Overall control of nitrogen metabolism in *Lactococcus lactis* by CodY, and possible models for CodY regulation in Firmicutes

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CodY, a pleiotropic transcriptional regulator conserved in low G + C species of Gram-positive bacteria, was previously described to be the central regulator of proteolysis in *Lactococcus lactis*. In this study, over 100 potential CodY targets were identified by DNA-microarray analysis. Complementary transcriptional analysis experiments were carried out to validate the newly defined CodY regulon. Moreover, the direct role of CodY in the regulation of several target genes was demonstrated by gel retardation experiments. Interestingly, 45 % of CodY-dependent genes encode enzymes involved in amino acid biosynthesis pathways, while most of the other genes are involved in functions related to nitrogen supply. CodY of *L. lactis* represents the first example of a regulator in Gram-positive bacteria that globally controls amino acid biosynthesis. This global control leads to growth inhibition in several amino-acid-limited media containing an excess of isoleucine. A conserved 15 nt palindromic sequence (AATTTTCNGAAATT), the so-called CodY-box, located in the vicinity of the –35 box of target promoter regions was identified. Relevance of the CodY-box as an operator for CodY was demonstrated by base substitutions in gel retardation experiments. This motif is also frequently found in the promoter region of genes potentially regulated by CodY in other Gram-positive bacteria.

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INTRODUCTION

In prokaryotes, the metabolism of amino acids is regulated by many specific mechanisms, including biochemical control of enzymes and of their synthesis in response to the availability of particular metabolites. In addition to these targeted regulatory mechanisms, more broad-range regulatory systems have been described, allowing a coordination of the whole cell machinery in response to the environment. This higher hierarchical mechanism might be carried out at the transcriptional level by global regulators, also called pleiotropic regulators. Examples of such regulators are now available for different bacteria, the paradigm being *Escherichia coli*, where seven regulatory proteins (CRP, FNR, IHF, FIS, ArcA, NarL and Lrp) directly modulate the expression of 51 % of the genes (Arfin *et al.*, 2000; Hung *et al.*, 2002; Kang *et al.*, 2005; Martinez-Antonio & Collado-Vides, 2003; Zheng *et al.*, 2004). Broad-range regulators have also been described in Gram-positive bacteria, examples being *Bacillus subtilis* CcpA and CodY (Blencke *et al.*, 2003; Molle *et al.*,

2003; Moreno *et al.*, 2001; Yoshida *et al.*, 2001). CcpA is involved in catabolic repression and controls the expression of about 10 % of *B. subtilis* genes (Blencke *et al.*, 2003; Moreno *et al.*, 2001; Yoshida *et al.*, 2001). The role exerted by this global regulator can be compared to that of CRP in *E. coli* (Moreno *et al.*, 2001). CodY represses the expression of a wide variety of genes involved in macromolecular degradation, nutrient transport, amino acid catabolism, branched-chain amino acid (BCAA) biosynthesis, genetic competence, antibiotic synthesis, motility and chemotaxis (Bergara *et al.*, 2003; Debarbouille *et al.*, 1999; Ferson *et al.*, 1996; Fisher *et al.*, 1996; Inaoka & Ochi, 2002; Inaoka *et al.*, 2003; Kim *et al.*, 2003; Lazazzera *et al.*, 1999; Mirel *et al.*, 2000; Molle *et al.*, 2003; Serror & Sonenshein, 1996a, b; Shivers & Sonenshein, 2004; Slack *et al.*, 1995; Wray *et al.*, 1997). In controlling expression of these late-exponential-phase and early-stationary-phase genes, the regulation by CodY allows cells to adapt to general nutrient limitation (Molle *et al.*, 2003). The role of CodY has been compared with that of Lrp from *E. coli*, although these regulators do not share any structural similarities (Molle *et al.*, 2003; Shivers & Sonenshein, 2004).

CodY is a highly conserved repressor protein present in a variety of low-G + C species of Gram-positive bacteria

Abbreviations: BCAA, branched-chain amino acid(s); RT-QPCR, real-time quantitative PCR.

Microarray datasets and supplementary tables are available with the online version of this paper.

(Ratnayake-Lecamwasam *et al.*, 2001). In *B. subtilis*, CodY has been shown to bind GTP, which enhances its regulatory activity. It follows that in cases of nutrient limitation leading to a drop in GTP in the cell, CodY repression is alleviated, allowing the expression of many genes involved in cell adaptation to this poorer environmental condition. Many CodY-dependent genes are also regulated by additional mechanisms, allowing their control in response to other environmental factors (Brandenburg *et al.*, 2002; Debarbouille *et al.*, 1999; Ferson *et al.*, 1996; Kim *et al.*, 2003; Mader *et al.*, 2004; Mirel *et al.*, 2000; Nakano *et al.*, 1991; Oda *et al.*, 2000; Ogura *et al.*, 2001; Wray *et al.*, 1997; Yoshida *et al.*, 2003). In *B. subtilis*, CodY has been shown to directly interact with, in addition to GTP, BCAA, in particular isoleucine (Shivers & Sonenshein, 2004). Interestingly, studies in *Lactococcus lactis* have shown that in this bacterium, CodY responds to BCAA, but not GTP, suggesting that the activity of CodY is modulated differently between these two bacteria (den Hengst *et al.*, 2005; Guedon *et al.*, 2001b; Petranovic *et al.*, 2004). In *L. lactis*, CodY has been found to repress genes involved in the utilization of proteins in the medium, including the cell-wall proteinase (*prtP*), peptide transporters (*opp*, *opt*), peptidases (*pepC*, *pepN*) and aminopeptidases (*araT*, *bcaT*) (Chambellon & Yvon, 2003; Guedon *et al.*, 2001a, b; Sanz *et al.*, 2001). CodY thus appears to be involved in external nitrogen supply, and its activity may be modulated by an intracellular pool of BCAA that would constitute an indicator of the nitrogen status of the cell (Guedon *et al.*, 2001b; Petranovic *et al.*, 2004). Compared to *B. subtilis* where the targets of CodY have been extensively studied, the global role of CodY appears to be limited in *L. lactis*.

In this study, more than 100 potential CodY-regulated genes have been characterized in *L. lactis*. Interestingly, most of them encode proteins involved in amino acid biosynthesis and in functions related to nitrogen supply, showing that *L. lactis* CodY globally controls nitrogen metabolism. To the best of our knowledge, *L. lactis* CodY is the first regulator shown to control nitrogen metabolism genes so extensively in a bacterium. A conserved 15 nt palindromic sequence located in the vicinity of the -35 box of CodY-regulated genes was characterized.

METHODS

Growth conditions, bacterial strains and media. *Escherichia coli* TG1 was used for plasmid propagation (Gilson, 1984). *E. coli* and *B. subtilis* strains were grown in Luria-Bertani (LB) medium at 37 °C under vigorous aeration (Maniatis *et al.*, 1982). *L. lactis* strains were grown at 30 °C without aeration in M17 or in a chemically defined medium (CDM) containing 0.5 g trehalose l⁻¹ as carbon source and all amino acids except aspartic acid and glutamic acid as nitrogen source (Sissler *et al.*, 1999; Terzaghi & Sandine, 1975). When required, 1.8% Casitone (Sigma) was added (CDM + Cas) (Guedon *et al.*, 2001a). CDM6 that derived from CDM contains only glutamate, isoleucine, leucine, methionine, serine and valine as source of amino acids (Lapujade *et al.*, 1998). To test the growth, precultures of *L. lactis* in CDM were washed twice in Ringer's solution and inoculated at 1% in fresh CDM or its derivatives. Growth

was measured kinetically with a Microbiology Reader Bioscreen C (LabSystems). When required, X-Gal (20 µg ml⁻¹), erythromycin (5 µg ml⁻¹ for *L. lactis*, 100 µg ml⁻¹ for *E. coli*, 1 µg ml⁻¹ for *B. subtilis*), ampicillin (100 µg ml⁻¹ for *E. coli*), spectinomycin (200 µg ml⁻¹ for *B. subtilis*) and neomycin (5 µg ml⁻¹ for *B. subtilis*) were added.

DNA manipulation procedures and strain construction.

