

# Growth phase-associated changes in the proteome and transcriptome of *Lactobacillus rhamnosus* GG in industrial-type whey medium

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## Summary

The growth phase during which probiotic bacteria are harvested and consumed can strongly influence their performance as health-promoting agents. In this study, global transcriptomic and proteomic changes were studied in the widely used probiotic *Lactobacillus rhamnosus* GG during growth in industrial-type whey medium under strictly defined bioreactor conditions. The expression of 636 genes ( $P \leq 0.01$ ) and 116 proteins ( $P < 0.05$ ) changed significantly over time. Of the significantly differentially produced proteins, 61 were associated with alterations at the transcript level. The most remarkable growth phase-dependent changes occurred during the transition from the exponential to the stationary growth phase and were associated with the shift from glucose fermentation to galactose utilization and the transition from homolactic to mixed acid fermentation. Furthermore, several genes encoding proteins proposed to promote the survival and persistence of *L. rhamnosus* GG in the host and proteins that directly contribute to human health showed temporal changes in expression. Our results suggest that *L. rhamnosus* GG has a highly flexible and adaptable metabolism and that the growth stage during which bacterial cells

are harvested and consumed should be taken into consideration to gain the maximal benefit from probiotic bacteria.

## Introduction

*Lactobacillus rhamnosus* GG (ATCC 53103) is one of the most intensively studied probiotic strains worldwide. It has most of the characteristics generally desired for a good probiotic bacterium, including the ability to survive passage through and to colonize the human gastrointestinal tract (GIT) (Goldin *et al.*, 1992; Alander *et al.*, 1997; 1999). Adhesion capacity is a prerequisite for colonization, and *L. rhamnosus* GG has been shown to adhere efficiently to both intestinal mucus and epithelial cells (Jacobsen *et al.*, 1999; Tuomola *et al.*, 2000; Ouwehand *et al.*, 2001). Consumption of *L. rhamnosus* GG promotes human health by reducing the risk of nosocomial rotavirus-related diarrhoea in infants (Szajewska *et al.*, 2001) and shortening the duration of acute diarrhoea (Szajewska *et al.*, 2007, recently reviewed by Guarino *et al.*, 2009). In addition to its influence on the GIT, *L. rhamnosus* GG have other beneficial effects, including reducing upper respiratory tract infections among children in day care (Hatakka *et al.*, 2001; Hojsak *et al.*, 2010), decreasing the risk of developing atopic eczema (Kalliomäki *et al.*, 2001; 2003; 2007), alleviating the symptoms of eczema (Majamaa and Isolauri, 1997; Isolauri *et al.*, 2000) and decreasing the risk of dental caries in children (Näse *et al.*, 2001). So far, the molecular mechanisms identified as being responsible for the health-promoting effects of lactobacilli mainly relate to immunomodulation, intestinal epithelial barrier protection and microbial balance maintenance (reviewed by Lebeer *et al.*, 2008; 2010). The recently published genome sequence of *L. rhamnosus* GG (Kankainen *et al.*, 2009) will boost mechanistic and functional genomic research of this well-known probiotic strain.

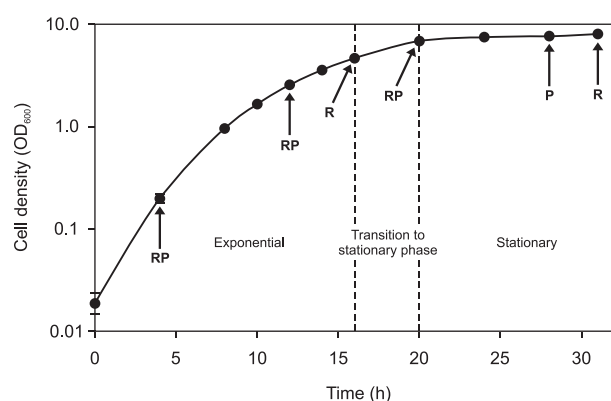
In industrial production, dairy strains, including probiotics, are commonly harvested during the late exponential or stationary growth phase to ensure high cell numbers. However, the desired probiotic-associated factors are not necessarily expressed at the highest levels during the stationary phase of growth. For example, cell surface

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properties of *L. rhamnosus* GG have been shown to change during growth, influencing the strain's ability to adhere to epithelial cells (Deepika *et al.*, 2009). Furthermore, consumption of another widely recognized probiotic bacterium, *Lactobacillus plantarum*, at different growth phases (mid-exponential or stationary) induced different transcriptional responses in human duodenal mucosa (van Baarlen *et al.*, 2009). Hence, when aiming to optimize the health-promoting properties of probiotic bacteria in large-scale industrial cultivations, it is essential to determine the most favorable growth conditions and phase for cell harvesting.

Functional genomics and proteomics analyses are useful tools for identifying the molecular mechanisms behind the health-promoting features of probiotic lactic acid bacteria. Transcriptomics provides detailed information about global changes in gene expression under particular conditions. Furthermore, microarrays enable reliable measurement of the expression of all genes on the array, regardless of the subcellular location of their corresponding proteins. However, only changes in mRNA level can be measured with this tool, while most biological phenomena result from protein function. In addition, changes occurring at the post-transcriptional level (due to proteolysis, charged modification, etc.) can be investigated only with proteomic tools. Therefore, it is advisable to use both transcriptomic and proteomic approaches when linking genomic sequences to potential biological functions. Transcript or protein level approaches have been used to investigate global changes in other probiotic lactic acid bacteria during growth transitions (Cohen *et al.*, 2006; Koistinen *et al.*, 2007; Azcarate-Peril *et al.*, 2009). For example, transcriptomic analysis of *Lactobacillus acidophilus* cultured in skim milk indicated temporal expression changes in genes involved in the proteolytic system, carbohydrate utilization, adhesion and cellular responses to stress (Azcarate-Peril *et al.*, 2009). In *L. plantarum*, protein expression profiles demonstrated differences in several anabolic and stress response pathways during different growth phases (Cohen *et al.*, 2006). A comparison of the proteomes of two *L. plantarum* strains during growth demonstrated the growth phase-dependent expression of proteins associated with energy metabolism, such as glycolysis, the phosphoketolase pathway and ribose metabolism (Koistinen *et al.*, 2007).

The global omics-level analyses conducted on the probiotic *L. rhamnosus* GG so far include two proteomic studies (Koskenniemi *et al.*, 2009; Sánchez *et al.*, 2009) and a study combining both proteomic and transcriptomic approaches (Koskenniemi *et al.*, 2011). Sánchez and colleagues (2009) tested different extraction methods for surface-associated proteins, and could identify 18 different possibly surface-associated proteins of *L. rhamnosus* GG. Comparison of the proteomes of



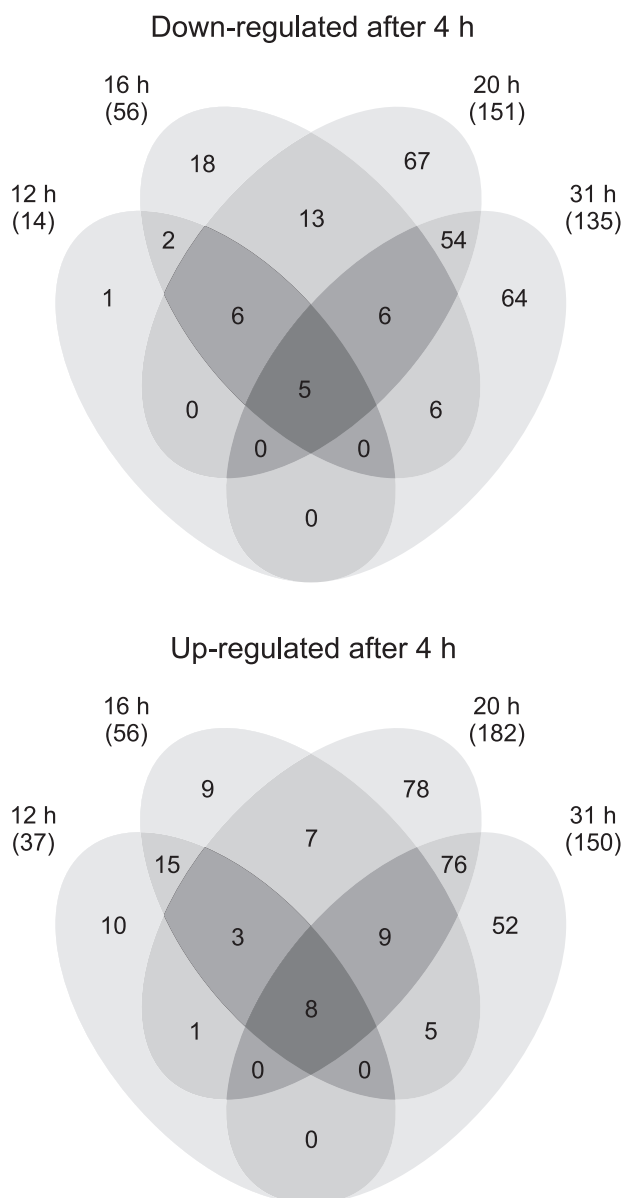
**Fig. 1.** Growth curve of *L. rhamnosus* GG in whey medium at 37°C at pH 5.8. Cell density values represent the mean of four individual pH-controlled bioreactor cultivations, and error bars represent the standard deviation. RNA samples were taken at five different time points representing mid-exponential, late exponential, stationary transition point, early stationary and late stationary phases, while protein samples were collected at four different time points (the same as the RNA sampling phases, excluding the stationary transition point). The time points at which the RNA (R) and protein (P) samples were taken are indicated by arrows.

*L. rhamnosus* GG grown in an industrial-type whey medium (the same as used in this study) and in a laboratory medium demonstrated fundamental effects of culture conditions on the protein production of *L. rhamnosus* GG (Koskenniemi *et al.*, 2009). In a recent study, transcript and protein level responses of *L. rhamnosus* GG towards bile were shown to include diverse and specific changes in general stress responses as well as alterations in cell envelope-related functions (Koskenniemi *et al.*, 2011). The present study involves complementary approaches, i.e. DNA microarray analyses combined with two-dimensional difference gel electrophoresis (2-D DIGE), to assess global changes in the transcriptome and proteome of probiotic *L. rhamnosus* GG during growth in industrial-type whey medium under strictly controlled bioreactor conditions. In particular, this study aimed to determine whether potential probiotic-associated factors were affected in a growth phase-dependent fashion during fermentation in whey.

