion was the probable means to avoid intracellular accumulation of pyruvic acid, a direct consequence of biotin limitation, and ensure reoxidation of NADH produced by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). Acetate and lactate were assumed to be the products of pyruvate metabolism by pyruvate oxidase (EC 1.2.2.2) and lactate dehydrogenase respectively, as described for *Lactobacillus plantarum* when cultivated with carbon sources supporting low growth rates under aerobic growth conditions (Sedewitz *et al.*, 1984). As described for *C. glutamicum* R under oxygen-deprived conditions, phosphotransacetylase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1) and CoA transferase (EC 2.8.3.-)

could also be involved in acetate production (Yasuda *et al.*, 2007). The second phase extended from 7.5 to 11 h of cultivation. Growth rate decreased (0.194 ± 0.02 g dry cells $\text{h}^{-1} \text{l}^{-1}$), while q_{glc} slightly increased [2.98 ± 0.04 mmol (g dry cells) $^{-1} \text{h}^{-1}$] and acetic acid was consumed and exhausted at a high specific rate [$q_{\text{ace}} = 8.38 \pm 0.1$ mmol (g dry cells) $^{-1} \text{h}^{-1}$]. L-Lactic acid was also consumed but at a low specific rate [$q_{\text{lac}} = 0.815 \pm 0.03$ mmol (g dry cells) $^{-1} \text{h}^{-1}$]. Consumption of acetic acid and L-lactic acid to a lesser extent occurred when significant amounts of glucose were still present in the culture medium (10 g l^{-1}). The slower rate of growth was attributable to the decreased rate of carbon assimilation into biomass [$Y_{\text{X/C}} = 7.73 \pm 0.37$ g dry cells (mol carbon consumed) $^{-1}$] due to the use of acetate as the preferred carbon source. During this phase, q_{glc} and q_{ace} were similar to the corresponding values determined by Gerstmeir *et al.* (2003) with a mixture of glucose and acetate as carbon sources and biotin excess [$q_{\text{glc}} = 2.16$ mmol (g dry cells) $^{-1} \text{h}^{-1}$ and $q_{\text{ace}} = 8.1$ mmol (g dry cells) $^{-1} \text{h}^{-1}$]. Acetate consumption was then probably mediated by the standard acetate utilization pathway that bypasses the pyruvate bottleneck (Gerstmeir *et al.*, 2003).

During the third phase, from 11 to 16.5 h of cultivation, growth rate decreased (0.113 ± 0.02 g dry cells $\text{h}^{-1} \text{l}^{-1}$) and glucose and L-lactic acid were progressively consumed with specific rates of 3.87 ± 0.03 mmol (g dry cells) $^{-1} \text{h}^{-1}$ and 2.12 ± 0.4 mmol (g dry cells) $^{-1} \text{h}^{-1}$ respectively. Acidification of the medium occurred and pH fell to about 5. During this phase, $Y_{\text{X/C}}$ was slightly higher than during the second phase [9.98 ± 0.05 g dry cells (mol carbon consumed) $^{-1}$]. After 25 h cultivation, glucose and L-lactic acid were exhausted from the medium and no more growth occurred. L-Lactic acid consumption was essentially catalysed by Lld, the membrane-bound and FMN-dependent lactate dehydrogenase (EC 1.1.2.3) encoded by *cg3227* (data not shown).

L-Lactate dehydrogenase in *C. glutamicum*

Of the phenomena seen during biotin-limited growth on glucose, L-lactic acid overflow was the most intriguing to us. We therefore focused our work on the identification and characterization of the *C. glutamicum* gene and the corresponding protein involved in L-lactic acid production under these conditions. Whole-genome screening enabled us to focus on the annotated lactate dehydrogenase-encoding genes *cg0763*, *cg1027*, *cg3219* and *cg3227*. Of these genes, *cg3219* was the best candidate as the corresponding protein YP_227149 exhibited all the features expected for a cytoplasmic, L-lactic acid-producing, L-lactate dehydrogenase (Clarke *et al.*, 1989). The *cg3219* gene was disrupted and the behaviour of the corresponding mutant (RES167::pMM29) was investigated in biotin-limited batch cultures as previously described. The growth profile of the Ldh-deficient mutant was similar to that of the wild-type strain. L-Lactic acid was no longer synthesized but acetic acid production was doubled (2.9 ± 0.53 g

l^{-1}). In addition, no Ldh activity was detected in crude extracts obtained from the mutant strain. This confirmed the conclusion of Inui *et al.* (2004) that *cg3219* was the sole L-lactate dehydrogenase-encoding *ldh* gene and was not essential for growth on glucose. Growth of this mutant and of the parental wild-type strain were compared in biotin-limited batch cultures with L-lactate as a carbon source. No difference was seen between the two strains, indicating that *ldh* was also not essential for growth on L-lactate.