Plasmids and total DNA were prepared as described previously (Sperandio *et al.*, 2005). All enzymes for DNA technology were used according to the manufacturers' specifications. Electrotransformation of *E. coli* and *L. lactis* was performed as described by Maniatis *et al.* (1982) and Holo & Nes (1989), respectively. The oligonucleotides used in this work were synthesized by Sigma Genosys (Table 1). DNA sequencing was performed on both strands using a fluorescent sequencing procedure (Perkin-Elmer Biosystems).

The chromosomal *codY* gene deletion of *L. lactis* IL1403 was constructed as follows. DNA fragments of 611 bp and 662 bp carrying, respectively, the upstream and the downstream region of the *codY* gene were generated by PCR. These fragments contain a *Bam*HI restriction site at their 3' and 5' ends, respectively, designed in *codY* up and *codY* down primers (Table 1). The PCR-amplified products were digested with *Bam*HI, ligated and cloned into the pGEM-T easy cloning vector (Promega). This plasmid was fused to the lactococcal thermosensitive vector pGhost 9 at the *Spe*I site to yield pJIM1080 *codY* deletion vector. In *L. lactis* IL1403, the *codY* gene was replaced by the inactive copy carried by pJIM1080 by double crossing over as described by Biswas *et al.* (1993), to give strain JIM8558. PCR amplification and Southern blotting were performed to verify the effective *codY* gene deletion in strain JIM8558.

RNA isolation. Total RNA was isolated from IL1403 wild-type and JIM8558 *codY* mutant strains cultivated in CDM + Cas. When the OD₆₀₀ reached 0.4, cells of 25 ml portions of the cultures were pelleted and rapidly frozen in nitrogen and stored at -80 °C. Total RNA was extracted from cell pellets as described by Sperandio *et al.* (2005) by using the High Pure RNA Isolation Kit (Roche). RNA concentration was determined by absorbance at 260 nm, and quality of RNA preparations was checked (i) by determining the ratio A₂₆₀/A₂₈₀, (ii) by visualizing the integrity of the 23S and 16S rRNA bands on an agarose gel and (iii) by verifying the absence of DNA contamination by PCR.

DNA-microarray analysis and validation of data. Genome-wide expression profiles were established by using commercial DNA microarray (Eurogentec, HO50C) containing most of the *L. lactis* IL1403 genes (2072 ORFs spotted in duplicate). Synthesis of CyDye fluorescently labelled cDNAs, DNA microarray hybridizations and washings were performed as previously described (Sperandio *et al.*, 2005). DNA microarrays were scanned through two channels for the respective fluorescent dyes using a confocal laser scanner (Virttek) to monitor the fluorescence intensities. For each spot, signal and local background intensities were determined with Imagene Software (version 5.1; BioDiscovery Inc.). A local background-subtracted signal value was calculated for each spot. Expression profiles were performed with RNA extracted from two independent cultures of strains IL1403 and JIM8558 to reduce culture-dependent or growth artefacts. Each RNA was labelled twice in order to perform a Dye-Swap. Raw data from four slides were normalized with the preP software (Garcia de la Nava *et al.*, 2003) by using a local normalization (LOWESS method) to reduce fluorescence bias introduced by the labelling reactions as well as by the differences in fluorescence intensity between the two dyes. A ratio corresponding to the mean of normalized signal and a z-test were calculated to compare expression profiles of strain IL1403 and its derivative *codY* mutant as described by Garcia de la Nava *et al.* (2003). Logarithmic representations of the data are presented in Fig. 1, allowing rapid visual inspection of

Table 1. Oligonucleotide primers used for PCR amplification

The *Bam*HI restriction sites designed for *codY* gene deletion in *L. lactis* strain IL1403 are in bold. The substitution bases designed into the CodY-box of the *gltA*, *cysD* and *ilvD* genes are underlined.

Gene name	Sequence of primer pairs
Primers for <i>codY</i> gene deletion	
<i>codY</i> up	5'-ATCACATGTCAAGGGTTATATTGA-3'/5'-TTTCAAGTAAT GGATCC ACTTTGT-3'
<i>codY</i> down	5'-TAAACTTGC AGGATCCA ATTTCTAAA-3'/5'-CATCTAACATTTCAACCATATCAATG-3'
Primers for QPCR	
<i>ahpF</i>	5'-GTGCAAGTGCAGCTATTTATGC-3'/5'-CTGACGACCAAGTTGAACTCCT-3'
<i>asnB</i>	5'-TCGTGAAGTGGCAGATTTCTTA-3'/5'-CCAATAGAAGCCCGAATAGTTG-3'
<i>ctrA</i>	5'-GGACCTTAGCCATTGTTGCTAT-3'/5'-CTTGACGACGACGAAGTGAATA-3'
<i>cysD</i>	5'-CAGCGACTCAAGCGATGTACTA-3'/5'-TACTCCCAAGTGTCCCAAGTG-3'
<i>fhuD</i>	5'-AACTCCTTAATCGCGAAGATGA-3'/5'-TCCTTGGAGTTCAAGAAGTGAA-3'
<i>glgD</i>	5'-CTTAACAGAGGTTTCGTCCGATT-3'/5'-ACAGATTGCGTTTCTCCTTCAT-3'
<i>glpF2</i>	5'-GATTGGACCTACGACAGGAATC-3'/5'-TTGGTGCAACAACAGGATACTC-3'
<i>gltA</i>	5'-TGCCATTACACAGAGGTAGAGG-3'/5'-GCAGCAGCAATTGTAGCATAAG-3'
<i>gltS</i>	5'-CAGTCAATTCAAAGGAGCAACA-3'/5'-GCTGCGACATAACTCTTAGCAA-3'
<i>ilvD</i>	5'-ATTAATTGGAGCAGGACTTGGA-3'/5'-CTAAACCAATCAACCCACCTTC-3'
<i>lctO</i>	5'-GACCTGCTTCAATTGATGTCCT-3'/5'-ACAACATCTGCTCCTTGTGCTA-3'
<i>optS</i>	5'-TCTTTAAACAAGCACGGACAGA-3'/5'-TTTGACGACTCCTGCTCTGTTA-3'
<i>ptsH</i>	5'-CCACATCGTTGTAGAACTGGT-3'/5'-CGAGTGACATAACACCCATGAT-3'
<i>purD</i>	5'-GCCCAACAAATGTTAGTCCAAT-3'/5'-TCTTTGTAGCTCCAGGCAATTC-3'
<i>purH</i>	5'-CCATGAATTAGTGGATGCAATG-3'/5'-GAAATTATCTCAACGGGTGGAA-3'
<i>serC</i>	5'-AGGACCCAGTGTACTTCCAAAG-3'/5'-AATCGTTCTCAGCCTCATCAAT-3'
<i>yaiF</i>	5'-CCAGGACAATTTGTAGCTGTTT-3'/5'-CCAATAAACCCCTGAATGAACA-3'
<i>yncA</i>	5'-CCTTCTTCTGGTGTAAGCATT-3'/5'-CCTCTGTTCTGAATTGGACGTAT-3'
<i>yohC</i>	5'-TCCTTGCTGACCTTCTACTTCC-3'/5'-CAGCCCACTTCCAAGTTCTATC-3'
<i>ypdC</i>	5'-CTCACAAGCATCATCACTTGGT-3'/5'-TCATGAGGATTCTCCCTACCAT-3'
<i>yshA</i>	5'-GGAATCATCACGTCAGCACTTA-3'/5'-TGTAAGGAACAGTCCCAAGGTT-3'
<i>yxdG</i>	5'-CCAATTCTGTGCTTAGCAGGAT-3'/5'-CGTAAAGCCTGAACTGAGAAGC-3'
Primers for gel retardation probe	
<i>asnB</i>	5'-AGTTTATCTTGAAACTTGGGTCA-3'/5'-CGATGCTCAATCAATTCCAAATTAC-3'
<i>ctrA</i>	5'-GGCGCCAAAAGTACTGACAGAACTC-3'/5'-GATTATAGTGTATCTGCATCTCG-3'
<i>cysD</i>	5'-CATGGACTTGCAAAAGTGTCG-3'/5'-CAACCTTGAGTTGTATTTTTTATTTCTAC-3'
<i>gltA</i>	5'-GTTGACCTTCATGTGGTTAAAGGTG-3'/5'-GTAAATTCAGTGAATTTGTGAA-3'
<i>ilvD</i>	5'-GAAAACCTTATTAATGAAAAGATACTTACTG-3'/5'-GAGTTCAGTCAACTTTTCCG-3'
<i>pepN</i>	5'-GACAAAATAGGGGATAATTGCAATTG-3'/5'-CTTGGTTTACGGTCAATATCGAGG-3'
<i>thrA</i>	5'-GATTTGCGAAGCTGATGCGAGAGAC-3'/5'-GTCTCTGAACAAATTAACATACA-3'
Primers for substitution analysis in the CodY-box of <i>gltA</i>, <i>cysD</i> and <i>ilvD</i> promoter region	
<i>gltA</i> -M1	5'-GTTGACCTTCATGTGGTTAAAGGTG-3'/5'-CGAAAATTATATATTTCTGAAAACCGAAATATTT-3' and 5'-AAATATTTCCGGTTTTCAGAAAATATATAATTTTCG-3'/5'-GTAAATTCAGTGAATTTGTGAA-3'
<i>gltA</i> -M2	5'-GTTGACCTTCATGTGGTTAAAGGTG-3'/5'-CGAAAATTATATATTTCT ACAA ATTTAAATATTT-3' and 5'-AAATATTTAAATTT GTAG AAAATATATAATTTTCG-3'/5'-GTAAATTCAGTGAATTTGTGAA-3'
<i>gltA</i> -M3	5'-GTTGACCTTCATGTGGTTAAAGGTG-3'/5'-CGAAAATTATATATTT CCAT GAAAATTTAAATATTT-3' and 5'-AAATATTTAAATTT CATG GAATATATAATTTTCG-3'/5'-GTAAATTCAGTGAATTTGTGAA-3'
<i>gltA</i> -M4	5'-GTTGACCTTCATGTGGTTAAAGGTG-3'/5'-CGAAAATTATATG CTT CTGAAAATTTAAATATTT-3' and 5'-AAATATTTAAATTT CAGAA GCATATAATTTTCG-3'/5'-GTAAATTCAGTGAATTTGTGAA-3'
<i>cysD</i> -M1	5'-CATGGACTTGCAAAAGTGTCG-3'/5'-GATAGAATTTCTGAATTTCTTATAATTA-3' and 5'-TAATTATAAGAAATTCAGGAAATCTATC-3'/5'-CAACCTTGAGTTGTATTTTTTATTTCTAC-3'
<i>cysD</i> -M2	5'-CATGGACTTGCAAAAGTGTCG-3'/5'-GATAGAATTTCTG CA ATTTCTTATAATTA-3' and 5'-TAATTATAAGAAAT GCAG AAAATCTATC-3'/5'-CAACCTTGAGTTGTATTTTTTATTTCTAC-3'
<i>ilvD</i> -M1	5'-GAGTTCAGTCAACTTTTCCG-3'/5'-TATAGACTATATAG CAT ATTCTGACAAATT-3' and 5'-AATTGTGAGAATAT GCT ATATAGTCTATA-3'/5'-GAAAACCTTATTAATGAAAAGATACTTACTG-3'
<i>ilvD</i> -M2	5'-GAGTTCAGTCAACTTTTCCG-3'/5'-TATAGACTATATAGAATAT CCT GACAAATT-3' and 5'-AATTGTGAGATATTTCTATATAGTCTATA-3'/5'-GAAAACCTTATTAATGAAAAGATACTTACTG-3'