## Results and discussion

### *Expression profile of L. rhamnosus* GG during growth in industrial-type whey medium

In the present study, 2-D DIGE and whole-genome DNA microarrays were used to determine how global gene expression in the widely used probiotic, *L. rhamnosus* GG, changed over time during growth in industrial-type whey medium under strictly defined bioreactor conditions (Fig. 1). Overall, the transcript levels of 636 genes were significantly changed ( $\geq 2$ -fold difference,  $P \leq 0.01$ ) at

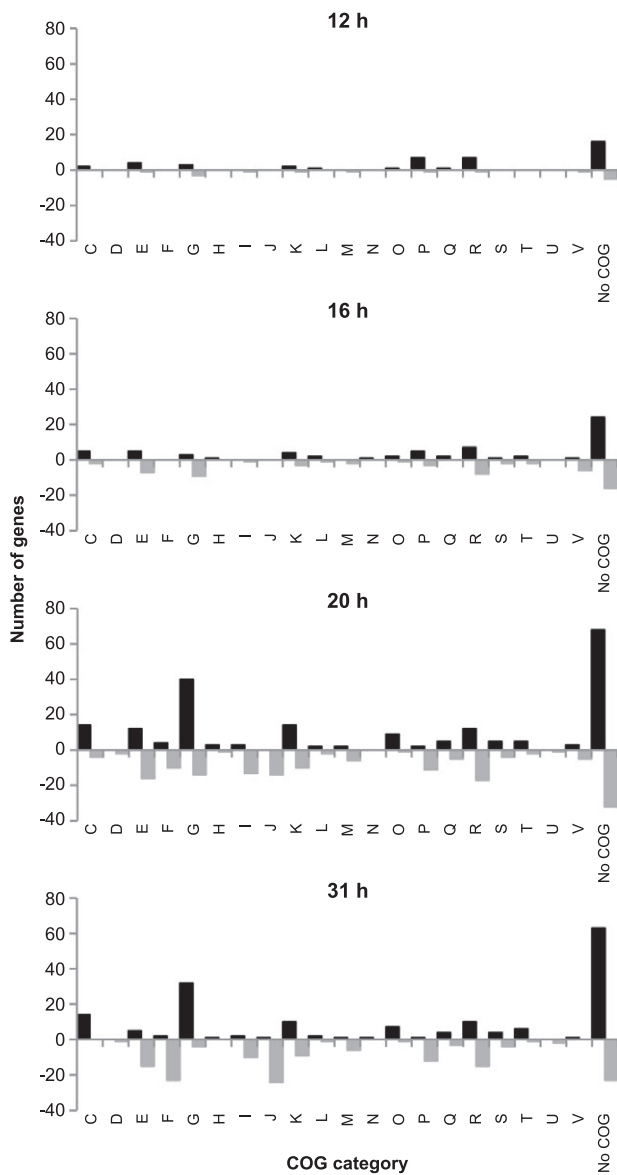


**Fig. 2.** Venn diagrams showing the numbers of significantly differentially transcribed genes ( $\geq 2$ -fold difference,  $P \leq 0.01$ ) during growth in whey. Time points at 12 h, 16 h, 20 h and 31 h were compared with the time point at 4 h (4 h, mid-exponential; 12 h, late exponential; 16 h, stationary transition point; 20 h, early stationary; and 31 h, late stationary phase).

one or more time points, representing 22% of the complete genome of *L. rhamnosus* GG (Fig. 2). This is comparable to results from a previous study, where 21% of the ORFs of the *L. acidophilus* genome were differentially expressed during shift from exponential to stationary growth phase in skim milk (Azcarate-Peril *et al.*, 2009). Expression profiles of the 636 significantly differentially expressed genes were clustered into 16 groups (Fig. S1, Table S1). A clear difference could be seen between the transcription profiles of the mid-exponential (4 h) and sta-

tionary growth phases (20 and 31 h), although only a few genes were down- (five genes) or upregulated (eight genes) during the whole progression of growth (Fig. 2). Interestingly, clusters of orthologous groups (COGs) classification of significantly differentially expressed genes showed that the majority of genes regulated at time points 12, 16, 20 and 31 h, compared with the 4 h time point, could not be included in any COG category (Fig. 3). At the 20 h time point, Carbohydrate transport and metabolism (G), Energy production and conversion (C) and Transcription (K) seemed to be coordinately upregulated when compared with 4 h, while most of the downregulated genes were involved in Amino acid transport and metabolism (E) or were categorized as 'General function prediction only' (R). At 31 h, most of the upregulated genes were assigned to the G and C categories, and the majority of downregulated genes belonged to the Translation, ribosomal structure and biogenesis (J) and Nucleotide transport and metabolism (F) categories.

Changes in protein production in *L. rhamnosus* GG during growth were studied at 4, 12, 20 and 28 h. Using the 2-D DIGE technique, proteome composition at each time point was compared with the 20 h time point in three separate DIGE experiments (Table S2). The 20 h time point was selected as the baseline because it was assumed to involve the broadest selection of different proteins. In total, 267 protein spots showing a statistically significant difference ( $\geq 1.5$ -fold difference,  $P < 0.05$ ) in abundance were detected and cut out from the gels, and 201 of these could be identified using MALDI-TOF mass spectrometry and/or LC-MS/MS (Fig. S2, Tables 1–3 and Tables S3–S5). In seven cases, two proteins were identified from one spot. Conversely, 30 proteins were differentially abundant and identified in more than one experiment, and 26 proteins were found to occur as different isoforms, i.e. they were identified in two or more separate spots in the same experiment. Overall, the identified protein spots represented 116 distinct gene products. The numbers of differentially abundant protein spots between the time points 4 versus 20 h, 12 versus 20 h and 20 versus 28 h were 132, 46 and 23 respectively. The most remarkable growth phase-dependent changes in protein abundance occurred in the set of carbohydrate metabolism proteins (COG category G), consistent with the RNA results. The abundance of these proteins was continuously changing during the fermentation process. For most other proteins, the differences were most obvious when comparing the mid-exponential and early stationary phases (4 h and 20 h). Altered abundances were detected among others in proteins from COG categories F (Nucleotide transport and metabolism), E (Amino acid transport and metabolism) and J (Translation, ribosomal structure and biogenesis).



**Fig. 3.** COGs classification of significantly differentially expressed genes at 12, 16, 20 and 31 h compared with 4 h. Black bars indicate upregulated genes, while grey bars represent downregulated genes. COG functional categories are as follows: C, Energy production and conversion; D, Cell cycle control, cell division, chromosome partitioning; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate transport and metabolism; H, Coenzyme transport and metabolism; I, Lipid transport and metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L, Replication, recombination and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification, protein turnover, chaperones; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function prediction only; S, Function unknown; T, Signal transduction mechanisms; U, Intracellular trafficking, secretion and vesicular transport; V, Defence mechanisms.

In conclusion, the microarray analyses coupled with proteomics revealed that the transcription of 636 genes and the production of 116 proteins were altered in *L. rhamnosus* GG during the progression of growth from the mid-exponential to the late stationary growth phases. Of these changes, a total of 61 could be confirmed by gene expression analysis at both the transcript and protein level.

#### *Genes and proteins involved in central metabolic pathways are modulated in a growth-dependent manner*

**Carbohydrate transport and metabolism.** The whey medium used in this study contains glucose and galactose derived from hydrolysed lactose. In *L. rhamnosus* GG, there are two different pathways for the catabolism of the galactose moiety of lactose. The Leloir pathway (proteins encoded by the *galKETRM* operon and *pgm*) converts galactose to glucose-6-phosphate, and the tagatose-6-phosphate pathway (proteins encoded by the *lacCDBAR* operon) metabolizes galactose to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Kankainen *et al.*, 2009). Genes encoding enzymes from both the Leloir pathway and the tagatose-6-phosphate pathway showed a significant increase in expression at the mRNA level when the culture shifted from the exponential to the stationary phase of growth (Fig. 4A, Table S1). The same expression pattern was also evident at the protein level, as galactose utilization enzymes were more abundant in the early stationary growth phase (20 h) than in the exponential growth phase (4 or 12 h) (Tables 1 and 2). Furthermore, chemical analyses of the cultures showed that glucose was no longer detectable (detection limit 0.05%) in the early stationary phase of growth while galactose was still found in high amounts (1.0%). Therefore, increased expression of genes involved in galactose utilization at the stationary-phase transition point was expected because *L. rhamnosus* GG first metabolizes the readily fermented glucose moiety; only after that does it metabolize the less easily exploited galactose portion of the hydrolysed lactose in the whey medium. Applying a proteomic approach, Cohen and colleagues (2006) also detected upregulation of the Leloir pathway in *L. plantarum* WCFS1 during stationary growth in the laboratory MRS medium, which possibly contained trace amounts of galactose. Our transcript results also indicated that genes encoding components of the phosphotransferase system (PTS), currently annotated as galactitol-specific (LGG\_00343, LGG\_00345–00346), were significantly upregulated upon entry into the stationary growth phase and clustered together with *lacCDBAR* genes (cluster 2 in Fig. S1). Increased expression of the IIA component (LGG\_00345) of that PTS system was also detected at the protein level (Tables 1 and 2). These results suggest that



**Table 1.** Proteins with differing abundance between the mid-exponential (4 h) and early stationary (20 h) growth phases and transcription differences in the corresponding genes.