Enzymic characteristics of the *C. glutamicum* Ldh were analysed. Substrate specificity assays showed that the enzyme was specific for the reduction of pyruvic acid to L-lactic acid, with NADH as the exclusive cofactor. The optimal pH for the reaction was 6.6. No reaction was detected when the enzyme was assayed for L-lactic acid oxidation, irrespective of the pH tested and the cofactor used. Moreover, no signal was detected when activity staining was performed (Selander *et al.*, 1986). These results indicated that Ldh operated only in the direction of pyruvic acid reduction to L-lactic acid. To our knowledge, only the *Clostridium acetobutylicum* Ldh has previously been experimentally demonstrated to be irreversible (Contag *et al.*, 1990). The K_m value was calculated as 7.3 mM for pyruvic acid and the V_{max} value was calculated as $120 \mu\text{mol min}^{-1}$. Out of the different effectors tested, only fructose 1,6-bisphosphate had a marked effect on the reaction. In the presence of 10 mM fructose 1,6-bisphosphate, V_{max} increased to $450 \mu\text{mol min}^{-1}$. This activation of Ldh by fructose 1,6-bisphosphate has been described for the enzymes of a great number of Gram-positive bacteria (Garvie, 1980) and was not specific to the *C. glutamicum* enzyme.

Time-course of expression of *ldh*

The time-course expression of *ldh* was followed during biotin-limited growth (Fig. 1). As Muffler *et al.* (2002) suggested an upregulation of *ldh* during growth on glucose when compared to acetate, strain RES167::pAG1022 was cultivated in biotin-limited conditions with either glucose or acetate. Both Ldh and β -Gal specific activities were measured. During growth on glucose, Ldh specific activity increased from 0.3 ± 0.02 mmol min^{-1} (mg protein) $^{-1}$ to about 3.1 ± 0.04 mmol min^{-1} (mg protein) $^{-1}$ during the first 9 h and progressively decreased after to a value of 2.6 ± 0.05 mmol min^{-1} (mg protein) $^{-1}$. Simultaneously, β -Gal specific activity increased from 23.7 ± 1.3 U (mg protein) $^{-1}$ to 88.3 ± 2.7 U (mg protein) $^{-1}$ during the first 9 h and then progressively decreased to a value of 64 ± 0.5 U (mg protein) $^{-1}$. The maxima of both Ldh and β -Gal specific activities corresponded to the moment when L-lactic acid consumption began. These results indicated a transcriptional induction of *ldh* during the first 9 h followed by a partial transcriptional repression. Even if a putative post-translational regulation of Ldh was possible, as the *C. glutamicum* Ldh was shown to be susceptible to serine phosphorylation under certain conditions (Bendt

et al., 2003), this phenomenon was unlikely to be involved in this case. During growth on acetate, the levels of Ldh [$0.4 \pm 0.01 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$] and β -Gal [$15.3 \pm 0.8 \text{ U} (\text{mg protein})^{-1}$] remained low throughout the cultivation time and no L-lactic acid was detected in the culture medium. Glutamate dehydrogenase (Gdh) and isocitrate dehydrogenase (Icd) activities were measured as internal controls because both activities were constant as a function of time and almost identical irrespective of the use of acetate or glucose as a carbon source (Börmann *et al.*, 1992; Eikmanns *et al.*, 1995). The values determined were 2.05 ± 0.3 and $2.1 \pm 0.2 \text{ mmol NADPH oxidized min}^{-1} (\text{mg protein})^{-1}$ for Gdh during growth on glucose and acetate respectively and 1.87 ± 0.1 and $1.86 \pm 0.1 \text{ mmol NADP reduced min}^{-1} (\text{mg protein})^{-1}$ for Icd during growth on glucose and acetate respectively. It was thus concluded that *ldh* regulation was specific and not the result of a general change in the transcriptional profile.