Fig. 1. (a, b, c) Logarithmic-scale plots of spot intensities determined from microarray hybridizations. Comparison of logarithmic plot of spot mean normalized intensities of array hybridizations with two independent samples (a) from the wild-type strain (WT1 versus WT2) and (b) from the *codY* mutant strain (*codY*1 versus *codY*2). Each spot intensity corresponds to the mean of four different spot normalized intensity values. (c) Logarithmic plot of spot mean normalized intensities of array hybridizations from the wild-type and the *codY* mutant strains (WT versus *codY*). Each spot intensity corresponds to the mean of eight different normalized intensity values. (d) Scatter plot comparative analysis of microarray and RT-PCR experiments. Each data point represents the logarithmic ratio of the signal intensity determined for 17 *CodY*-dependent genes (*ahpF*, *asnB*, *ctrA*, *cysD*, *glgD*, *glpF2*, *gltA*, *gltS*, *ilvD*, *lctO*, *optS*, *serC*, *yaiF*, *yncA*, *yohC*, *yshA*, *yxdG*) for the wild-type and the *codY* mutant strains, as determined by both RT-QPCR and microarray analyses. R^2 , Pearson correlation coefficient.

data quality and reproducibility (Fig. 1a, b) and evaluation of overall modifications of pattern expression induced by *codY* mutation (Fig. 1c). Genes considered as significantly differentially expressed correspond to those for which (i) expression is, at least in one channel, twofold above the background, (ii) the *P*-value is lower than 0.35 and (iii) the differential expression level is higher than 2 (induced gene) or lower than 0.5 (repressed gene). Raw datasets are available as supplementary material with the online version of this paper. Additional analyses were performed to confirm the significance of the DNA-microarray data including (i) consistency with previous knowledge of the *CodY* regulated genes, (ii) consistency with the expression levels between genes belonging to the same clusters and (iii) additional measures by real-time quantitative PCR (RT-QPCR).

Twenty genes (*pi127*, *pi241*, *pi319*, *pi326*, *fluD*, *mesJ*, *purC*, *purD*, *purE*, *purF*, *purH*, *purK*, *purL*, *purM*, *purN*, *ptsH*, *rpmGA*, *ypgD*, *yphC* and *ypdC*) were excluded as probable artefacts (they belong to large clusters not differentially expressed, with expression levels just above the threshold; independent additional measures did not confirm DNA-microarray data). These 20 genes are thus not presented in Table 2.

RT-PCR amplification. RT-QPCR was performed using first-strand DNA as template. cDNAs were synthesized from 20 µg RNA samples as described above but without using aminoallyl-dUTP. Specific primers for each studied gene (Table 1) were designed using eprimers software (<http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html>). PCR was performed using the ABsolute QPCR SYBR Green MIX (ABgene) and was run on an ABI 7700 instrument (Applied Biosystems) as described previously (Sperandio *et al.*, 2005). For each strain, cDNA was synthesized from RNA extracted from three independent cultures and performed in triplicate for each gene. Internal control and normalization of the results were done with the *tuf* gene encoding the elongation factor TU.

Crude extract preparation and gel retardation assay. The protein extracts used for the biochemical assays were prepared from *B. subtilis* $\Delta codY$ carrying pHT315-P_{xyl} (JIM8168) or pHT315-P_{xyl}-*codY*_{lactis} plasmid (JIM8169) (Petranovic *et al.*, 2004). In strain JIM8169, the *L. lactis* *CodY* regulator is overproduced from the inducible *xylA* promoter while the control strain JIM8168 does not contain the *codY* gene. Cells were grown in 250 ml LB medium in the presence of 20 mM xylose. At culture saturation, cells were harvested and washed once with TN buffer and then resuspended in 1.5 ml lysis buffer (Yoshida *et al.*, 1999). The cells were disrupted by sonication (30 min) with a Vibra Cell Disrupter (Biolock Scientific). After centrifugation, 40% glycerol was added to the supernatant and the preparation was used as the *CodY*-enriched (JIM8169) or control (JIM8168) extract.

DNA probes of about 250 bp corresponding to the promoter regions of *cysD*, *gltA*, *ilvD*, *serC*, *thrA*, *pepN* and *asnB* were amplified by PCR using specific primers (Table 1) and labelled at the 5' end with [γ - 32 P]ATP by the T4 polynucleotide kinase (NEB). Fragments carrying *ilvD*, *cysD* and *gltA* promoter regions with specific substitutions were obtained by PCR using mutated primers (Table 1). Unincorporated nucleotides were removed with the NucleoSpin PCR purification kit (Macherey Nagel). *CodY*-DNA complexes were formed in 10 µl by incubating the 32 P-labelled probes (10 fmol DNA) with different amounts of *B. subtilis* crude extract (2.5–20 µg) in binding buffer [20 mM Tris (pH 7.8), 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol] in the presence of 0.1 g poly(dI-dC) l⁻¹ and 10 mM isoleucine (pH 7.8). Reaction mixtures were incubated at 25 °C for 10 min and electrophoresis was carried out at 4 °C in TAM buffer as previously described (Sperandio *et al.*, 2005).