Locus ID	Gene name	Function	Proteomic (fold change)		Transcriptomic (Log <sub>2</sub> R) 20 h/4 h
			Spot No.	20 h/4 h	
<b>Amino acid metabolism</b>					
LGG_00568	<i>cysK</i>	Cysteine synthase	1	1.9	0.518
LGG_01158	<i>pepD</i>	Dipeptidase A	2	-2	-0.507
LGG_01201	<i>sufS</i>	Cysteine desulfurase	3	1.6	-0.281
LGG_01478	<i>pepO</i>	Endopeptidase O	4	1.6	1.788
LGG_02639	-	Aminotransferase	5	1.7	3.741
LGG_02708	<i>selA</i>	Pyridoxal phosphate-dependent enzyme	6 <sup>a</sup>	2.5	3.039
<b>Carbohydrate metabolism</b>					
<b>Galactose metabolism</b>					
LGG_00341	<i>lacC</i>	Tagatose-6-phosphate kinase	7	3.4	7.214
LGG_00653	<i>galK</i>	Galactokinase	8	2.5	2.755
LGG_00664	<i>lacC</i>	Tagatose-6-phosphate kinase	9	7.9	6.897
LGG_00665	<i>lacD</i>	Tagatose 1,6-diphosphate aldolase	10	4.4	6.910
LGG_00666	<i>lacB</i>	Galactose-6-phosphate isomerase subunit B	11,12	23.9	6.923
LGG_00667	<i>lacA</i>	Galactose-6-phosphate isomerase subunit A	13,14	31.9	6.988
LGG_00668	<i>lacR</i>	Lactose phosphotransferase system repressor	15	15	7.055
LGG_01062	<i>galU</i>	UTP-glucose-1-phosphate uridylyltransferase	16,17	2.1	0.872
LGG_02575	<i>lacD</i>	Tagatose 1,6-diphosphate aldolase	18	4	-0.07
<b>Glycolysis/gluconeogenesis</b>					
LGG_00524	<i>fbA</i>	Fructose-bisphosphate aldolase	19	-1.5	-1.97
LGG_00921	<i>pgm</i>	Phosphoglucosmutase	20	1.5	1.686
LGG_00933	<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	21-25	2.9	0.644
LGG_00933	<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	26,27	-1.8	0.644
LGG_00934	<i>pgk</i>	Phosphoglycerate kinase	28,29,30 <sup>a</sup>	3.1	0.286
LGG_00935	<i>tpiA</i>	Triosephosphate isomerase	31	2.2	0.147
LGG_00935	<i>tpiA</i>	Triosephosphate isomerase	32	-1.9	0.147
LGG_00936	<i>eno</i>	Enolase	33	2	0.672
LGG_01375	<i>pyk</i>	Pyruvate kinase	34-36	4.2	-2.004
<b>Others</b>					
LGG_00052	<i>eda</i>	2-Dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase	37	-1.7	0.64
LGG_00321	<i>deoC</i>	Deoxyribose-phosphate aldolase	38	1.9	0.859
LGG_00342	<i>srlD</i>	Sorbitol-6-phosphate 2-dehydrogenase	39,40	53.6	7.877
LGG_00757	<i>adhE</i>	Aldehyde-alcohol dehydrogenase	41-44	18	5.86
LGG_01322	<i>pdhC</i>	Pyruvate dehydrogenase complex E2 component, dihydrolipoylysine-residue acetyltransferase	45	-1.6	0.796
LGG_01360	<i>fruB</i>	1-Phosphofructokinase	46	-3	-1.659
LGG_01421	<i>pfkB</i>	Formate acetyltransferase	47-49	4.7	4.288
LGG_01914	<i>citF</i>	Citrate lyase, alpha subunit, Citrate CoA-transferase	50	2.8	-2.673
LGG_02025	<i>glgD</i>	Glucose-1-phosphate adenylyltransferase regulatory subunit	51	1.9	3.588

Table 1. cont.

Locus ID	Gene name	Function	Proteomic (fold change)		Transcriptomic (Log <sub>2</sub> R) 20 h/4 h
			Spot No.	20 h/4 h	
Lipid metabolism					
LGG_00509	<i>dhaK</i>	Dihydroxyacetone kinase	52,53	3.4	1.109
LGG_02113	<i>fabZ</i>	(3R)-hydroxymyristoyl-acyl-carrier-protein] dehydratase	54	-5.7	-6.321
LGG_02115	<i>fabF</i>	3-Oxoacyl-acyl-carrier-protein] synthase II	55,56	-2.8	-5.969
LGG_02118	<i>fabK</i>	Enoyl-(Acyl-carrier-protein) reductase II	57	-1.9	-5.285
Membrane transport					
LGG_00345	<i>gatA</i>	PTS system, galactitol-specific IIA component	58	5.5	7.069
LGG_00951	<i>malE</i>	ABC transporter, sugar transporter, periplasmic component	59 <sup>a</sup>	1.9	-0.74
LGG_01940	<i>oppD</i>	ABC transporter, oligopeptide transporter ATPase component	60	3.2	2.51
LGG_01941	<i>oppD</i>	ABC transporter, oligopeptide transporter ATPase component	61	2.3	2.733
LGG_02838	<i>manA</i>	PTS system, mannose-specific IIB component	62,63	1.8	1.199
Metabolism of cofactors and vitamins					
LGG_02764	<i>entB</i>	Isochorismatase family protein	64	4.7	3.538
LGG_02765	<i>pncB</i>	Nicotinate phosphoribosyltransferase	65	4.7	3.707
LGG_01258	<i>nifS</i>	Aminotransferase class V	66	-3.2	-0.546
Nucleotide metabolism					
Purine metabolism					
LGG_00249	<i>guaB</i>	Inosine-5'-monophosphate dehydrogenase	67	-2.1	-1.349
LGG_01803	<i>purD</i>	Phosphoribosylamine-glycine ligase	30 <sup>a</sup>	1.7	-1.771
LGG_01805	<i>purH</i>	Bifunctional purine biosynthesis protein purH	68,69	2.3	-1.698
LGG_01807	<i>purM</i>	Phosphoribosylformylglycinamide cyclo-ligase	70 <sup>a</sup>	2	-1.574
LGG_01808	<i>purF</i>	Amidophosphoribosyltransferase	71,72	2.8	-1.625
LGG_01809	<i>purL</i>	Phosphoribosylformylglycinamide synthase II	73	3.8	-1.653
LGG_01812	<i>purC</i>	Phosphoribosylaminimidazole-succinocarboxamide synthase	74	2.5	-1.466
LGG_01813	<i>purK</i>	Phosphoribosylaminimidazole carboxylase, ATPase subunit	6 <sup>a</sup> ,75,76	2.8	-1.539
LGG_01968	<i>guaA</i>	GMP synthase	77	1.8	-1.916
LGG_02466	<i>adk</i>	Adenylate kinase	78	-1.7	-1.865
Pyrimidine metabolism					
LGG_01175	<i>upp</i>	Uracil phosphoribosyltransferase	79	-1.6	-1.009
LGG_01456	<i>carB</i>	Carbamoyl-phosphate synthase, large subunit	80,81	-2.3	-3.819
LGG_01458	<i>pyrC</i>	Dihydroorotase	82	-1.9	-3.269
LGG_01459	<i>pyrB</i>	Aspartate carbamoyltransferase	83	-1.6	-3.143
LGG_01461	<i>pyrF</i>	Pyrimidine operon regulatory protein, bifunctional protein pyrF	84	-2.2	-2.752
LGG_02546	<i>pyrG</i>	CTP synthase	85	-2	-0.964
Others					
LGG_01474	<i>nrpE</i>	Ribonucleoside-diphosphate reductase, alpha subunit	86,87	-2.3	-1.339
Peptidoglycan biosynthesis					
LGG_00254	<i>dacA</i>	D-alanyl-D-alanine carboxypeptidase	59 <sup>a</sup>	1.9	0.149
LGG_00662	-	Beta-lactamase class C-related penicillin-binding protein	88	7.3	6.844
LGG_01282	<i>murD</i>	UDP-N-acetylmuramoylalanine-D-glutamate ligase	89	-1.7	-0.545
LGG_01768	<i>murC</i>	UDP-N-acetylmuramate-L-alanine ligase	90	-1.9	-1.239

Table 1. cont.

Locus ID	Gene name	Function	Proteomic (fold change)		Transcriptomic (Log <sub>2</sub> R) 20 h/4 h
			Spot No.	20 h/4 h	
<b>Protein synthesis</b>					
LGG_01628	<i>rpsB</i>	SSU/30S ribosomal protein S2P	70 <sup>a</sup>	2	-1.408
LGG_01628	<i>rpsB</i>	SSU/30S ribosomal protein S2P	91,92	-2.1	-1.408
LGG_01690	<i>rplU</i>	LSU/50S ribosomal protein L21P	93	-2.5	-0.991
LGG_02493	<i>fusA</i>	Protein translation elongation factor G (EF-G)	94	-1.7	-1.288
<b>Stress</b>					
LGG_01367	<i>clpB</i>	ATP-dependent chaperone ClpB	95	1.7	0.783
LGG_01604	<i>dnaK</i>	Chaperone protein dnaK	96	-1.5	-0.027
LGG_02151	<i>usp</i>	Universal stress protein, UspA family	97	5.6	-0.364
LGG_02239	<i>groEL</i>	60 kDa chaperonin GROEL	98,99	2.3	0.058
LGG_02499	<i>clpC</i>	ATP-dependent Clp protease ATP-binding subunit	100	4.5	0.25
LGG_02806	<i>htrA</i>	Serine protease	101	-1.6	-0.454
<b>Transcription</b>					
LGG_02461	<i>rpoA</i>	DNA-directed RNA polymerase, subunit alpha	102	-1.8	-1.536
LGG_02498	<i>rpoB</i>	DNA-directed RNA polymerase, beta chain	103,104	-2.5	-0.533
<b>Translation</b>					
LGG_00848	<i>leuS</i>	Leucyl-tRNA synthetase	105	-2.4	-0.541
LGG_01261	<i>valS</i>	Valyl-tRNA synthetase	106	-2.1	-0.427
LGG_01786	<i>argS</i>	Arginyl-tRNA synthetase	107	-1.8	-0.827
LGG_02332	<i>glxX</i>	Glutamyl-tRNA synthetase	108	-1.6	-1.553
LGG_02584	<i>metG</i>	Methionyl-tRNA synthetase/protein secretion chaperonin, CsaA	109,110	-2	-0.586
<b>Miscellaneous</b>					
LGG_00226	-	Pyridoxine 5'-phosphate oxidase V related favin-nucleotide-binding protein	111	-3.4	-3.048
LGG_00491	-	NADH peroxidase	112	4.1	-0.197
LGG_00615	<i>yeaE</i>	Aldo/keto reductase	113	2.1	0.153
LGG_00634	-	Dyp-type peroxidase family protein	114	2.5	2.248
LGG_00663	-	Conserved protein	115	4.9	6.808
LGG_00740	<i>gph</i>	Hydrolase, haloacid dehalogenase-like family	116	-1.8	-0.495
LGG_00744	<i>dkgA</i>	Aldo/keto reductase	117	1.6	0.71
LGG_00899	<i>secA</i>	Protein translocase subunit secA	118	-2.1	-0.076
LGG_01265	<i>mreB</i>	Rod shape-determining protein MreB	119	1.6	-0.331
LGG_01311	<i>ykqC</i>	Metallo-beta-lactamase superfamily protein	120	-2	0.493
LGG_01327	<i>typA</i>	GTP-binding protein TypA	121	-1.8	-2.590
LGG_01395	-	Conserved protein	122	3.4	3.504
LGG_01433	-	Nitroreductase	123	2.3	1.344
LGG_01465	<i>fns</i>	Formate-tetrahydrofolate ligase	124	1.9	-1.209
LGG_01468	-	Nitroreductase family protein	125,126	-3.6	-3.852
LGG_01821	<i>ptsH</i>	Phosphocarrier protein HPr	127	2.2	0.884
LGG_01837	<i>mvaS</i>	Hydroxymethylglutaryl-CoA synthase	128	-1.5	0.071
LGG_02050	<i>glf</i>	UDP-galactopyranose mutase	129	-1.7	-1.711
LGG_02098	-	Conserved protein	130	2.4	-0.526
LGG_02415	<i>cueO</i>	Multicopper oxidase	131	3.9	1.73
LGG_02630	<i>yghZ</i>	Aldo/keto reductase (oxidoreductase)	132	2.6	1.3

a. Two proteins were identified from these spots. It remains unclear which of these proteins was more abundant.