The time-course regulation of *ldh* and the L-lactate variations in the culture medium could be explained as follows. During the first hours of cultivation, the pool of glycolytic intermediates, including fructose 1,6-bisphosphate and pyruvate, was high due to the bottleneck created at the pyruvate node by the low flux supported by pyruvate carboxylase under biotin limitation. Of the glycolytic intermediates accumulated, one was probably involved somehow in the increase of *ldh* expression. Biotin limitation also led to an increase of the NADH/NAD ratio, due to glyceraldehyde-3-phosphate dehydrogenase activity and a suspected diminished flux through the Krebs cycle. Both fructose 1,6-bisphosphate and a high NADH/NAD ratio have been described as activators of Ldh activity (Garvie, 1980; Garrigues *et al.*, 1997). Increase in *ldh* expression and Ldh activation then allowed the efficient conversion of pyruvate into lactate and a progressive decrease of the intracellular concentration of glycolytic intermediates and the NADH/NAD ratio. This led to a partial repression of *ldh* expression and a diminished flux through Ldh. This hypothesis was substantiated, as the intracellular concentration of fructose 1,6-bisphosphate actually decreased progressively during the cultivation time as shown in Fig. 1(b). After 9 h cultivation, L-lactic acid consumption was essentially catalysed by Lld, the membrane-bound and FMN-dependent lactate dehydrogenase encoded by *cg3227*, expression of which was induced by the excreted L-lactic acid (Georgi *et al.*, 2008). During this second phase, the reducing equivalents that had been used first for L-lactic acid synthesis were redirected to the respiratory chain to gain ATP and pyruvic acid was redirected to glycolysis.

Identification of the *ldh* transcriptional regulator

In order to explain the increase in *ldh* transcription during growth on glucose and its low, constitutive transcription during growth on acetate, we had to postulate the existence of a transcriptional regulator binding to the *ldh* promoter.

A DNA-affinity approach (Gabrielsen *et al.*, 1989; Rey *et al.*, 2003; Engels *et al.*, 2004) was used to purify such a protein, with the *ldh*-*cg3220* intergenic region as a target. Protein purification was performed with dialysed crude extracts from cells grown on glucose or acetate, harvested after 7.5 h cultivation when lactate reached its maximum concentration on glucose. Two proteins from the extract of acetate-grown cells (YP_224408 and YP_226173) and three from the extract of glucose-grown cells (YP_226173, YP_227153 and YP_227238) appeared to bind efficiently to the fragment, as shown in Fig. 2. It is noteworthy that all these proteins can bind the DNA fragment in the absence of added metabolites. Unequivocal protein identification was performed by MALDI-TOF mass spectrometry as described in Methods. All these proteins were predicted to be potential transcriptional repressors, protein YP_226173 being present in both extracts. As the target DNA fragment contained both the *ldh* promoter and the promoter of the diverging *cg3220* putative gene, it was impossible to unambiguously identify the *ldh* regulator. Defined in-frame deletion mutants for each of the candidate genes (*cg2115*, *cg3224*, *cg0146*, *cg3315*) were established by a gene replacement strategy (Schäfer *et al.*, 1994). As all these genes were predicted to belong to operon structures (Baumbach *et al.*, 2007), extreme care was taken to avoid polar effects of these deletions. After transformation of the four deleted strains with pAG1022, β -Gal (data not shown) and Ldh activities were analysed in extracts from glucose-grown or acetate-grown cells. Ldh specific activity in the $\Delta cg2115$ strain was about $12 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$ irrespective of the carbon source, whereas Ldh specific

Fig. 2. SDS-PAGE of *C. glutamicum* RES167 proteins eluted from a DNA-affinity chromatography experiment using the *ldh* promoter/operator as a probe and cell extracts from glucose-grown cells (lane 1) and acetate-grown cells (lane 3). Lane 2, molecular mass marker.

activity was not affected in the other mutant strains ($\Delta cg3224$, $\Delta cg0146$ and $\Delta cg3315$) when compared to the wild-type one. These results clearly show that the YP_226173 protein encoded by *cg2115* was the repressor of *ldh*. Time-course analysis of *ldh* expression in the $\Delta cg2115$ strain grown on glucose was investigated. The Ldh specific activity remained constant, indicating that YP_226173 was also responsible for the transcriptional repression observed after 9 h cultivation. The *cg2115* gene was recently identified as *sugR*, SugR being the repressor of *ptsG*, *ptsF*, *ptsI*, *ptsH* and *ptsS*, the genes encoding the glucose-, fructose- and sucrose-specific PTSs and the general components EI and Hpr (Engels & Wendisch, 2007; Gaigalat *et al.*, 2007; Tanaka *et al.*, 2008a). Comparison of Ldh activities in extracts from the wild-type [3.1 ± 0.04 mmol min⁻¹ (mg protein)⁻¹] and the $\Delta sugR$ [12 ± 0.7 mmol min⁻¹ (mg protein)⁻¹] strains showed that *ldh* was not completely derepressed during growth on glucose.