Bioinformatic procedures. iMoMi is a multigenomic relational database around which are implemented different utilities to facilitate the search of operator sequences (N. Pons and others,

Table 2. CodY-regulated genes identified by DNA-microarray experiments

Function	Gene	Assignment	Organization*	Ratio†	CodY-box‡	P-value
Amino acid biosynthesis						
Aromatic	<i>aroH</i>	Phospho-2-dehydro-3-deoxyheptonate aldolase	<i>aroH-lctO</i>	3.7	—	5E-02
	<i>lctO</i>	L-Lactate oxidase	<i>aroH-lctO</i>	3.5	NA	2E-01
Aspartate	<i>asnB</i>	Asparagine synthetase B	<i>asnB</i>	12.9	aatttcagacaatt	2E-03
	<i>dapB</i>	Dihydrodipicolinate reductase	<i>dapB-yqaB</i>	3.2	aatttctgaataat	1E-01
	<i>yqaB</i>	Acetyltransferase	<i>dapB-yqaB</i>	2.8	NA	2E-01
	<i>lysA</i>	Diaminopimelate decarboxylase	<i>lysA</i>	3.9	aattgtcagaatata	3E-02
	<i>hom</i>	Homoserine dehydrogenase	<i>hom-thrB</i>	2.7	aattatcttaaaatt	1E-01
	<i>thrB</i>	Homoserine kinase	<i>hom-thrB</i>	3.1	NA	1E-01
	<i>thrA</i>	Aspartokinase/homoserine dehydrogenase	<i>thrA</i>	2.4	tatttattgaaaatt	3E-01
	<i>thrC</i>	Threonine synthase	<i>thrC-yvhA</i>	2.2	agtttacagaatatt	3E-01
	<i>yvhA</i>	Na ⁺ driven efflux pump homologue	<i>thrC-yvhA</i>	2.6	NA	2E-01
	<i>ychH</i>	2,3,4,5-Tetrahydropyridine-2-carboxylate N-succinyltransferase	<i>ychH</i>	2.0	aattttccaaatatt	4E-01
Glutamate	<i>gltD</i>	Glutamate synthase small subunit	<i>glt(B)D</i>	49.6	aattgtcagaatata	3E-07
	<i>gltA</i>	Citrate synthase	<i>gltA-citB-icd</i>	78.8	aattttcagaaaaata	7E-08
			<i>gltA-citB-icd</i>		aattttcggaataaa	
	<i>citB</i>	Aconitase	<i>gltA-citB-icd</i>	58.0	NA	4E-07
	<i>icd</i>	Isocitrate dehydrogenase	<i>gltA-citB-icd</i>	64.0	NA	3E-09
Branched chain	<i>pycA</i>	Pyruvate carboxylase	<i>pycA</i>	2.3	—	3E-01
	<i>leuB</i>	3-Isopropylmalate dehydrogenase	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	15.8	attttattgacaatt	7E-04
	<i>leuC</i>	3-Isopropylmalate dehydratase small subunit	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	15.8	NA	1E-03
	<i>leuD</i>	3-Isopropylmalate dehydratase large subunit	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	13.4	NA	3E-03
	<i>ilvD</i>	Dihydroxy-acid dehydratase	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	80.0	aatttctgacaat	1E-07
	<i>ymeB</i>	ABC transporter ATP-binding protein	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	21.7	NA	3E-04
	<i>ilvB</i>	Acetolactate synthase large subunit	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	53.0	NA	3E-07
	<i>ilvN</i>	Acetolactate synthase small subunit	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	114.7	NA	4E-09
	<i>ilvC</i>	Ketol-acid reductoisomerase	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	63.4	NA	3E-08
	<i>aldB</i>	α -Acetolactate decarboxylase	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	2.3	NA	1E-01
	<i>aldR</i>	Putative regulator	<i>leu(A)BCD-ymeB-ilvDBNC(A)-aldBR</i>	3.1	NA	1E-01
	<i>bcaT</i>	Branched-chain amino acid aminotransferase	<i>bcaT</i>	2.2	aatttcgatgaatatt	3E-01
	<i>hisC</i>	Histidinol phosphate aminotransferase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	13.6	ataattcagataatt	3E-03
	<i>hisZ</i>	ATP phosphoribosyltransferase regulatory subunit	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	11.9	NA	4E-03
	<i>hisD</i>	Histidinol dehydrogenase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	5.2	NA	5E-02
Histidine	<i>hisB</i>	Imidazoleglycerol phosphate dehydratase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	3.4	NA	1E-01
	<i>ymdC</i>	Aminoglycoside 3-phosphotransferase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	5.4	NA	5E-02
	<i>hisA</i>	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	6.6	NA	2E-02
	<i>hisF</i>	Cyclase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	3.4	NA	1E-01

Table 2. cont.

Function	Gene	Assignment	Organization*	Ratio†	CodY-box‡	P-value
Serine	<i>hisI</i>	Phosphoribosyl-AMP cyclohydrolase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	5.4	NA	6E-02
	<i>hisK</i>	Histidinol phosphatase	<i>hisCZGD-(ymdA)-hisB-ymdC-hisHAFIK</i>	6.3	NA	2E-02
	<i>cysD</i>	O-Acetylhomoserine thiolase	<i>cysD</i>	5.4	aattttctgaattc	4E-02
	<i>serC</i>	Phosphoserine aminotransferase	<i>serCAB</i>	5.8	aattatcagaaaatt	2E-02
	<i>serA</i>	D-3-Phosphoglycerate dehydrogenase	<i>serCAB</i>	7.1	NA	1E-02
Undefined	<i>serB</i>	Phosphoserine phosphatase	<i>serCAB</i>	6.9	NA	1E-02
	<i>bar</i>	Acyltransferase	<i>bar</i>	2.6	—	3E-01
	<i>yncA</i>	Acetyltransferase	<i>yncA</i>	2.6	tatattctgacaatt	2E-01
Nitrogen source uptake and peptide degradation						
Peptide degradation	<i>pepC</i>	Aminopeptidase C	<i>pepC</i>	2.8	attttttgataatt	8E-02
			<i>pepC</i>		aactctacgaaaatt	
Amino acids, peptides and amines	<i>pepN</i>	Aminopeptidase N	<i>pepN</i>	2.7	—	9E-02
	<i>ctrA</i>	Cationic amino acid transporter	<i>ctrA</i>	43.9	aatttactgacaagt	9E-08
			<i>ctrA</i>		aattgtcagacaatt	
	<i>gltS</i>	Glutamate or arginine ABC transporter substrate-binding protein	<i>gltS-argE</i>	0.3	—	1E-02
	<i>oppD</i>	Oligopeptide ABC transporter ATP-binding protein	<i>oppDFBCA</i>	5.6	—	4E-02
	<i>oppF</i>	Oligopeptide ABC transporter ATP-binding protein	<i>oppDFBCA</i>	3.0	NA	2E-01
	<i>oppA</i>	Oligopeptide ABC transporter substrate-binding protein	<i>oppA</i>	5.1	aattattggagaatt	6E-02
	<i>optS</i>	Oligopeptide ABC transporter substrate-binding protein	<i>optS</i>	62.2	tattttcattaaatt	2E-08
			<i>optS</i>		aattttcagaataat	
	<i>optA</i>	Oligopeptide ABC transporter substrate-binding protein	<i>optABCDF</i>	3.5	aattttctgaaaatt	3E-02
Other transport systems			<i>optABCDF</i>		aattttcagaaaaca	
			<i>optABCDF</i>		aattttctgacaata	
	<i>optB</i>	Oligopeptide ABC transporter permease protein	<i>optABCDF</i>	3.8	NA	8E-02
	<i>optC</i>	Oligopeptide ABC transporter permease protein	<i>optABCDF</i>	3.9	NA	1E-01
	<i>optD</i>	Oligopeptide ABC transporter ATP-binding protein	<i>optABCDF</i>	4.4	NA	4E-03
	<i>optF</i>	Oligopeptide ABC transporter ATP-binding protein	<i>optABCDF</i>	5.0	NA	1E-02
	<i>ydGC</i>	Amino acid permease	<i>ydGC</i>	2.8	aatcatctgataatt	2E-01
	<i>ydGB</i>	Amino acid permease	<i>ydGC</i>	2.1	NA	3E-01
	<i>yibG</i>	Amino acid permease	<i>yibG</i>	4.1	—	8E-02
	<i>yshA</i>	Amino acid permease	<i>yshA</i>	0.1	aattttcagaacaag	3E-12
	<i>cadA</i>	Cadmium efflux ATPase	<i>cadA</i>	0.5	aattttcagaaatgc	2E-04
	<i>feoA</i>	Ferrous ion transport protein A	<i>feoAB-ybiK</i>	2.1	atttttcagaaaatt	3E-01
	<i>feoB</i>	Ferrous ion transport protein B	<i>feoAB-ybiK</i>	2.7	NA	2E-01
	<i>ybiK</i>	Conserved potential membrane protein	<i>feoAB-ybiK</i>	2.9	NA	2E-01
	<i>lmrP</i>	Integral membrane protein LmrP	<i>lmrP-yweA</i>	5.9	—	3E-02

Table 2. cont.