**Table 2.** Proteins with differing abundance between the late exponential (12 h) and early stationary (20 h) growth phases and transcription differences in the corresponding genes.

Locus ID	Gene name	Function	Proteomic (fold change)		Transcriptomic (Log <sub>2</sub> R)
			Spot No.	20 h/12 h	20 h/12 h
Carbohydrate metabolism					
Galactose metabolism					
LGG_00653	<i>galK</i>	Galactokinase	133,134	4	4.176
LGG_00654	<i>galE</i>	UDP-glucose 4-epimerase	135	3.1	3.817
LGG_00655	<i>galT</i>	Galactose-1-phosphate uridylyltransferase	136	3.7	3.831
LGG_00664	<i>lacC</i>	Tagatose-6-phosphate kinase	137	5.3	6.928
LGG_00665	<i>lacD</i>	Tagatose 1,6-diphosphate aldolase	138	2.3	6.461
LGG_00666	<i>lacB</i>	Galactose-6-phosphate isomerase subunit B	139,140	30.1	6.675
LGG_00667	<i>lacA</i>	Galactose-6-phosphate isomerase subunit A	141	26.8	6.561
LGG_00668	<i>lacR</i>	Lactose phosphotransferase system repressor	142	15.9	6.699
LGG_02575	<i>lacD</i>	Tagatose 1,6-diphosphate aldolase	143,144	2.1	-1.145
Glycolysis/gluconeogenesis					
LGG_00921	<i>pgm</i>	Phosphoglucosomutase	145	1.9	1.331
LGG_00933	<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	146,147	1.6	0.239
LGG_00933	<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	148	-2.9	0.239
LGG_01375	<i>pyk</i>	Pyruvate kinase	149,150	2	-2.596
LGG_01375	<i>pyk</i>	Pyruvate kinase	151	-1.8	-2.596
Others					
LGG_00342	<i>srlD</i>	Sorbitol-6-phosphate 2-dehydrogenase	152,153	26.1	6.56
LGG_00373	<i>rbsK</i>	Ribokinase	154	2.1	4.074
LGG_00757	<i>adhE</i>	Aldehyde-alcohol dehydrogenase	155-157	5.6	2.279
LGG_01421	<i>pflB</i>	Formate acetyltransferase	158	2.2	3.134
LGG_02523	<i>ldh</i>	L-lactate dehydrogenase	159 <sup>a</sup>	-1.6	-3.868
Membrane transport					
LGG_00345	<i>gatA</i>	PTS system, galactitol-specific IIA component	160	13	6.286
Metabolism of cofactors and vitamins					
LGG_02764	<i>entB</i>	Isochorismatase family protein	161	2.6	1.32
LGG_02765	<i>pncB</i>	Nicotinate phosphoribosyltransferase	162,163	3.3	1.623
Nucleotide metabolism					
Purine metabolism					
LGG_00249	<i>guaB</i>	Inosine-5'-monophosphate dehydrogenase	164	-1.6	0.419
LGG_01808	<i>purF</i>	Amidophosphoribosyltransferase	165	1.7	-2.035
Peptidoglycan biosynthesis					
LGG_00662	-	Beta-lactamase class C-related penicillin-binding protein	166	5.2	6.994
Stress					
LGG_01823	<i>clpE</i>	ATP-dependent clp protease ATP-binding subunit	167	1.8	0.722
LGG_02151	<i>usp</i>	Universal stress protein, UspA family	168	2.3	-2.413
LGG_02499	<i>clpC</i>	ATP-dependent Clp protease ATP-binding subunit	169	1.9	0.399
Translation					
LGG_00767	<i>alaS</i>	Alanyl-tRNA synthetase	170	-1.5	-0.758
LGG_01019	<i>gatA</i>	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A	171	-1.7	-0.603
Miscellaneous					
LGG_00226	-	Pyridoxine 5'-phosphate oxidase V-related favin-nucleotide-binding protein	172	-2.1	-1.605
LGG_00491	-	NADH peroxidase	173	1.9	-2.539
LGG_00634	-	Dyp-type peroxidase family protein	174	1.5	1.042
LGG_00763	<i>ytqI</i>	Phosphoesterase, DHH family protein	159 <sup>a</sup>	-1.6	-0.088
LGG_01395	-	Conserved protein	175	2.4	3.379
LGG_01465	<i>fhs</i>	Formate-tetrahydrofolate ligase	176	-1.5	-1.411
LGG_01468	-	Nitroreductase family protein	177	-1.6	-1.985
LGG_02630	<i>yghZ</i>	Aldo/keto reductase (oxidoreductase)	178	1.6	-0.024

a. Two proteins were identified from this spot. It remains unclear which of these proteins was more abundant.

this particular PTS is involved in the transportation of galactose, and revision of its present annotation could be considered. It is noteworthy that current annotations of PTS transporters do not necessarily indicate their true role in the metabolism of particular sugars because the speci-

ficiencies of many lactobacilli PTS transporters are incorrectly annotated (Francl *et al.*, 2010), and current computational methods are unreliable for the prediction of substrate specificity. Therefore, further experiments are needed to characterize the PTS transporter specificities of *L. rham-*



**Table 3.** Proteins with differing abundance between the early stationary (20 h) and late stationary (28 h) growth phases and transcription differences in the corresponding genes.

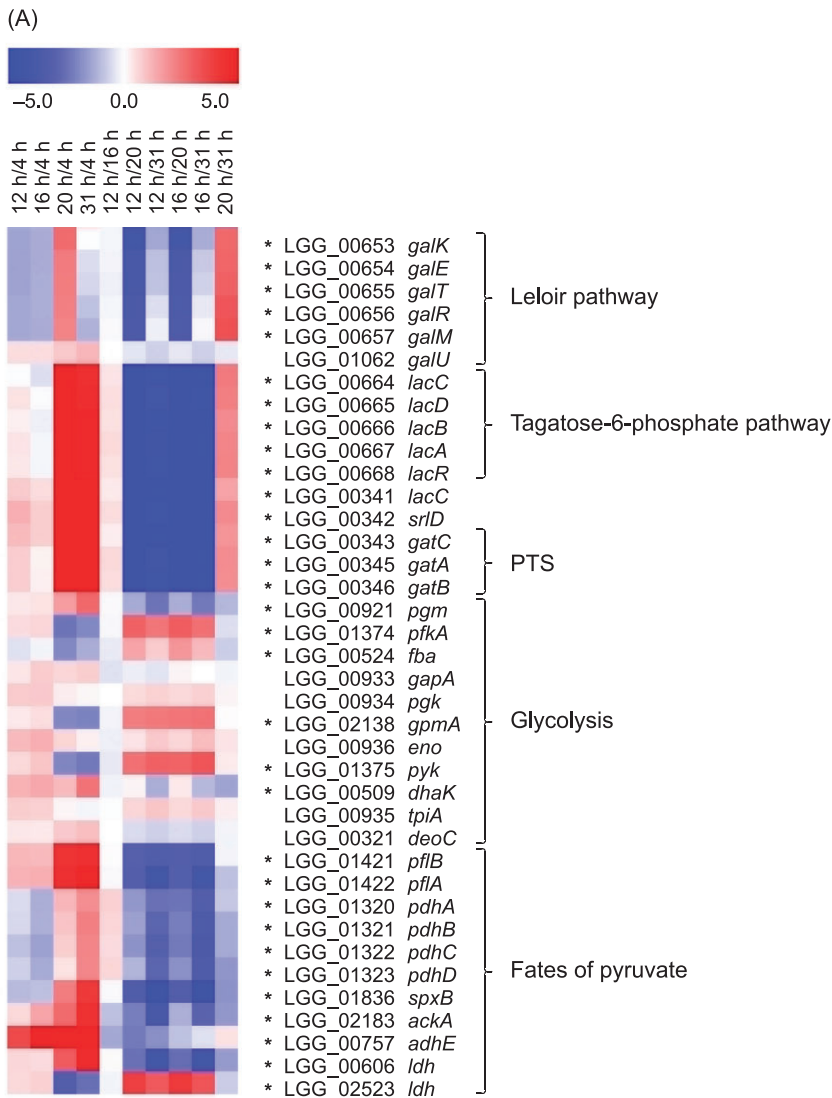
Locus ID	Gene name	Function	Proteomic (fold change)		Transcriptomic (Log <sub>2</sub> R)
			Spot No.	28 h/20 h	31 h/20 h
Amino acid metabolism					
LGG_02639	–	Aminotransferase	179	2.1	0.824
Carbohydrate metabolism					
Galactose metabolism					
LGG_00341	<i>lacC</i>	Tagatose-6-phosphate kinase	180	2.1	–1.548
LGG_00664	<i>lacC</i>	Tagatose-6-phosphate kinase	181	2.3	–2.407
LGG_00665	<i>lacD</i>	Tagatose 1,6-diphosphate aldolase	182	2	–2.313
LGG_00666	<i>lacB</i>	Galactose-6-phosphate isomerase subunit B	183,184	2.6	–2.027
LGG_00667	<i>lacA</i>	Galactose-6-phosphate isomerase subunit A	185,186	2	–2.163
LGG_00668	<i>lacR</i>	Lactose phosphotransferase system repressor	187	2.3	–2.18
Glycolysis/gluconeogenesis					
LGG_00921	<i>pgm</i>	Phosphoglucumutase	188	1.6	1.085
LGG_01375	<i>pyk</i>	Pyruvate kinase	189	–1.5	–0.329
Others					
LGG_00342	<i>srlD</i>	Sorbitol-6-phosphate 2-dehydrogenase	190,191	2.4	–2.053
LGG_00757	<i>adhE</i>	Aldehyde-alcohol dehydrogenase	192,193	2.5	–0.466
LGG_01421	<i>pfkB</i>	Formate acetyltransferase	194	1.5	0.176
LGG_01876	<i>pck</i>	Phosphoenolpyruvate carboxykinase (ATP)	195	1.8	0.693
LGG_02693	<i>xyIB</i>	Alcohol dehydrogenase	196 <sup>a</sup>	1.5	1.601
Membrane transport					
LGG_02421	<i>mtsB</i>	ABC transporter, ATPase component	197 <sup>a</sup>	–1.5	–0.438
Peptidoglycan biosynthesis					
LGG_00662	–	Beta-lactamase class C-related penicillin-binding protein	198	2.6	–2.265
Nucleotide metabolism					
Pyrimidine metabolism					
LGG_01625	<i>pyrH</i>	Uridylate kinase	197 <sup>a</sup>	–1.5	0.296
Miscellaneous					
LGG_01016	<i>ligA</i>	NAD-dependent DNA ligase	199	–1.6	–0.432
LGG_01061	<i>mvaK</i>	Phosphomevalonate kinase	200	1.6	–0.477
LGG_01395	–	Conserved protein	201	2.5	0.926
LGG_02124	<i>yqhD</i>	Iron-containing alcohol dehydrogenase	196 <sup>a</sup>	1.5	0.64

a. Two proteins were identified from these spots. It remains unclear which of these proteins was more abundant.