Recently, Georgi *et al.* (2008) have shown that YP_227153 corresponded to LldR, the transcriptional regulator of *lld* (*cg3227*) that encodes the membrane-bound L-lactate dehydrogenase (EC 1.1.2.3) involved in L-lactate consumption in *C. glutamicum* (Stansen *et al.*, 2005). Georgi *et al.* (2008) have shown that the sequence 5'-TGGTCTGACCA-3' was essential for the binding of LldR on the *cg3226-lld* promoter. The presence of the highly similar motif 5'-TGGTCAGACCA-3' in the *ldh-cg3220* intergenic region sequence, overlapping the -35 region of *Pldh*, explained the binding of LldR in our experiment. As described before, the deletion of *lldR* (*cg3224*) does not affect *ldh* expression, indicating that LldR is not involved in *ldh* regulation.

Given the presence of the genes coding for general PTSs EI (*ptsI*) and HPR (*ptsH*), fructose-specific PTS EII (*ptsF*), 1-phosphofructokinase (*pfkB*), the transcriptional regulator FruR (*fruR*) and a putative phosphofructokinase (*cg2116*) in the chromosomal vicinity of *sugR*, fructose was further considered as an additional carbon source of interest to study *ldh* expression. Ldh activities were comparable in extracts from cells grown on fructose [11.2 ± 0.5 mmol min⁻¹ (mg protein)⁻¹], irrespective of the genetic background. This indicated that *ldh* was fully derepressed when cells were grown in the presence of fructose and/or in the absence of SugR.

As *fruR* was a paralogue of *sugR* (DeoR-like protein, 51 % identity at the protein level with SugR) and was proven to be involved in the transcriptional regulation of *ptsI*, *ptsH* and the *cg2118-cg2119-cg2120* operon (Tanaka *et al.*, 2008b), genes also under the control of SugR, we tested the effect of the FruR deficiency on the *ldh* expression level. *C. glutamicum* RES::pAG1035 was cultivated with glucose or fructose as carbon sources and the Ldh activity was measured in both conditions. When compared to Ldh activities from the wild-type strain grown in the same conditions, no difference was seen, indicating that FruR was not involved in *ldh* regulation.

Because of the common features of fructose and sucrose metabolism in *C. glutamicum* (Dominguez & Lindley, 1996), Ldh specific activity was also measured in extracts from cells grown on sucrose [11.5 ± 0.6 mmol min⁻¹ (mg protein)⁻¹]. In these conditions, *ldh* was also derepressed.

Growth of the $\Delta sugR$ mutant was similar to that of the wild-type strain on minimal medium containing acetate, glucose, fructose and sucrose, indicating that *sugR* was not essential for growth on these different carbon sources, as mentioned by Engels & Wendisch (2007).

Identification of the SugR^{His}-binding site

To demonstrate direct binding of the SugR protein to the *ldh* promoter by band-shift assays, a SugR^{His} protein was constructed and purified as described in Methods.

To test the *in vivo* functionality of SugR^{His} in *C. glutamicum*, the RES::pAG1034 strain was constructed as described in Methods. This strain and the wild-type strain were grown on glucose and on fructose, and Ldh specific activities were measured and compared. Ldh specific activities were 3.1 ± 0.04 mmol min⁻¹ (mg protein)⁻¹ and 11.2 ± 0.5 mmol min⁻¹ (mg protein)⁻¹ for RES167::pAG1034 grown on glucose and fructose respectively and 2.9 ± 0.1 mmol min⁻¹ (mg protein)⁻¹ and 10.9 ± 0.4 mmol min⁻¹ (mg protein)⁻¹ for RES grown on glucose and fructose respectively. This indicated that the recombinant SugR^{His} was fully functional *in vivo* in *C. glutamicum*.

DNA band-shift assays were performed with the purified SugR^{His} protein from *E. coli* and a DNA fragment corresponding to the full-length *ldh-cg3220* intergenic region. The 205 bp DNA was obtained by PCR with oligonucleotides Ldh-10 and Ldh-11, and 1 pmol of this fragment was incubated with different amounts of SugR^{His} protein (0, 30, 60, 90, 120 and 150 pmol). The assays were then separated by non-denaturing agarose gel electrophoresis and visualized by fluorescence after ethidium bromide staining of gels (Fig. 3a). The presence of purified SugR^{His} protein caused a complete mobility shift at the concentration of 90 pmol, a 90-fold molar excess that probably represented only a 10-fold molar excess of native protein, as purified DeoR-type repressors seemed to be mainly octamers (Zeng *et al.*, 2000; Mortensen *et al.*, 1989).