Function	Gene	Assignment	Organization*	Ratio†	CodY-box‡	P-value
	<i>yweA</i>	Membrane protein	<i>lmrP-yweA</i>	8.7	NA	8E-03
	<i>ynaD</i>	ABC transporter ABC-binding and permease protein	<i>ynaDC</i>	2.1	aatttttagaatatt	3E-01
	<i>ynaC</i>	ABC transporter ABC-binding and permease protein	<i>ynaDC</i>	2.4	NA	2E-01
	<i>yudJ</i>	YitT family, probable bacterial ABC transporter permease	<i>yudJ</i>	2.5	—	2E-01
Intermediary carbon metabolism						
Glycogen and internal polysaccharides	<i>ygjD</i>	4- α -Glucanotransferase	<i>ygjD-malQ-glgCDAP-apu</i>	3.6	aattttctgaaaaat	1E-01
	<i>glgC</i>	Glucose-1-phosphate adenylyltransferase	<i>ygjD-malQ-glgCDAP-apu</i>	8.9	NA	3E-03
	<i>glgD</i>	Glucose-1-phosphate adenylyltransferase	<i>ygjD-malQ-glgCDAP-apu</i>	7.0	NA	2E-03
	<i>glgA</i>	Glycogen synthase	<i>ygjD-malQ-glgCDAP-apu</i>	8.3	NA	7E-03
	<i>glgP</i>	Glycogen phosphorylase	<i>ygjD-malQ-glgCDAP-apu</i>	7.6	NA	4E-03
	<i>apu</i>	Amylopullulanase	<i>ygjD-malQ-glgCDAP-apu</i>	7.8	NA	8E-03
	<i>amyL</i>	α -Amylase	<i>amyL</i>	4.0	aaatttcagaaatt	4E-02
Organic acid and alcohol	<i>ymhC</i>	Potential glycosyl transferase	<i>ymhC</i>	7.8	aattttctgaaatt	1E-02
	<i>glpK</i>	Glycerol kinase	<i>glpKDF2</i>	3.4	—	1E-01
	<i>glpD</i>	Glycerol-3-phosphate dehydrogenase	<i>glpKDF2</i>	4.7	NA	5E-02
	<i>glpF2</i>	Glycerol uptake facilitator protein	<i>glpKDF2</i>	4.7	NA	4E-02
	<i>ypjH</i>	Potential glycerol dehydrogenase	<i>ypjH</i>	35.9	aattttctaataatt	9E-06
	<i>als</i>	α -Acetolactate synthase	<i>als</i>	2.3	—	3E-01
	<i>mleS</i>	Malolactate enzyme	<i>mleSP</i>	2.4	—	2E-01
	<i>mleP</i>	Malate transporter	<i>mleSP</i>	2.3	NA	3E-01
	<i>xylH</i>	4-Oxalocrotonate tautomerase	<i>xylH</i>	2.2	—	3E-01
Regulatory functions						
	<i>gadR</i>	Positive regulator	<i>gadR</i>	0.3	—	1E-11
	<i>glnB</i>	Nitrogen regulatory protein P-II	<i>(amtB)-glnB</i>	9.9	aattgtcagaaaata	7E-03
	<i>hslA</i>	HU-like DNA-binding protein	<i>hslA</i>	2.5	—	3E-01
	<i>ymhA</i>	Probable regulatory protein	<i>ymhA</i>	5.7	—	3E-02
	<i>yohC</i>	Transcriptional regulator	<i>yohC</i>	10.4	—	3E-03
Other cellular processes						
Communication/bacteriocin	<i>yfgC</i>	LacA bacteriocin homologue	<i>yfgC</i>	4.5	—	8E-02
	<i>yaiE</i>	Transporter	<i>yaiEF</i>	17.8	aatttttttaaaattt	9E-04
	<i>yaiF</i>	CAAX protease family protein	<i>yaiEF</i>	12.1	NA	4E-03
	<i>yxdF</i>	CAAX protease family protein	<i>yxdFG</i>	54.3	—	2E-06
	<i>yxdG</i>	Transporter	<i>yxdFG</i>	48.4	NA	2E-06
	<i>yaiH</i>	CAAX protease family protein	<i>yaiH</i>	23.9	aattgtgcgaaaatt	1E-04
	<i>ahpC</i>	Alkyl hydroperoxide reductase	<i>ahpCF</i>	2.2	—	3E-02
Detoxification	<i>ahpF</i>	Alkyl hydroperoxide reductase	<i>ahpCF</i>	2.7	NA	1E-01
	<i>yneH</i>	Arsenate reductase ArsC family protein	<i>yneHG</i>	2.7	aattttctaaattt	2E-01

Table 2. cont.

Function	Gene	Assignment	Organization*	Ratio†	CodY-box‡	P-value
Other	<i>yneG</i>	ArsD repressor family	<i>yneHG</i>	2.4	NA	3E-01
	<i>dfpB</i>	Flavoprotein	<i>dfpB</i>	0.1	aatttttagtaata	3E-05
	<i>ftsW1</i>	Cell division protein	<i>ftsW1</i>	2.2	-	1E-01
	<i>udp</i>	Uridine phosphorylase	<i>udp</i>	6.3	aattgtctgaatat	2E-02
	<i>yeiD</i>	Prophage pi3 protein homologue	<i>yeiD</i>	4.3	aattgtctgaaat	9E-02
	<i>yfiD</i>	Prophage pi3 protein homologue	<i>yfiD</i>	4.9	aattatcatagaatt	5E-02
	<i>yneC</i>	Conserved unknown protein	<i>yneCB</i>	2.3	-	3E-01
	<i>yneB</i>	Esterase/lipase	<i>yneCB</i>	4.2	NA	9E-02
	<i>yohD</i>	Probable membrane protein	<i>yohD</i>	2.4	aattttactaaagt	3E-01
	<i>yrdB</i>	Probable membrane protein/ <i>yreF</i> CW anchored	<i>yrdB-(yreF)</i>	2.7	aaatttcgaaaaata	2E-01
	<i>yvfA</i>	Potential copper chaperone	<i>yvfA</i>	0.1	-	1E-05

*Gene clusters are deduced from sequence analysis. Underlined genes are those whose differential expression was validated independently. Genes in parentheses are absent on the DNA microarray.

†Ratio for signal measured in the IL1403 *codY* mutant versus the wild-type strain.

‡Potential CodY DNA-binding box found in the promoter region of the cluster or subcluster; NA, not applicable due to absence of promoter region; -, no CodY DNA-binding box identified in the promoter region of the cluster.

unpublished). A first utility allows automatic extraction of orthologous gene promoter regions from a set of genomes. In this work we used a set of 14 genomes from low-G + C % Gram-positive bacteria including *L. lactis* IL1403 (accession no. AE005176), *Streptococcus agalactiae* NEM316 (accession no. AL732656), *Streptococcus pneumoniae* R6 (AE007317), *Streptococcus mutans* UA159 (AE014133), *Streptococcus pyogenes* MGAS315 (AE014074), *Streptococcus thermophilus* CNRZ 1066 (CP000024), *Staphylococcus aureus* Mu50 (BA000017), *Staphylococcus epidermidis* ATCC 12228 (AE015929), *Bacillus cereus* ATCC 14579 (AE016877), *B. subtilis* 168 (AL009126), *Listeria monocytogenes* EGD-e (NC_003210), *Enterococcus faecalis* VE583 (AE016830), *Lactobacillus plantarum* WCFS1 (AL935263), *Lactobacillus johnsonii* NCC 533 (AE017198) and *Clostridium acetobutylicum* ATCC 824 (AE001437). A second utility allows the extraction of a promoter set from microarray data. It is then possible to achieve automatically a search of motifs by combining the two utilities, by first selecting a set of promoters from the microarray data (here from IL1403 versus its *codY* derivative) and then their orthologous genes in the selected set of genomes. The last step is to search motifs by an appropriate procedure; the MEME algorithm (Bailey & Elkan, 1994) was used in the present work. Finally, an extensive search for a motif in all promoter regions of the selected genomes (Dsouza *et al.*, 1997) was done.