*nosus* GG; for example, this could be done by studying transcript expression profiles in response to different carbohydrates.

The abundance of several glycolytic enzymes and the transcription of the corresponding genes changed in a growth phase-dependent fashion. Genes coding for 6-phosphofructokinase (*pfkA*), fructose-bisphosphate aldolase (*fba*), phosphoglyceromutase (*gpmA*) and pyruvate kinase (*pyk*) were significantly downregulated during the stationary-phase transition (Fig. 4A, Table S1). At the protein level, Fba was more abundant in the mid-exponential growth phase than in the early stationary phase, consistent with the mRNA results (Table 1). No clear trend in the abundance changes of glycolytic proteins was seen during growth, however. Many glycolytic proteins (GapA, Pgc, TpiA, Eno, Pyk) were detected in several parallel protein spots (two to seven spots) on 2-D gels, and in some of these spot sets (GapA, TpiA, Pyk), both a decrease and an increase in abundance were detected (Tables 1 and 2). For instance, five glyceraldehyde-3-phosphate dehydrogenase (GapA) isoforms were more

abundant during the early stationary growth phase (compared with 4 h) while two other isoforms were more abundant during the mid-exponential growth phase (compared with 20 h). Post-translational protein modifications such as phosphorylation (Cozzone, 1998) and methionine formylation (Bandow *et al.*, 2003) may alter the migration of proteins in 2-D gels, which could also explain the appearance of parallel spots in this case. In *Lactococcus lactis*, glycolysis has been shown to be regulated at the transcriptional level only to a lesser degree, and post-translational modifications might be more important for regulation (discussed in Soufi *et al.*, 2008). In *Lactococcus*, *Escherichia coli* and *Bacillus*, several glycolytic proteins have been shown to be phosphorylated (Eymann *et al.*, 2007; Macek *et al.*, 2007; 2008; Soufi *et al.*, 2008), and characterization of the phosphoproteome suggests conservation of this type of modification of glycolytic enzymes in *L. rhamnosus* GG (J. Koponen, K. Laakso, K. Koskeniemi, M. Kankainen, K. Savijoki, T.A. Nyman, S. Tynkkynen, N. Kalkkinen and P. Varmanen, in preparation). Transcripts of genes coding for GapA, Pgc, TpiA and Eno were only slightly



**Fig. 4.** Gene expression patterns of selected genes and operons during growth in whey coding for (A) carbohydrate and pyruvate metabolic proteins, (B) proteinases, peptidases and amino acid transporters and (C) probiotic-associated factors. Gene expression changes (averages of three biological replicates) between paired time points are represented colorimetrically, with dark red indicating an expression ratio of 5.0 and dark blue indicating an expression ratio of -5.0 on a log<sub>2</sub> scale. The first four lanes show results of comparison of time points 12, 16, 20 and 31 h to the time point 4 h corresponding to the mid-exponential phase. Statistically significant changes ( $P$ -value  $\leq 0.01$ ) are marked with asterisks.

upregulated in the early stationary growth phase compared with the exponential growth phase in our study, suggesting that they are constitutively expressed (Fig. 4A, Tables 1 and 2).

Dihydroxyacetone kinase (DhaK), which catalyses the phosphorylation of dihydroxyacetone, was more abundant in early stationary-phase cells than in exponential-phase cells at both the transcript and protein levels (Fig. 4A, Table 1). Dihydroxyacetone phosphate is channelled to glycolysis via the activity of triosephosphate isomerase (TpiA), which catalyses the formation of glyceraldehyde-3-phosphate from dihydroxyacetone phosphate. Deoxyribose-phosphate aldolase (DeoC, LGG\_00321), which converts deoxyribose-5-phosphate to glyceraldehyde-3-phosphate (a glycolytic intermediate), and its concomitant gene were upregulated upon entry into the stationary phase of growth (Fig. 4A, Table 1). One of the most highly differentially expressed

proteins and genes was sorbitol-6-phosphate 2-dehydrogenase (SrlD, LGG\_00342), which channels sorbitol-6-phosphate to glycolysis via the conversion of sorbitol-6-phosphate to fructose-6-phosphate (Fig. 4A, Tables 1–3). The expression of SrlD increased over time, especially when the culture shifted from the exponential growth to the stationary phase. Transcription of the genes encoding the glucitol/sorbitol-PTS (LGG\_02717–02719) was induced when the culture shifted from exponential growth to stationary growth (Table S1). These results suggest that *L. rhamnosus* GG starts to use alternative energy sources other than the previously mentioned galactose, namely sorbitol, dihydroxyacetone and deoxyribose-5-phosphate, at the beginning of the stationary phase after the exhaustion of glucose. In *Lactobacillus casei*, the sorbitol operon is repressed by glucose (Alcántara *et al.*, 2008), and this seems to also apply for *L. rhamnosus* GG because the upregulation of SrlD

(B)

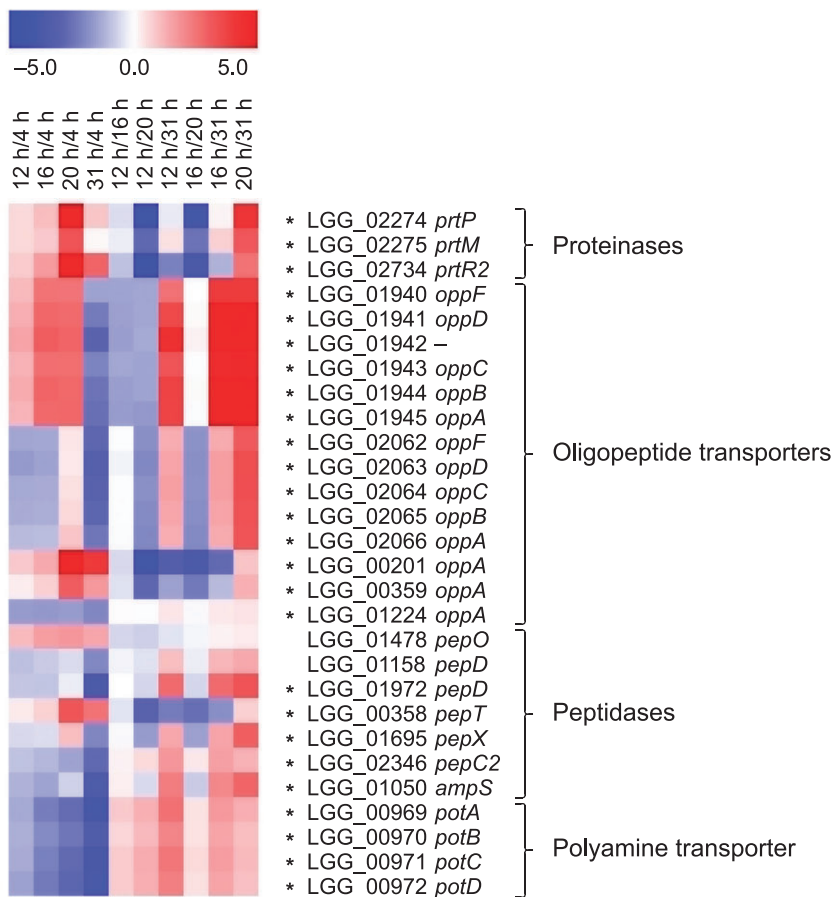


Fig. 4. cont.

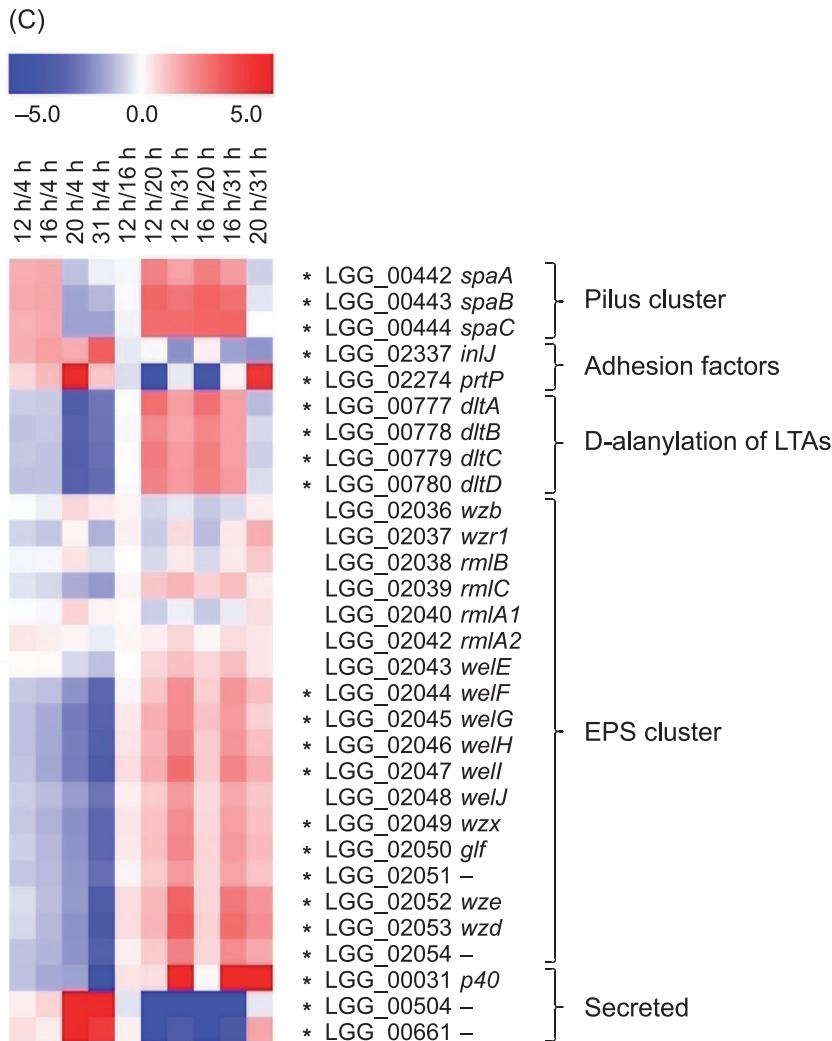
occurs when glucose has been used up (according to chemical analyses, detection limit 0.05%).

In summary, during the transition to the stationary growth phase, the utilization of glucose as the main carbon and energy source was replaced in *L. rhamnosus* GG by alternative pathways for carbohydrate metabolism, such as galactose utilization, using the tagatose-6-phosphate and the Leloir pathways.