To determine the exact location of the SugR-binding site in relation to the *ldh* promoter, the transcriptional start site of *ldh* was identified by using the 5' RACE method. The transcriptional start site was located 88 bp upstream of the annotated start codon. The determination of the transcriptional start site allowed the identification of -35 (5'-TGACCA-3') and -10 (5'-CATAAT-3') regions, the sequences of which were in good accordance with the features of corynebacterial promoter sequences (Pátek *et al.*, 2003). Interestingly, a 28 bp DNA sequence overlapping the -35 and -10 regions of the *C. glutamicum* *ldh*

promoter exhibited 93 % sequence similarity to the corresponding region of the *C. efficiens* *ldh* gene, whereas the surrounding sequences shared only 23 % sequence similarity.

To obtain a more precise location of the SugR-binding site, EMSAs were performed with three subfragments of the *ldh* promoter. These fragments were obtained by PCR with the oligonucleotides Ldh-26 and Ldh-11, Ldh-24 and Ldh-11 or Ldh-27 and Ldh-11. With respect to the transcriptional start site, these fragments extended from -60 to $+67$, from -35 to $+67$ and from -10 to $+67$ respectively. As a control, an internal fragment of *ldh* was amplified with oligonucleotides Ldh-1 and Ldh-2 and tested for SugR binding. The results (Fig. 3b), summarized in Fig. 4(a), showed that the SugR-binding site on the *ldh* promoter overlapped the -35 and -10 regions of the *ldh* promoter, a typical location of DNA-binding motifs for repressors (Gralla & Collado-Vides, 1996).

Comparing the *ldh* promoter region in *Corynebacterium* species with MEME software revealed a consensus motif of 27 bp (Fig. 4a), which strictly corresponded to the motif identified from EMSAs, in the *C. glutamicum* *ldh* promoter. The *C. efficiens* and *C. diphtheriae* motifs were strongly conserved (respectively 89 % and 82 % identity) and displayed the same structure as the *C. glutamicum* one. For those three species the motif began between the putative -35 and -10 regions and totally overlapped the -10 region. The motif found for *C. jeikeium* had low identity (48 %) and its structure was not coherent with that of the *C. glutamicum* motif.

Using Scan For Matches software, we found in the promoter regions of the *ptsG*, *cg2118-ptsF* and *sugR-cg2116* genes of *C. glutamicum* motifs partially similar to the 25 bp motif experimentally identified in the *ldh* promoter (Fig. 4b). For the *ptsG* promoter, only one of the motifs we found partially overlapped the motif found by Engels & Wendisch (2007). For the *cg2118-ptsF* promoter, both motifs we found more or less overlapped the motifs found by Gaigalat *et al.* (2007).

The analysis of putative targets of SugR in *C. glutamicum* genome revealed a 9 bp consensus motif (5'-TCGGACATA-3') for its binding site, which was totally or partially conserved in *Pldh* (identity 100 %), *PptsG* (100 %), *PptsS* (89 %), *PptsH* (89 %), *PsugR-cg2116* (67 %), *Pcg2118-ptsF* (67 %), *PptsI* (55 %), but was not present in *PscrB* (Fig. 4b). This 9 bp consensus motif was consistent with the SugR-binding site experimentally determined on a *ptsI-fruR* promoter fragment by Tanaka *et al.* (2008a). For *Pldh*, *PptsG* and *Pcg2118-ptsF*, this 9 bp motif was a part of the experimentally determined motif. Thus, it was likely that the SugR-binding sites contained this 9 bp motif.

From these results two models of structure for SugR-binding sites were built. The first one came from the *ldh*, *sugR-cg2116*, *cg2118-ptsF*, *ptsH* and *ptsS* promoters, and consisted of about 20–25 bp, more or less overlapping the