RESULTS

Determination of CodY-regulated genes

In order to identify genes regulated by CodY, the expression of 2072 genes was quantified by the use of commercial *L. lactis* DNA microarrays. The experiment was carried out by comparing the expression profiles from *L. lactis* IL1403 and its derivative deleted for the *codY* gene grown in repressive conditions for CodY. For this purpose, total RNA was isolated from the two strains grown in CDM + Cas and harvested in the mid-exponential growth phase. Fig. 1 presents an overall view of the modifications of the expression pattern consequent to *codY* inactivation. Table 2 presents 110 out of the 130 genes that displayed (i) a significant signal, (ii) a differential transcript level higher than twofold and (iii) a *P*-value lower than 0.35. Twenty genes were rejected on the basis of experiments and analysis performed to validate the DNA-microarray data (see Methods).

The reliability of the DNA-microarray data was tested by (i) corroborating the data with previous knowledge on CodY-regulated genes, (ii) checking the consistency of expression levels within each cluster and (iii) performing additional measures by RT-QPCR. First, except for *araT*, *pepX* and *dtpT*, all genes already known to be regulated by CodY, such as *pepC*, *pepN*, *bcaT* and the genes of the *opp* and *opt* operon, were retrieved by our DNA-microarray analysis (Chambellon & Yvon, 2003; Guédon *et al.*, 2001a, b; Sanz *et al.*, 2001). Second, the change in the expression level of 75 clustered genes was consistent with their genetic organization. Only four genes out of these 23 clusters (*argE*, *oppB*, *oppC* and *malQ*) were rejected by our criteria, but manual inspection revealed that their scores were just below the threshold used. Last, the relative expression level of 17 genes was measured by RT-QPCR from new cultures in CDM + Cas of the wild-type and the *codY* mutant strains

(Fig. 1d). The ratio values obtained in these experiments were similar to that obtained previously by DNA microarray, with a Pearson correlation coefficient of 0.91.

Of the 110 genes, 21 were not tested by the above criteria. Among these, 13 had a highly significant differential expression (P -value < 0.05), suggesting that they are also likely to be regulated by CodY. We decided to include them in further analysis. From the clustering of the 110 genes, we deduced that CodY regulates 61 transcriptional units either directly or indirectly. As expected for a repressor, most of these genes (104, $\sim 95\%$) are negatively controlled. Among these, 56 are downregulated more than fourfold, indicating their tight control by CodY.

Analysis of the *L. lactis* CodY regulon

Analysis of the potential functions of the 110 genes reveals that 18 putative transcription units, which contain a total of 50 genes, encode proteins involved in amino acid biosynthesis pathways or are clustered with this class of genes. This set of repressed genes includes the pathway for biosynthesis of the sulfur amino acids, histidine, lysine, threonine, asparagine, aromatic amino acids, serine, glycine, glutamate and BCAA (Table 2). It also includes four genes organized in two clusters linked to the TCA cycle (*pycA*, *gltA*, *citB*, *icd*) that have, as a major role in *L. lactis*, glutamate and aspartate synthesis from central metabolism. Moreover, 19 genes encode proteins involved in peptide and amino acid transport (*ctrA*, *gltS*, *oppDFCBA*, *optSABCDF*, *ydgCB*, *yibG*, *yshA*) and peptide degradation (*pepC*, *pepN*). All these genes are repressed by CodY except two, *gltS* and *yshA*, encoding amino acid transporters. In addition to the above genes, involved in nitrogen supply, CodY also represses 17 genes from central and intermediary metabolism such as the glycerol assimilation pathway (*glgKDF2*, *ypjH*), organic acid fermentation (*mleSP*, *lctO*, *aldB*) and polysaccharide metabolism (*amyL*, *ygiD-malQ-glgCDAP-apu*, *xylH*). Last, the transcript level of six genes involved in regulatory processes is also modified by *codY* inactivation. Three are related to nitrogen metabolism (*gadR*, *aldR*, *glnB*), one encodes HslA, a histone-like DNA-binding protein, and two are still uncharacterized (*yohC* and *ymhA*).

Interestingly, CodY represses the expression of six genes (*yfgC*, *yaiEF*, *yxdFG* and *yaiH*) which might be involved in cell communication or in bacteriocin production. YfgC shares 31 % identity with the LacA lactacin RM, a lactococcal bacteriocin (Yarmus *et al.*, 2000). YaiF, YxdF and YaiH have all the features of the CAAX amino-terminal protease family proteins, while YaiE and YxdG are transport and binding proteins. The CAAX proteases are membrane-bound metalloproteases involved in protein and/or peptide modification (Pei & Grishin, 2001). In *Lactobacillus plantarum*, one such protein is associated with a cluster involved in bacteriocin production (Pei & Grishin, 2001).

Specific CodY binding to the promoter region of regulated genes

In order to show that CodY directly regulates transcription of the newly identified CodY-dependent genes, we carried out gel retardation experiments with a $\Delta codY$ *B. subtilis* crude protein extract from the JIM8169 cells producing high amounts of *L. lactis* CodY (Petranovic *et al.*, 2004). For this purpose, we used radiolabelled fragments of 250 bp, corresponding to several *L. lactis* promoter regions. Since it was shown that isoleucine enhanced CodY affinity for its targets in *L. lactis* and in *B. subtilis* (den Hengst *et al.*, 2005; Shivers & Sonenshein, 2004), we tested the effect of each single BCAA on the formation of the CodY-*ilvD* promoter region complex (Fig. 2). Whereas the migration of the *ilvD* probe was not modified in the presence of 10 μ g CodY-containing extract, addition of isoleucine induced the formation of the CodY-*ilvD* promoter fragment complex, as observed by the mobility shift of the probe. The effect of leucine or valine was lower or insignificant, respectively. These results confirmed that isoleucine is the main signal for CodY regulation and that crude cell extracts can be used to test CodY targets in *L. lactis*. Further binding experiments were carried out in the presence of 10 mM isoleucine.

Probes corresponding to *ilvD*, *ctrA*, *gltA*, *asnB*, *thrA*, *pepN* and *cysD* (Fig. 3a, b, c, d, e, f, respectively, and not shown for *cysD*) were retarded in the presence of 2.5 μ g CodY-containing extract and the shift reached a maximum with 20 μ g total proteins (lanes 2–5). As control, no band shift

Fig. 2. Effect of BCAA on CodY DNA binding to *ilvD* promoter region. A labelled DNA fragment containing the *ilvD* promoter region (10 fmol) was incubated with or without 10 μ g CodY-enriched crude extract from *B. subtilis* cells in the presence or not of 10 mM of one of the three BCAA (I, isoleucine; L, leucine; V, valine) and analysed on non-denaturing polyacrylamide gel electrophoresis.

Fig. 3. Gel mobility shift assay for CodY binding to different regulated gene promoter regions. The promoter regions of CodY-dependent genes were PCR amplified and labelled. Radiolabelled DNA probes (10 fmol) [*ilvD* (a), *ctrA* (b), *gltA* (c), *asnB* (d), *thrA* (e), *pepN* (f)] were incubated with *B. subtilis* crude extracts at various concentrations, and analysed by non-denaturing PAGE (see Methods for details). Lane 1 contains no protein. Lanes 2–5 contain 2.5, 5, 10 and 20 µg CodY-enriched crude extract, respectively. Lane 6 contains 20 µg control crude extract.

was detected with 20 µg crude extract of the $\Delta codY$ *B. subtilis* control strain JIM8168 (lane 6). Finally, no band shift could be detected if the CodY-independent *metA* promoter was used as probe in the presence of CodY-containing extract (data not shown). These data demonstrate the formation of a specific CodY complex with seven promoter regions and suggest the direct control of these regions by CodY.

Effect of isoleucine-dependent CodY repression on amino acid metabolism

The repression of CodY on such a large panel of genes involved in nitrogen supply as a function of the concentration of isoleucine, as single signal, is surprising. Indeed, in the presence of an excess of isoleucine, it might be expected that CodY would interfere with the biosynthesis of many other amino acids, and therefore have a negative effect on growth. Growth inhibition phenomena due to isoleucine (hereafter designated isoleucine inhibition) were therefore sought.