**Energy production and conversion (pyruvate metabolism).** Under normal glucose fermentation conditions, lactic acid bacteria reduce pyruvate to lactate by a NAD<sup>+</sup>-dependent lactate dehydrogenase. In addition, lactic acid bacteria may have alternative means of utilizing pyruvate, including the pyruvate-formate lyase system, the pyruvate dehydrogenase pathway, the pyruvate oxidase pathway and the diacetyl/acetoin pathway (Axelsson, 2004). Genes encoding pyruvate-formate lyase (LGG\_01421–01422), aldehyde-alcohol dehydrogenase (LGG\_00757), pyruvate dehydrogenase (LGG\_01320–01323) and enzymes of the pyruvate oxidase pathway (LGG\_01836, pyruvate oxidase and LGG\_02183, acetate kinase) were induced at the

stationary-phase transition point (Fig. 4A, Table S1). The PflB subunit of pyruvate-formate lyase and aldehyde-alcohol dehydrogenase (AdhE) were also more abundant in stationary-phase cells than in exponential-phase cells at the protein level, supporting the transcript results (Table 1–3). However, proteomic analyses indicated that pyruvate dehydrogenase (PdhC) was more abundant in the exponential growth phase than in the stationary growth phase, in contrast with the transcript results (Table 1). A gene encoding lactate dehydrogenase (LGG\_00606) clustered together with the pyruvate-formate lyase and acetate kinase genes, and its expression was significantly increased over time (cluster 4 in Fig. S1). Expression of another gene coding for a lactate dehydrogenase (LGG\_02523) was, however, decreased upon entry into the stationary growth phase, and the same tendency was seen at the protein level (12 h versus 20 h) (Table 2). Thus, our results suggest that *L. rhamnosus* GG changes from homolactic fermentation to a mixed acid fermentation when the culture reaches the stationary phase, and the end-products formed, in addition to lactate, are formate, acetate and ethanol. This is probably due to the shift detected from glucose

Fig. 4. cont.



to galactose utilization in *L. rhamnosus* GG at the stationary-phase transition point. The same trend has been observed in *Lactococcus* strains, which shift from homolactic to mixed acid fermentation when glucose is limited or during galactose fermentation (reviewed by Neves *et al.*, 2005). In *L. plantarum* cells, more lactate dehydrogenase was produced in the exponential than in the stationary growth phase (Cohen *et al.*, 2006), supporting our conclusions.

**Nucleotide transport and metabolism.** The abundance of pyrimidine biosynthetic gene cluster (*pyr*) transcripts was reduced upon entry into the stationary phase of growth, and the downregulation of the pyrimidine biosynthesis pathway was also detected at the protein level (Table 1 and Table S1). In addition, the expression of several other genes and gene products involved in nucleotide metabolism were decreased over time in whey. This is probably due to protracted growth and, thus, reduced requirements for pyrimidine nucleotides. However, in the purine biosyn-

thesis pathway, no clear downregulation was observed. At the mRNA level, the purine biosynthesis operon (*purBCD-FHKLM* and *guaAB*) was expressed at lower levels in the stationary growth phase than in the exponential growth phase, while the PurCDFHKLM and GuaA proteins were more abundant at 20 h (early stationary) than at 4 h (mid-exponential) (Table 1 and Table S1). The whey medium used here does not supply *L. rhamnosus* GG cells with purines. It has previously been shown that in *L. rhamnosus* GG cells grown in whey medium, during the stationary phase, the abundance of Pur proteins is elevated compared with cells grown in medium rich in purines (Koskeniemi *et al.*, 2009). Therefore, our results suggest that under the growth conditions described here, purine biosynthesis in *L. rhamnosus* GG cells remains active during the switch from the exponential to the stationary phase. Furthermore, the identified growth phase-associated changes in Pur protein abundance were not associated with changes in the corresponding transcript levels, suggesting either the involvement of post-transcriptional

regulation mechanisms or that these proteins have a long half-life.

**Lipid transport and metabolism.** Fatty acids are constituents of phospholipids and glycolipids found in the cytoplasmic membrane of bacteria. Our results revealed that fatty acid biosynthetic genes (LGG\_02110–02122) were strongly repressed at the stationary-phase transition point (Table S1). Similarly, the abundance of the proteins, FabZ, FabF and FabK, which are involved in the biosynthesis of long-chain saturated fatty acids, were decreased over time (Table 1). Furthermore, the level of transcripts encoding cyclopropane-fatty-acyl-phospholipid synthase (*cfa*, LGG\_02109), which participates in the cyclopropanation of fatty acids, was reduced upon entry into the stationary phase of growth (Table S1). Fatty acids are energetically the most expensive membrane lipid components to produce, and their production is tightly regulated to match the growth rate of bacterial cells (Zhang and Rock, 2009). Therefore, our observations probably result from the deceleration of the growth rate in the stationary phase. Applying a proteome-level approach, Cohen and colleagues (2006) observed similar downregulation of several enzymes involved in lipid metabolism in *L. plantarum* during the shift from the exponential to the stationary phase of growth.

#### *Proteolytic systems, amino acid transport and metabolism*

*Lactobacillus rhamnosus* GG has a limited capacity to synthesize amino acids; therefore, it requires exogenous amino acids and peptides for growth (Kankainen *et al.*, 2009). In this study, several components of the proteolytic system were identified as exhibiting growth phase-dependent induction at both the transcript and the protein level, including proteinases, which degrade proteins into oligopeptides, peptide transporter systems involved in oligopeptide uptake, and peptidases, which degrade oligopeptides into shorter peptides and amino acids (reviewed by Savijoki *et al.*, 2006). The whey medium used in this study contains free amino acids and oligopeptides derived from hydrolysed casein and whey protein and probably also traces of non-hydrolysed proteins. During growth in whey medium, *L. rhamnosus* GG first consumes the accessible free amino acids and, after that, oligopeptides, which require only minimal processing to liberate the free amino acids. Expression of genes encoding cell-envelope proteinase PrtP, maturation protein PrtM and proteinase PrtR increased during growth of *L. rhamnosus* GG in whey medium until the early stationary growth phase, suggesting that the non-hydrolysed or partially hydrolysed proteins in whey medium may be utilized after the exhaustion of other amino acid sources (Fig. 4B,

Table S1). In protein gels, no differentially produced proteinases were detected. However, it is well known that high-molecular-weight proteins, hydrophobic proteins and proteins with extreme pI values are under-represented on 2-D gels possibly explaining the lack of identification of PrtP (206.6 kDa), PrtR (155.8 kDa) and PrtM (pI value 10.3). It has previously been shown that whey-grown *L. rhamnosus* GG is unable to hydrolyse intact casein (Kankainen *et al.*, 2009), and the role of the proteinases in the utilization of intact or partially hydrolysed whey proteins remains to be elucidated. Consistent with our findings, in *L. acidophilus* cultivated in milk, the expression of genes encoding PrtP and PrtM was increased over time (Azcarate-Peril *et al.*, 2009).

The genome of *L. rhamnosus* GG holds three complete oligopeptide ABC transporter operons (LGG\_01652–01656, LGG\_01940–01945 excluding LGG\_01942, and LGG\_02062–02066) (Kankainen *et al.*, 2009). Each consists of five genes encoding oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC) and two ATP-binding proteins (OppD and OppF). Of these operons, LGG\_01940–01945 was found to be differentially expressed at both the mRNA and protein levels. Expression of the whole operon at the mRNA level was increased until the early stationary growth phase (Fig. 4B, Table S1), and the ATPase components, OppD and OppF, were more abundant in the early stationary growth phase than in the mid-exponential growth phase at the protein level, supporting the transcript results (Table 1). LGG\_02062–02066 transcripts were more abundant in the mid-exponential growth phase (4 h) than at the 12, 16 and 31 h time points but less abundant at 4 h than in the early stationary growth phase (20 h) (Fig. 4B, Table S1). Transcription of these genes was therefore transient, with a short peak in the early stationary growth phase. Differential production of the corresponding proteins was not observed possibly because the fold changes were below threshold value of 1.5. In addition to the three oligopeptide-binding proteins (OppA) encoded within the complete Opp operons, four additional *oppA* genes are present in the *L. rhamnosus* GG genome (Kankainen *et al.*, 2009). Of these, three *oppA* genes were significantly differentially expressed in a growth phase-dependent manner (Fig. 4B, Table S1). Based on these observations, it could be speculated that different oligopeptide transport systems may have different specificities, and while one Opp transporter system is active in the exponential growth phase, another might be active in the stationary phase of growth. Similar results were obtained in a microarray study of *L. acidophilus* grown in milk, which showed that different Opp genes were expressed at different growth stages (Azcarate-Peril *et al.*, 2009).

At the mRNA level, the genes encoding tripeptidase PepT (LGG\_00358), proline-specific aminopeptidase



PepX (LGG\_01695), dipeptidase PepD (LGG\_01972), aminopeptidases PepC2 (LGG\_02346) and AmpS (LGG\_01050) were significantly differentially expressed at different growth phases (Fig. 4B, Table S1). The gene coding for PepT clustered together with the OppA-encoding gene (LGG\_00359) and was maximally expressed in *L. rhamnosus* GG at the early stationary phase of the growth curve (cluster 10 in Fig. S1). The transcript encoding PepX clustered together with the ORFs LGG\_02062–02066 (the Opp transporter system), and its expression was transiently elevated at 20 h (cluster 6 in Fig. S1). The expression levels of *pepD*, *pepC2* and *ampS*, as well as genes involved in polyamine transport (*potABCD*, LGG\_00969–00972), were decayed during growth (Fig. 4B, Table S1). Proteomic results indicated that endopeptidase PepO (LGG\_01478) was produced at higher levels in the early stationary cultures than in the mid-exponential cultures while dipeptidase PepD (LGG\_01158) was produced more in mid-exponential cells compared with early stationary cells (Table 1). The same pattern was seen at the mRNA level, although it was not statistically significant (Fig. 4B). These findings suggest that different peptidases are utilized specifically in different growth phases, probably depending on their specificities for the particular peptides available at each stage.