Fig. 3. Binding of SugR^{His} to various target DNA fragments. (a) EMSA using the *ldh* promoter region (1 pmol) from *C. glutamicum* (PCR fragment obtained with oligonucleotides Ldh-10 and Ldh-11) and increasing amounts of purified His-tagged SugR protein (SugR^{His}). Lanes: 1, control with no added SugR^{His}; 2–6, increasing amounts of SugR^{His} (30–150 pmol, in 30 pmol steps). (b) EMSA using subfragments of the *ldh* promoter region (0.5 pmol), control DNA (0.5 pmol) from *C. glutamicum* and SugR^{His} (50 pmol). Lanes: 1, Ldh-27–Ldh-11 fragment (SugR^{His} heat inactivated); 2, Ldh-27–Ldh-11 fragment (native SugR^{His}); 3, Ldh-24–Ldh-11 fragment (SugR^{His} heat inactivated); 4, Ldh-24–Ldh-11 fragment (SugR^{His} heat inactivated); 5, Ldh-26–Ldh-11 fragment (native SugR^{His}); 6, Ldh-26–Ldh-11 fragment (SugR^{His} heat inactivated); 7, Ldh-1–Ldh-2 (control DNA) fragment (SugR^{His} heat inactivated); 8, Ldh-1–Ldh-2 fragment (native SugR^{His}).

$-35/-10$ region. The second one came from the *ptsG* promoter and consisted of about 25 bp located upstream from the -35 region and which did not overlap the $-35/-10$ region of the promoter. Nevertheless, in each case, the locations of DNA-binding motifs are typical of those observed for repressors (Gralla & Collado-Vides, 1996).

To confirm our hypothesis on the SugR-binding site on *Pldh* in *C. glutamicum*, we constructed pAG1033, a derivative of pAG1022 (*lacZ* under the control of the *ldh* promoter) in which the 9 bp consensus binding site 5'-

Fig. 4. The organization of promoters of genes containing determined or predicted SugR-binding motifs, and construction of a consensus sequence of the binding motifs. The double-stranded sequence of each promoter is shown. The predicted -10 and -35 promoter regions and the transcriptional start sites are in bold. The translational start codons of the corresponding genes are underlined. The experimentally determined SugR-binding motifs are shown as grey boxes with a black border. The SugR-binding motifs predicted by sequence comparisons are shown as transparent boxes with a black border. A frequency plot of the deduced consensus sequence of all SugR-binding motifs predicted by sequence comparison constructed by means of the WebLogo tool (Crooks *et al.*, 2004) is also shown. The overall height of each stack of letters indicates the sequence conservation at each position of the motif, whereas the height of each symbol within the stack reflects the relative frequency of the corresponding nucleotide at that position. (a) Comparisons of *ldh* promoters in *C. glutamicum*, *C. efficiens* and *C. diphtheriae*. (b) Comparisons of *ldh*, *cg2118-ptsF*, *ptsS*, *sugR-cg2116*, *ptsH* and *ptsG* promoters in *C. glutamicum*.

TCGGACATA-3' described in Fig. 4(b) was replaced by 5'-AGCCTGTAT-3'. Strains RES167::pAG1022 and RES167::pAG1033 were grown on glucose or fructose as carbon source and β -Gal activities were measured. β -Gal activities were 83.2 ± 2.5 U (mg protein) $^{-1}$ and 231 ± 7 U (mg protein) $^{-1}$ for RES167::pAG1022 grown on glucose and fructose respectively; 187 ± 8 U (mg protein) $^{-1}$ and 220 ± 7.5 U (mg protein) $^{-1}$ for RES167::pAG1033 grown on glucose and fructose respectively. This clearly indicated that the 9 bp consensus binding site 5'-TCGGACATA-3' was involved in the *in vivo* binding of SugR to the *ldh* promoter.

Note that in *PptsS* and *Pcg2118-ptsF*, the motifs similar to the 9 bp consensus motif we identified were quite different from those found by Engels & Wendisch (2007). The motif they mentioned for *Pcg2118-ptsF* (5'-TGTGCAAC-3') was 250 bp upstream of the one we identified, and for *PptsS* the sequence 5'-TGTGCAAC-3' they mentioned was not detectable.

Another interesting feature was that the *sugR* and the *cg2118* coding sequences exhibit a strong nucleotide similarity in a 41 bp region included in the HTH_DEOR-encoding domains of both genes. Moreover, this region contains the 9 bp consensus motif for SugR binding on *Pldh*. This feature has been shown to exist in various genes encoding HTH regulatory proteins (Roy *et al.* 2002), the internal binding motif being involved in autoregulation of some of these regulators.