First, the omission of asparagine from CDM increased the lag phase of *L. lactis* NCDO 2118 from 3 to 15 h. This lag was reduced to 9 h if isoleucine was removed as well, showing that isoleucine interfered negatively with asparagine biosynthesis. Second, *L. lactis* NCDO 2118 was not able to grow in CDM containing isoleucine, leucine, valine, glutamate, serine and methionine as nitrogen source

(CDM6). Surprisingly, its growth could be restored by the addition of asparagine, histidine or lysine plus threonine, which are not required in CDM. Interestingly, if isoleucine was omitted from CDM6, the additional amino acids were no longer required, suggesting an isoleucine CodY-mediated growth repression.

Identification of a CodY regulatory box

We have shown that CodY regulator directly controls several promoter regions by band-shift experiments. Since most regulators recognize a motif to promote their binding to specific sites, we searched for sequence patterns conserved among CodY-regulated genes in order to detect a CodY regulatory box (CodY-box). For this purpose, the Extract-Motif program using the MEME algorithm (Bailey & Elkan, 1994; N. Pons and others, unpublished) was used to find a conserved motif in the region upstream of the start codon of the *optS*, *ilvD*, *gltA*, *asnB*, *ctrA*, *gltB*, *yaiH* and *amtB* genes. A conserved 15 nt sequence with AATTKTCAGAMWAWW as degenerate consensus was identified in the promoter region of these genes (Fig. 4). This motif is found twice in the *gltA* and *optS* promoter regions. Interestingly, the motif is located in the vicinity of the potential promoter sequences and often overlaps them (Fig. 4).

We next searched for the strict palindromic AATTTTCN-GAAAATT sequence (three mismatches accepted) in all

<i>optS1</i>	GTATTTTTTTATTTTCATTAAATTTATCAGAAAAATACAACAATATTCTTGACAATGATTTTTTGGAAAAAGTATAATAAGTCCACATTTAATTTTCAGAAATATGAA-N35-ATG	
<i>optS2</i>	AGTATAATAAGTCCACATTTAATTTTCAGAAATATGAAATTC-N30-ATG	
<i>ilvD</i>	GTATTTTATAGACTATATAGAATATTCTGACAAATATTGTATTTTCATTTTTTTAGTGATAAAATAGCTCTATGT-N30-ATG	
<i>gltA1</i>	TTTCTTCAAAAAATATTAAATTTTCAGAAAAATATAATTTTCGGAATAAAAGTGATAATAATGAAATAGATGGAGGCGTTT-ATG	
<i>gltA2</i>	TAAATTTTCAGAAATATATAATTTTCGGAATAAAAGTGATAATAATGAAATAGATGGAGGCGTTT-ATG	
<i>asnB</i>	CTATTTTTGTTTTATTATGAAATTTCCAGACAATTGTGATATAATTTGAAATTTATAATTATTATT-N10-ATG	
<i>ctrA</i>	GCATAAAGTTAGTTCTTGACAATTGTCAGACAATTCGGTAAATACAGTTTATTGAAATATTTA-N30-ATG	
<i>gltB</i>	TAAAAATTACATTTATATATAATTTGTCAGAAATATATTGATATTTTCATCGAAATTTAGTAGAATAAAAGA-N30-ATG	
<i>amtB</i>	TATATTTTATCATGATTAAATTTGTCAGAAAAATTTGATAAAATAATCTGTT-N30-ATG	
<i>yaiH</i>	AATTTAAGGGCTTCATTTGTAATTTGTCGAAAAATCTTGCAATTTATGTTTTTTTCGGAGTATACTGACATTATT-N174-ATG	
DEGENERATE	CONSENSUS	AATTKTCAGAMNAW
PALINDROMIC	CONSENSUS	AATTTTCNGAAAATT

Fig. 4. Sequence alignment of promoter regions of CodY-dependent genes. The ATG start codon of each gene is indicated in italics. The potential -10 and -35 boxes for each promoter are underlined. The identified CodY-boxes are shaded. The degenerate and the strict palindromic consensus are deduced from the alignment of the CodY-boxes. K (T or G), M (A or C), W (A or T), N (A, T, G or C).

potential promoter regions of the *L. lactis* IL1403 genome (Bolotin *et al.*, 2001). Interestingly, 40 out of the 64 promoter regions of CodY-regulated genes contain at least one CodY-box (Table 2). Moreover, these newly found motifs are located in most cases less than 20 nt from the predicted -35 or -10 boxes of the promoters, a location well in agreement with their potential regulatory role and therefore strengthening their significance.

To test the relevance of the CodY-box on CodY DNA binding, we used a gel retardation assay (Fig. 5). Replacement of the leading AA of the consensus and the A just preceding the consensus with CGG completely abolished the CodY binding to the *gltA* promoter, as did the replacement of the GAA with a TGG, while TC $\rightarrow$ GT and AT $\rightarrow$ GC substitutions modified significantly the migration of the complex. Moreover, while the addition of an excess of *gltA* unlabelled probe dramatically decreased CodY binding to the same labelled DNA fragment, no modification of the CodY binding was observed in the presence of an excess of *gltA* unlabelled probe carrying substitutions in the CodY-box (Fig. 5c). Finally, single substitutions in the *cysD* and *ilvD* CodY-boxes also affected the ability of CodY to form a complex with its targets (data not shown). These experiments indicate that the motif identified by the bioinformatic approach is a CodY DNA-binding site.

CodY-box in *B. subtilis* and other Gram-positive bacteria

To check for the existence of the CodY-box in bacteria other than *L. lactis*, we carried out a search for the occurrence of the AATTTTCNGAAAATT sequence (three mismatches accepted) in the intergenic regions of the *B. subtilis* genome. This search provided 248 hits in front of 228 genes (20 genes have two potential CodY-boxes). Importantly, among the 67 promoter regions determined as CodY targets by Chip-to-Chip experiments (Molle *et al.*, 2003), 25 displayed at least one CodY-box (see Supplementary Table 1, available with the online version of this paper).

A second search was carried out to find sets of orthologous genes that contain the CodY motif in the genomes of *L. lactis*, *Str. agalactiae*, *Str. pneumoniae*, *Str. mutans*, *Str. pyogenes*, *Staphylococcus aureus*, *Staph. epidermidis*, *B. cereus*, *B. subtilis*, *Listeria monocytogenes*, *E. faecalis* and *C. acetobutylicum* (see Supplementary Table 2, available with the online version of this paper). Strikingly, CodY motifs are present upstream of almost all BCAA biosynthesis genes, specific BCAA transporters and oligopeptide transporters, which are known CodY targets in *L. lactis* and *B. subtilis*. Furthermore, several other amino acid biosynthesis genes, previously shown to be CodY-dependent in *L. lactis*, are also preceded by a CodY-box in most genomes (aspartokinase, aspartate β -semialdehyde dehydrogenase, homoserine dehydrogenase, phosphoserine aminotransferase, threonine synthase and O-acetylhomoserine thiolase). Among transporter genes preceded by the CodY motif are *amtB*, encoding ammonium transporter, and several amino acid transporters of unknown specificity. Last, a CodY motif binding site is present in the close vicinity of the -35 box of the promoters controlling the transcription of CodY in the bacteria of the genera *Lactococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Clostridium*.

DISCUSSION

CodY is a transcriptional regulator found in many low G + C species of Gram-positive bacteria. Most studies of this regulator have been carried out in *B. subtilis* and *L. lactis*. In the former, CodY was shown to repress expression of a wide variety of genes involved in macromolecular degradation, nutrient transport, amino acid catabolism, BCAA biosynthesis, genetic competence, antibiotic synthesis and motility and chemotaxis (Molle *et al.*, 2003). In the latter, CodY was found to be the central regulator for proteolysis and peptide assimilation pathways (Chambellon & Yvon, 2003; Guedon *et al.*, 2001a, b; Sanz *et al.*, 2001). In this work, over 100 potential CodY targets were found by DNA microarrays, and a consensus for the CodY DNA-binding site was characterized.