#### General stress responses

Nutrient limitations activate a variety of bacterial stress responses (reviewed by De Angelis and Gobetti, 2004). Changes in environmental conditions also rapidly trigger stress-related proteolytic systems to cope with the accumulation of irreparably damaged proteins (Savijoki *et al.*, 2006). Not surprisingly, the abundance of several stress proteins and the transcription of genes encoding stress responsive proteins was elevated when the cells reached the stationary growth phase. At the mRNA level, transcripts encoding Clp protease subunits, ClpL and ClpE, endopeptidase subunits, HslV (ClpQ) and HslU (ClpY), and heat shock proteins, Hsp1 and Hsp3, were elevated in the stationary phase of growth. However, genes encoding universal stress protein (*usp*) and 33 kDa chaperonin (*hslO*) showed their highest expression in the exponential growth phase (Table S1). At the protein level, production of the universal stress protein, the chaperone protein GroEL and the stress-inducible Clp protease complex subunits ClpC and ClpB was increased over time while the chaperone protein DnaK and the stress-related serine protease HtrA were more abundant in the mid-exponential growth phase (4 h) than in the early stationary growth phase (20 h) (Table 1). The corresponding genes, *groEL*, *clpC*, *clpB*, *dnaK* and *htrA*, were not significantly differentially expressed. As chaperone proteins are required for *de novo* protein folding, the expression of chaperones and

their corresponding genes in the exponential growth phase, when essential nutrients are still sufficiently available, could be a response to the high protein synthesis rate. Because growth phase-associated changes in stress proteins were detectable at either the mRNA or protein level, but not at both levels, it is tempting to speculate that post-transcriptional regulation mechanisms are involved in the adaptation of *L. rhamnosus* GG to the stationary growth phase conditions. Recently, it has been demonstrated that most of the stress-related genes showed increasing levels of expression in the stationary growth phase in *L. acidophilus* (Azcarate-Peril *et al.*, 2009). Furthermore, in a proteomic study of *L. plantarum* WCFS1, it has been observed that some stress proteins were highly abundant in the late exponential and early stationary growth phases (ClpL and Hsp3, among others) while some were highly abundant during the exponential growth phase (ClpP, among others) (Cohen *et al.*, 2006). It should be noted that the earlier studies describe bacterial growth phase responses in flask culture conditions where gradual acidification of the cultures causes additional stress. These growth conditions were different from those of the present study where pH-controlled conditions were used.

#### Expression of genes mediating potential probiotic-associated traits changes over time in whey

*Lactobacillus rhamnosus* GG colonizes the human intestine and adheres efficiently to mucus and epithelial cells (Alander *et al.*, 1997; 1999; Laparra and Sanz, 2009). Cell surface factors, such as cell surface proteins, lipoteichoic acids (LTAs) and exopolysaccharides (EPSs), mediate adherence, biofilm formation and colonization by lactobacilli (reviewed by Lebeer *et al.*, 2008). Recently, MabA (modulator of adhesion and biofilm) encoded by LGG\_01865 was shown to contribute to the adhesion of *L. rhamnosus* GG to the host cells (Vélez *et al.*, 2010). Cell surface-exposed pilus, encoded by a gene cluster located in a genomic island unique to *L. rhamnosus* GG, was suggested to play a key role in the adhesion of *L. rhamnosus* GG to human intestinal mucus (Kankainen *et al.*, 2009). Our transcriptomic results showed that strain-specific pilus-encoding genes (*spaCBA*, LGG\_00442–00444) were active in the exponential phase of growth, and their expression was clearly reduced when the culture transitioned to the stationary growth phase (Fig. 4C, Table S1). In contrast, growth phase dependence in the expression pattern of *mabA* was not observed. However, several other genes encoding proteins predicted to contain an adhesion domain, such as LGG\_02337 (*inlJ*) and LGG\_02274 (*prtP*), were upregulated in *L. rhamnosus* GG cultures in the stationary growth phase compared with the exponential growth phase (Fig. 4C, Table S1). No changes in the correspond-

ing proteins were observed, which may be due to the hydrophobic nature or the low abundance of these proteins. Furthermore, pilus proteins may occur at heteropolymeric high-molecular-weight complexes (Mandlik *et al.*, 2008) that are not detectable using 2-D DIGE. In *L. lactis*, cell wall-anchored PrtP has been shown to have positive effect on cell adhesion to solid surfaces (Habimana *et al.*, 2007); however, its effect on adhesion to human cells or mucus remains to be elucidated. Pili of Gram-positive pathogens have been extensively investigated and have been shown to promote bacterial adhesion to host cells, play a role in biofilm formation and evoke host immune responses (reviewed by Mandlik *et al.*, 2008). In *L. rhamnosus* GG, it has also been demonstrated that pili play an important role in adhesion to Caco-2 epithelial cells and in biofilm formation (Lebeer *et al.*, 2011). In a virulent *Streptococcus pneumoniae* strain, pilus genes were found to be expressed in a growth phase-dependent manner, and the regulation of pilus genes was shown to be mediated by a two-component system (Song *et al.*, 2009). In our study, the expression pattern of genes encoding a two-component system (LGG\_02387–02388, cluster 14 in Fig. S1) was similar to that of the pilus-encoding genes (cluster 13 in Fig. S1, Table S1), but further studies are needed to establish whether this particular two-component system plays a role in the regulation of the pilus locus genes in *L. rhamnosus* GG or whether it is regulating one of the various other functions altered during exponential growth.

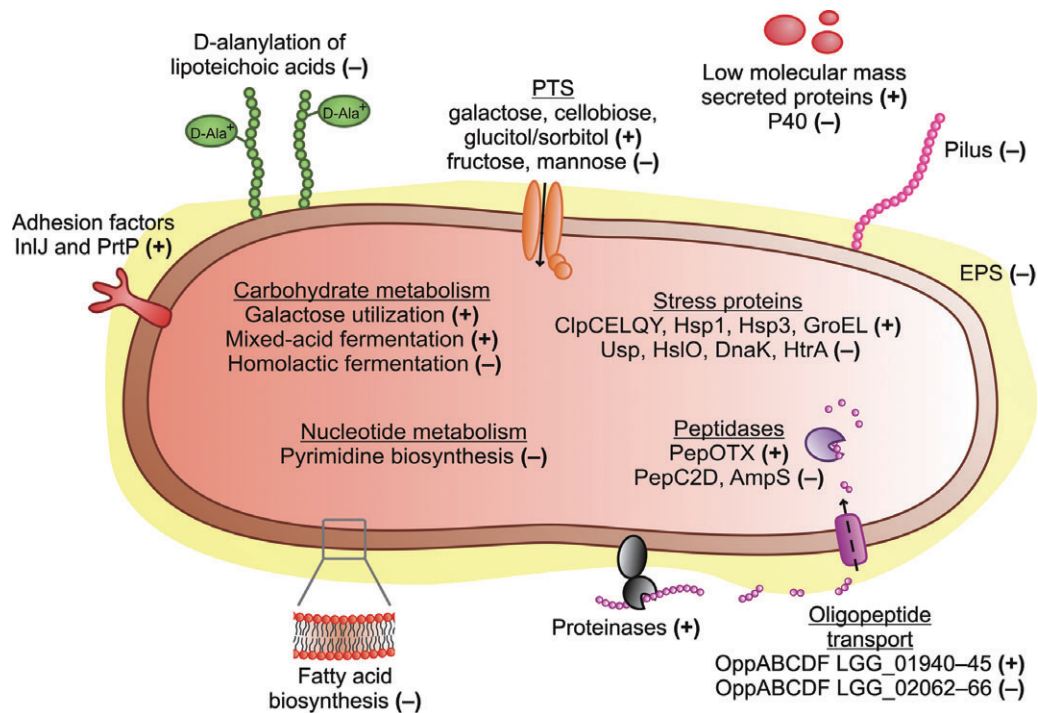
The expression of genes involved in D-alanylation of LTAs (*dlt* operon, LGG\_00777–00780, clusters 13 and 16 in Fig. S1) and most of the genes encoding enzymes required for EPS biosynthesis (LGG\_02044–02047 and LGG\_02049–02054, clusters 14 and 16 in Fig. S1) was reduced over time in whey (Fig. 4C, Table S1). Consistent with our findings, previous studies have demonstrated that EPS production is growth rate-dependent (Welman *et al.*, 2003; 2006). In *L. rhamnosus* GG, EPS plays a negative role in adhesion and biofilm formation, possibly by shielding adhesion factors such as pili (Lebeer *et al.*, 2009). However, EPS appears to play an important role in the protection against host defence mechanisms involving the innate immune system (Lebeer *et al.*, 2010). Because the balance between adhesion properties and resistance against the host immune system seems to be of the utmost importance for the adaptation of *L. rhamnosus* GG in the intestine, expression of pili and EPS biosynthetic genes are likely to be regulated accordingly. The products of the *dlt* operon are involved in the D-alanylation of secondary cell wall polymer LTA. D-alanyl ester substitutions for LTA add positive charges to otherwise negatively charged LTAs and, in this way, directly affect cell surface charges (Neuhaus and Baddiley, 2003). Lebeer and colleagues (2007) have shown that D-alanyl-

substituted LTAs serve a modulatory function in the adhesion of *L. rhamnosus* GG to Caco-2 cells and in biofilm formation and activate immune responses in the host. Claes and colleagues (2010) recently showed that deletion of *dltD* resulted in a significant increase in the probiotic efficacy of *L. rhamnosus* GG in a mouse model of colitis. Therefore, it is suggested that D-alanylation of LTAs may play a role in the survival and persistence as well as in the probiotic effects of *L. rhamnosus* GG in the host, and our results indicate that the expression of this probiotic-associated factor is growth phase-dependent.

In addition to adhesion factors such as pili and InlJ, contact between *L. rhamnosus* GG and host cells can also be mediated by secreted, small soluble proteins. For instance, secreted, low-molecular-weight proteins from *L. rhamnosus* GG have been shown to promote *in vitro* intestinal epithelial cell homeostasis through certain signalling pathways (Yan and Polk, 2002; Tao *et al.*, 2006; Yan *et al.*, 2007; Seth *et al.*, 2008). Expression of a gene coding for the NLP/P60 family secreted protein, p40 (LGG\_00031), was reduced during growth (Fig. 4C, Table S1). In contrast, the genes LGG\_00504 and LGG\_00661, coding for secreted proteins of low molecular mass (< 10 kDa), were strongly induced in stationary-phase cells (Fig. 4C, Table S1). Interestingly, a gene located next to one of these small-sized soluble proteins (LGG\_00503) and annotated as a myosin-cross-reactive antigen orthologue was also highly expressed in the stationary growth phase compared with the exponential growth phase. None of these responses was detected at the protein level, probably because cell surface-exposed and secreted proteins are usually hydrophobic and are precipitated within the first dimension once they have migrated to their isoelectric point.