Features of *sugR* transcription and expression

As the locus encompassing *sugR* and *cg2116* was predicted to be an operon (Baumbach *et al.*, 2007), its transcriptional organization was analysed by RT-PCR. Total RNA isolated from *C. glutamicum* was transcribed into cDNA by using primer Regg1-12. The resulting cDNA was used for PCR amplification with primers Regg1-12 and Regg1-3 or Regg1-12 and Regg1-11. The cDNA obtained with Regg1-12, annealing in *cg2116*, allowed the amplification of not only a *cg2116* internal fragment with Regg1-12 and Regg1-3, but also a fragment overlapping *sugR* and *cg2116* with Regg1-12 and Regg1-11. Both fragments were at the expected size. No PCR product was obtained when reverse transcriptase was omitted. From this experiment we concluded that *sugR*

and *cg2116* were organized as an operon, an observation that was also reported by Gaigalat *et al.* (2007).

The transcriptional start site of the *sugR-cg2116* operon was identified by using the 5' RACE method. The transcriptional start site was located 48 bp upstream of the annotated start codon. The sequences of the -35 (5'-TGAGGC-3') and -10 (5'-GACAAT-3') regions were also in accordance with the features of corynebacterial promoter sequences (Pátek *et al.*, 2003).

A transcriptional fusion with the *sugR-cg2116* promoter and a promoterless *lacZ* gene was constructed. The resulting plasmid pAG1030 was introduced in *C. glutamicum* as described in Methods. Strain RES167::pAG1030 was cultivated in biotin-limited batch cultures on acetate, glucose, fructose or sucrose, and β -Gal specific activities were measured. Specific activities were 4.86 ± 0.4 U (mg protein) $^{-1}$, 4.68 ± 0.3 U (mg protein) $^{-1}$, 4.73 ± 0.3 U (mg protein) $^{-1}$ and 9.32 ± 0.5 U (mg protein) $^{-1}$ in extracts from glucose-, fructose-, sucrose- and acetate-grown cells respectively. This indicated that SugR was present in all the conditions tested, as suggested by the DNA-affinity experiment described for the isolation of SugR (Fig. 2). From these measurements we also concluded that the *sugR-cg2116* operon was subject to transcriptional regulation during growth on acetate. The same result was obtained by Gaigalat *et al.* (2007) using real-time RT-PCR.

Identification of SugR co-inducers

To explain the differences in *ldh* expression in extracts from acetate-grown, glucose-grown, sucrose-grown and fructose-grown cells, we postulated that SugR binding on *Pldh* was affected by inducer molecules derived from sugar metabolism.

We therefore tested the *in vitro* prevention of SugR binding to the *ldh* promoter (PCR fragment obtained with oligonucleotides Ldh-10 and Ldh-11) with several metabolites, including fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate and glucose 6-phosphate, that have been previously shown (Engels & Wendisch, 2007; Gaigalat *et al.*, 2007) to prevent SugR binding to various *pts* promoters.

Fructose 1-phosphate fully inhibited SugR binding to *Pldh* at concentrations ≥ 100 μ M (Fig. 5a), as did fructose 1,6-

Fig. 5. Effector screening studies. EMSAs were conducted using the *ldh* promoter region (0.05 pmol) from *C. glutamicum* (PCR fragment obtained with oligonucleotides Ldh-10 and Ldh-11) incubated with 5 pmol purified SugR^{His} and increasing concentrations of different putative effectors. Effectors tested were: (a) fructose 1-phosphate, (b) fructose 1,6-bisphosphate, (c) fructose 6-phosphate and (d) glucose 6-phosphate. The non-complexed *ldh* promoter was used as a control (lane P).

bisphosphate at concentrations ≥ 10 mM (Fig. 5b). No effect was observed by using up to 20 mM of either fructose 6-phosphate (Fig. 5c) or glucose 6-phosphate (Fig. 5d). In addition, no influence on SugR binding was observed by using glyceraldehyde 3-phosphate, phospho(enol)pyruvate, pyruvate, glucose, fructose, sucrose, lactate or acetate (data not shown).

Fructose 1-phosphate could be considered the specific metabolite of fructose and sucrose metabolism in *C. glutamicum* for the following reasons. Fructose uptake in *C. glutamicum* involves two PTSs enabling phospho(enol)-pyruvate-dependent transport and phosphorylation of fructose to give fructose 1-phosphate (PTS^{Fru}) and fructose 6-phosphate (PTS^{Glc}). Fructose 1-phosphate is then converted to fructose 1,6-bisphosphate by the *cg2119*-encoded 1-phosphofructokinase (Moon *et al.*, 2005). Partitioning between the two systems was estimated as follows: fructose uptake due to PTS^{Fru} = $3.5 \text{ mmol (g dry cells)}^{-1} \text{ h}^{-1}$ and fructose uptake due to PTS^{Glc} = $0.9 \text{ mmol (g dry cells)}^{-1} \text{ h}^{-1}$ (Dominguez *et al.*, 1998). Sucrose metabolism in *C. glutamicum* involves a phospho(enol)-

pyruvate-dependent sucrose PTS to give intracellular sucrose 6-phosphate, and a sucrose-6-phosphate hydrolase that yields glucose 6-phosphate and fructose (Engels *et al.*, 2008). In the absence of fructokinase, further complete sucrose metabolism requires efflux of fructose and its reassimilation via the fructose PTS and the glucose PTS to a lesser extent as previously described (Dominguez & Lindley, 1996; Moon *et al.*, 2005).