Fig. 5. *In vitro* analysis of the role of the CodY-box in the DNA-binding ability of CodY. DNA probes (10×10^{-15} mol) were incubated without (–) or with (+) CodY-enriched crude extract (b, 20 μ g; c, 5 μ g) in the presence of 10 mM isoleucine and analysed by non-denaturing PAGE. (a) Sequence of the wild-type (*gltA_{wt}*) and mutated (*gltA_{M1}*, *M2*, *M3*, *M4*) *gltA* CodY DNA-binding box. (b) Gel mobility shift assay for CodY binding to *gltA_{wt}*, *gltA_{M1}*, *gltA_{M2}*, *gltA_{M3}* and *gltA_{M4}* promoter regions. (c) Gel mobility shift assay for CodY binding to *gltA_{wt}* promoter region in the presence of an excess (10×10^{-13} mol) of unlabelled *gltA_{wt}* or *gltA_{M3}* probes. ~, Contains, as negative control, 10 μ g control crude extract (without CodY_{*lactis*}).

Overall control of nitrogen metabolism by CodY

As already described, CodY controls the major genes involved in peptide assimilation, including their transport and their further degradation by peptidases (Guedon *et al.*, 2001b). Furthermore, CodY also controls the expression of five genes encoding transporters involved in the uptake of free amino acids. CodY thus exerts a control on most genes involved in external amino acid supply. Moreover, CodY also represses the expression of *de novo* amino acid biosynthesis genes. This control covers full sets of genes encoding amino acid biosynthesis pathways such as BCAA, glutamate-glutamine, histidine, serine, threonine, lysine and asparagine. It also applies to single steps, often located at the entry of the pathways, such as *aroH*, the first step of the common branch leading to aromatic amino acid biosynthesis, and *hom* and *cysD*, involved in methionine synthesis. Finally, the

CodY control is indirect on cysteine and glycine biosynthesis pathways, which use serine as precursor, and on proline, arginine and alanine, which require glutamate for their biosynthesis. Among these genes, we have shown that CodY binds to the promoter regions of *ilvD*, *gltA*, *thrA*, *pepN*, *ctrA*, *asnB* and *cysD*, confirming the direct control of CodY on their transcription. Finally, CodY also represses glycogen metabolism and glycerol assimilation pathways that may fuel the cell with a carbon skeleton for amino acid biosynthesis upon sugar starvation. These results indicate that CodY controls all pathways for nitrogen supply in *L. lactis* by modulating *de novo* amino acid biosynthesis and the import of secondary nutritional sources.

Strikingly, BCAA biosynthesis genes are among the most tightly regulated by CodY in *L. lactis* and are also controlled by CodY in *B. subtilis* (Molle *et al.*, 2003). BCAA are the

intracellular effectors activating CodY repression in *L. lactis* (Guedon *et al.*, 2001b; Petranovic *et al.*, 2004) and in *B. subtilis* (Shivers & Sonenshein, 2004). Thus, both in *L. lactis* and in *B. subtilis*, CodY exerts a feedback control on the pathway involved in the biosynthesis of its direct effectors. This control is direct since CodY binds to the promoter region of *ilvB*, *ilvD* and *ybgE* in *B. subtilis* (Shivers & Sonenshein, 2004) and *ilvD* in *L. lactis* (this work). Furthermore, isoleucine strongly increases the affinity of CodY for these promoters, confirming that BCAA exerts a similar effect on CodY in *L. lactis* and *B. subtilis* (Shivers & Sonenshein, 2004; this work).

The role of CodY in nitrogen supply is significantly extended in *L. lactis* compared to *B. subtilis* since in the latter, CodY controls the biosynthesis of BCAA, asparagine and glutamate precursors and amino acid and peptide transporters (Molle *et al.*, 2003). Previously, the role of CodY from *B. subtilis* has been compared to that of Lrp from *E. coli*, the leucine-responsive regulatory protein (Molle *et al.*, 2003). In this bacterium, Lrp alters the expression of about one-tenth of the genes, including several genes involved in the synthesis of amino acids and their transport and the *opp* operon (Hung *et al.*, 2002; Tani *et al.*, 2002). However, this control is less strong, it might be positive (e.g. for *leuD*, *serA* and *proB*) and does not encompass all pathways. To our knowledge, *L. lactis* CodY represents the first example of such a global and tight control on nitrogen metabolism by a single transcriptional regulator in bacteria.

Isoleucine signal and nitrogen metabolism

In vitro experiments showed that isoleucine is the most important effector among BCAA for CodY repression (this work; den Hengst *et al.*, 2005; Shivers & Sonenshein, 2004). Since the CodY signal in *L. lactis* is essentially mediated by isoleucine, excess of this amino acid in the environment leads to growth inhibition by blocking inappropriately CodY-dependent biosynthesis pathways. Several studies in chemically defined media have reported a CodY-dependent isoleucine growth inhibition in *L. lactis* linked to repression of the biosynthesis of valine and leucine (Goupil-Feuillerat *et al.*, 1997), asparagine, histidine, lysine and threonine (this work) and glutamate (Lapujade *et al.*, 1998). In the latter case, the growth defect was shown to be the result of a reduced synthesis of 2-oxoglutarate associated with a low level of the glutamate synthase (Lapujade *et al.*, 1998). These results can now be explained by the CodY-mediated tight control of the *gltA-citB-icd* and *gltBD* operons in the presence of isoleucine. Last, CodY-dependent isoleucine inhibition has also been observed in complex media such as milk (Chambellon & Yvon, 2003), showing that a strong imbalance of isoleucine leads to growth inhibition.

CodY targets in Gram-positive bacteria

Although extensively studied in *B. subtilis*, no consensus sequence has yet been found for the CodY-binding site and it has been suggested that CodY might not recognize a primary

sequence but tertiary DNA structures (Serror & Sonenshein, 1996a). In this work, we have characterized a 15 nt palindromic motif with AATTTTCNGAAAATT as consensus sequence. This motif is present in the promoter region of 63 % of the CodY-dependent transcriptional units identified by transcriptome analysis in *L. lactis* and of 40 % of the known CodY *B. subtilis* targets. Furthermore, a CodY-box is also located in its own promoter region and in those of BCAA biosynthesis, amino acid and oligopeptide transporter genes of Gram-positive bacteria of the genera *Staphylococcus*, *Bacillus*, *Listeria* and *Streptococcus*, suggesting the general significance of this motif in bacteria containing the CodY regulator.

Moreover, the CodY-box is found in the CodY-protected region from the *oppD* promoter region of *L. lactis* (two CodY-boxes) and from the *ilvB*, *citB*, *dpp*, *fla-che*, *comK* and *sfrA* promoter regions of *B. subtilis* (Bergara *et al.*, 2003; den Hengst *et al.*, 2005; Kim *et al.*, 2003; Serror & Sonenshein, 1996a, b; Shivers & Sonenshein, 2004). Moreover, mutations and deletions carried out on the promoter regions of *dppA*, *hut* and *ureA* of *B. subtilis* and *oppD* of *L. lactis* and that affect CodY regulation are within or immediately adjacent to the CodY-box (Eda *et al.*, 2000; Serror & Sonenshein, 1996a; Wray *et al.*, 1997; Wray & Fisher, 1994). To improve the relevance of our *in silico* analysis, biochemical experiments coupled with mutation analysis have been carried out on several CodY-dependent gene promoters of *L. lactis*. In each case, mutations affecting the characterized motif decreased or impaired the ability of CodY to form a complex with the promoter regions of its target genes, demonstrating the CodY operator role of the CodY-box.

Although many CodY-regulated gene promoters contain the CodY-box, 60 % and 30 % of the CodY targets from *B. subtilis* and *L. lactis*, respectively, have no CodY motif with less than three mismatches. This suggests that (i) additional degeneracy rules exist to define this motif, (ii) CodY may also recognize DNA structures as suggested by Serror & Sonenshein (1996a), or (iii) CodY indirectly controls their expression level. Notably, we have shown in this paper that the *pepN* promoter was a direct target of CodY, although it does not display the presently defined CodY-box, suggesting that additional work is required to fully understand the interaction of CodY with its targets.

Conclusion

We have shown that CodY controls amino acid supply in *L. lactis*, including protein degradation pathways, uptake of peptides and amino acids and *de novo* biosynthesis, providing here the first example of an overall control of nitrogen metabolism by a single regulator. Characterization of a CodY-box in *L. lactis* and its distribution in other Gram-positive bacteria suggest that CodY is generally involved in nitrogen control in Firmicutes. As CodY effector, isoleucine would have a predominant role in sensing nitrogen availability in these micro-organisms.

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