Although the internal concentration of fructose 1-phosphate in *C. glutamicum* is unknown, micromolar concentrations are probable in cells grown on fructose or sucrose. Then, given its strong effect *in vitro* at low concentrations on SugR binding to the *ldh* promoter, plus the complete derepression of *ldh* on both fructose and sucrose when compared to glucose as carbon sources, fructose 1-phosphate could be considered the most effective co-inducer of SugR for *ldh* expression.

To test the *in vivo* effect of fructose 1-phosphate on *ldh* expression, we constructed a *cg2120* mutant (RES167::pAG1032) devoid of the fructose-specific EII^{Fru} activity (EC 2.7.1.69). As fructose alone only supports weak growth of a *ptsF* mutant (Moon *et al.*, 2005), the wild-type and the RES167::pAG1032 strains were grown on minimal medium with either glucose or sucrose, or a mixture of glucose and fructose, as carbon sources and Ldh activities were measured in cell extracts. Ldh activities were $2.27 \pm 0.1 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$, $12.13 \pm 0.7 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$ and $11.02 \pm 1.1 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$ in extracts from the wild-type strain grown on glucose, sucrose and glucose/fructose respectively, and $2.76 \pm 0.2 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$, $2.43 \pm 0.2 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$ and $3.49 \pm 0.3 \text{ mmol min}^{-1} (\text{mg protein})^{-1}$ for the RES167::pAG1032 strain grown on glucose, sucrose and glucose/fructose respectively. This experiment confirmed that fructose 1-phosphate, the product of fructose phosphorylation by the fructose-specific EII^{Fru} component, was actually an efficient inducer of *ldh* transcription.

As fructose 1,6-bisphosphate should be present in fructose-grown or sucrose-grown cells, its contribution to *ldh* induction should not be neglected. However, its contribution to *ldh* induction is unlikely to exceed its contribution to *ldh* expression with glucose as a carbon source, otherwise the *ldh* expression would be as high with glucose as a carbon source as with sucrose in both the wild-type and the *ptsF* mutant strain.

On the other hand, fructose 1,6-bisphosphate, the main activator of Ldh activity, was a good candidate to explain *ldh* induction and the time-course variation of *ldh* expression during biotin-limited growth on glucose (Fig. 1a). This can be seen in Fig. 1(b), as the intracellular concentration of fructose 1,6-bisphosphate progressively diminished from about 10 mM, a concentration that led to a complete *in vitro* inhibition of the SugR binding to Pldh (Fig. 5b), to about 1 mM at the end of cultivation time of the wild-type strain on glucose, a concentration that did not allow the *in vitro* inhibition of SugR binding to Pldh.

As a striking difference, Engels & Wendisch (2007) identified fructose 6-phosphate as the only effector of SugR binding to the *ptsG* promoter, whereas Gaigalat *et al.* (2007) identified fructose 1-phosphate as the main effector of SugR binding to the promoters of *ptsI*, *ptsH*, *ptsG*, *ptsS* and the *cg2118-fruK-ptsF* operon, and fructose 1,6-bisphosphate and glucose 6-phosphate to a lesser extent. Gaigalat *et al.* (2007) suggested that SugR may be able to form complexes with individual binding sites with different sensitivities to sugar phosphates as described for the TrmB regulator of *Pyrococcus furiosus* (Lee *et al.*, 2005, 2007), and proposed mutational analyses of the SugR-binding sites to clarify this issue. Prior to this analysis, however, SugR binding on its various targets under the same *in vitro* binding conditions and with a native SugR protein should be analysed. A note of caution should be exercised, as Engels & Wendisch (2007) used an amino-terminally His-tagged SugR protein, Gaigalat *et al.* (2007) used a recombinant SugR obtained after thio-induced self-cleavage of an intein tag, and we used a recombinant carboxyterminal-His-tagged SugR, and these differences may have a subtle, yet important impact on SugR-binding properties.

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