### Concluding remarks

Consumption of probiotic bacteria harvested at different phases of growth has been shown to cause profoundly different mucosal responses in human (van Baarlen *et al.*, 2009). Accordingly, we assume that the expression status of the genes during the industrial cultivation of a probiotic reflects its activity in the GIT. We investigated how gene expression, at both the transcript and protein levels, in the probiotic bacterium, *L. rhamnosus* GG, changed over time during growth in industrial-type whey medium under controlled bioreactor conditions. In particular, we aimed to determine whether any traits that are associated with the probiotic activity of *L. rhamnosus* GG were affected during the fermentation of whey. Expression of 636 genes, many implicated in the probiotic-linked actions of *L. rhamnosus* GG, and 116 proteins were modulated in a growth-dependent manner and half of the changes in protein abundance were



**Fig. 5.** A model for physiological changes in *L. rhamnosus* GG during the progression of growth. Increased and decreased expression is indicated by plus symbol '+' and minus symbol '-' respectively.

associated with changes in transcript levels. Most of the observed growth phase-dependent changes at both the mRNA and protein level appeared during the shift from the exponential growth phase to the stationary growth phase. In particular, genes and proteins involved in the galactose utilization pathways were among the highest induced upon entry into stationary phase. Many transcripts and proteins involved in central metabolic pathways such as carbohydrate, nucleotide, lipid and pyruvate metabolism were differentially expressed during growth in whey (Fig. 5). Differential expression of distinct carbohydrate utilization and energy production pathways and several distinct PTS transporters observed in response to changes in nutritional environment (e.g. exhaustion of the preferred carbon source in the medium) indicate that *L. rhamnosus* GG has a flexible and adaptable metabolism. This adaptability might provide a competitive advantage for *L. rhamnosus* GG in the host's GIT. Furthermore, expression of many genes encoding adhesion factors and secreted proteins with low molecular masses were increased over time during the growth of *L. rhamnosus* GG in whey medium (Fig. 5), with the highest expression in the stationary phase. The expression patterns of genes encoding mucus-binding pili and secreted protein p40 differed from this general pattern (Fig. 5), having the highest expression values in the exponential phase. Like nutritional versatility, these adhesion and probiotic-

associated factors provide a competitive advantage for *L. rhamnosus* GG by promoting its survival and persistence in the GIT. At present, the number of probiotic-associated factors of *L. rhamnosus* GG that has been identified is still limited which makes it impossible to draw conclusions about the optimal harvesting phase for this probiotic bacterium. However, as soon as the key mediators of probiotic traits have been identified, the results presented here together with future studies involving growth conditions mimicking those in the GIT will be instrumental for these purposes.

## Experimental procedures

### *Growth conditions and chemical analyses*

*Lactobacillus rhamnosus* GG (ATCC 53103) was maintained in a laboratory culture collection as a glycerol stock at  $-70^{\circ}\text{C}$  and propagated at  $37^{\circ}\text{C}$  in MRS medium (Labema). Appropriate dilutions of a culture grown for 17 h were plated on MRS agar and incubated anaerobically at  $37^{\circ}\text{C}$  for 3 days. Single colonies from these plates were resuspended in MRS medium. One individual colony represented one biological replicate. Cells were passaged through two sequential subcultures in MRS medium at  $37^{\circ}\text{C}$  for 12 h and one subculture in industrial-type whey medium containing 5% hydrolysed whey, 0.6% casein hydrolysate and 0.0015%  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  at  $37^{\circ}\text{C}$  for 17 h. Four Biostat Q fermentation vessels (B. Braun Biotech International) containing 750 ml of whey medium were inoculated at 2% (v/v) with four individual cultures of



*L. rhamnosus* GG. The cultures were grown at 37°C and stirred constantly (150 r.p.m. min<sup>-1</sup>). The pH was maintained at 5.8 by automatic titration with 5% (v/v) ammonia. To monitor growth, samples were taken at regular intervals from each vessel, and their optical density at 600 nm was measured. The amount of glucose, galactose and lactose in whey medium during fermentation was determined by enzymatic methods based on ISO 5765-1: 2002 (IDF 79-1: 2002) and ISO 5765-2: 2002 (IDF 79-2: 2002) standards. Samples for RNA extraction were collected at the mid-exponential (4 h), late exponential (12 h), stationary transition point (16 h), early stationary (20 h) and late stationary (31 h) phases (Fig. 1). Protein samples were collected at the mid-exponential (4 h), late exponential (12 h), early stationary (20 h) and late stationary (28 h) phases (Fig. 1). Three parallel RNA samples and four parallel protein samples from each sampling point were included in the analyses.

### Transcriptomic methods

**Experimental design, RNA methods, cDNA synthesis, labeling and hybridization.** mRNA samples from three independent biological replicates, i.e. from three fermentations, for each time point were hybridized, each sample independently, to microarrays according to an ANOVA design where all possible direct pair-wise comparisons within biological replicates were conducted. A total of 30 hybridizations were performed. Dye-swaps were carried out over biological replicate sets.

Depending on the growth stage, 1–4 ml of culture was added to 2–8 ml of RNeasy Protect Bacteria reagent (Qiagen), and the samples were processed according to the manufacturer's instructions. Cell pellets were stored at –70°C for later RNA extraction. RNA was extracted and purified as described previously (Koskenniemi *et al.*, 2011).

For each sample, 5 µg of RNA was reverse transcribed to cDNA using the SuperScript Indirect cDNA Labelling System (Invitrogen) and fluorescently labelled with Cy3 or Cy5 mono-reactive dyes (Amersham Biosciences) according to previously published protocols (Koskenniemi *et al.*, 2011). The labelling efficiency was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and labelled cDNA samples were hybridized to microarrays according to Agilent's procedure, 'Two-Color Microarray-Based Gene Expression Analysis' (<http://www.agilent.com>).

**Scanning, image analysis and data analyses.** Microarrays were scanned at 5 µm resolution with a GenePix 4200 AL scanner (Axon Instruments). The fluorescence signal intensities of each feature were measured and addressed to genomic ORFs using GenePix® Pro 6.0 software (Axon Instruments/Molecular Devices Corp.) with default parameters. The computational alignments were further improved manually, and features that could not be verified were flagged.

This study was a part of a larger experiment, comprising a total of 66 two-color microarrays, which were analysed as an entity. After pre-processing, samples involved in this particular study were separated and analysed independently from the remaining data sets. All analyses were performed with the Bioconductor package for statistical analysis using the R programming language (Gentleman *et al.*, 2004). The

data were pre-processed with limma (Smyth and Speed, 2003). The foreground and background median intensity estimates were corrected for the background using the normexp-function (offset set to 50) (Ritchie *et al.*, 2007), normalized within arrays using loess (100 iterations, suspicious spots and probe sequences matching multiple hits or borders of the coding regions were down weight to zero, and intergenic probe sequences were down weight to 0.1) (Smyth and Speed, 2003) and normalized between arrays using quantile normalization (Yang and Thorne, 2003). Gene expression ratios were obtained by taking the average over the log<sub>2</sub>-transformed expression ratios of features describing the same gene and matching a single genetic coding locus. Expression ratios between conditions were calculated using linear models implemented in limma (Smyth, 2004). Gene expression ratios were calculated for 2798 genes out of the total 2944 genes predicted to be encoded by the genome (95% coverage). After pre-processing, samples involved in this study were separated from the remaining samples, and the statistical significance of the expression ratio of a gene between two conditions was analysed using the paired *t*-test method implemented in CyberT (the Bayesian prior estimate of within-treatment variance was set to five, and the window size was set to 101) (Baldi and Long, 2001). *P*-values were adjusted for multiple hypothesis correction using the Bonferroni method and the number of performed *t*-tests in total (61 556). Significantly differentially expressed genes with at least a twofold change in expression ratio between paired time points were clustered in MeV version 4.3 (Saed *et al.*, 2006) by using the *K*-means clustering algorithm and a Euclidean distance metric. Data for the classification of COGs were obtained from Kankainen and colleagues (2009). The statistical significance of groups was assessed using the hypergeometric distribution, and the results were corrected with the Bonferroni method. Microarray platform and data details are available at the Gene Expression Omnibus (GEO platform GPL10580 and series GSE28903).

### Proteomic methods

**Protein extraction and CyDye labeling.** Cells were harvested from 1–5 ml of samples by centrifuging at +4°C and washed twice with ice-cold 50 mM Tris-HCl pH 8 (Sigma-Aldrich). Bacterial cells were broken by bead beating (Koskenniemi *et al.*, 2009). The protein samples were processed using a Clean-up kit (GE Healthcare) and dissolved in 25–50 µl of a buffer containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma-Aldrich) and 30 mM Tris (Bio-Rad). Protein concentrations in the samples were determined using a 2-D Quant Kit (GE Healthcare).

Protein samples were adjusted to pH 8.5 by adding 2 M Tris. The samples were then labelled using Cy2, Cy3 and Cy5 dyes (CyDye DIGE Fluor minimal dyes; GE Healthcare), according to the Ettan™ 2-D DIGE protocol and Koskenniemi and colleagues (2009). The experiment was divided into three parts; each time point (4 h, mid-exponential; 12 h, late exponential; 28 h, late stationary growth state) was separately compared with the early stationary-phase (20 h) time point. Labelling was performed reciprocally so that both the

early stationary-phase samples and the other time point samples (4 h, 12 h or 28 h) were labelled with Cy3 and Cy5 to account for any preferential protein labelling by the CyDyes (labelling chart as Table S2). Cy2 was used for the internal pooled standard, consisting of equal amounts of each sample.

**2-DE and DeCyder analyses.** The labelled proteins were separated by isoelectric focusing using immobilized pH gradient strips (24 cm, pH 3–10 non-linear, Bio-Rad) and a Protean IEF Cell (Bio-Rad) for 80 000 Vh (Koskenniemi *et al.*, 2009). The strips were then equilibrated and loaded onto 12% acrylamide gels, and the gels were subjected to electrophoresis in an Ettan™ DALTsix Electrophoresis Unit (GE Healthcare) (Koskenniemi *et al.*, 2009). The gels were imaged using an FLA-5100 laser scanner (Fujifilm), and the images were cropped to identical sizes by removing areas extraneous to the proteins spots with ImageQuant™ TL 7.0 software (GE Healthcare). After scanning, the gels were fixed in 30% ethanol and 0.5% acetic acid for a minimum of 60 min and then silver stained (O'Connell and Stults, 1997). Image and statistical analyses for the cropped 2-D DIGE gels were performed using DeCyder™ 2D 7.0 software (GE Healthcare) (Koskenniemi *et al.*, 2009). Approximately 700–800 separate protein spots were detected on each gel. Protein spots were picked and identified if they demonstrated at least a 1.5-fold difference in average spot volume ratio (average ratio  $\geq 1.5$  or  $\leq -1.5$ ) between different time points in at least three out of four separate biological replicates. Statistical significance was calculated using the Student's *t*-test; a *P*-value value less than 0.05 was considered significant.

**Protein identification by mass spectrometry.** MS-compatible silver staining (O'Connell and Stults, 1997) was performed to visualize the protein spots for identification. Protein spots of interest were digested in-gel with trypsin and the peptides recovered as previously described (Koskenniemi *et al.*, 2009). The resulting peptides were analysed by peptide-mass fingerprinting (PMF) or by fragment ion analysis with LC-ESI-MS/MS as described in detail previously (Koskenniemi *et al.*, 2011).

The PMF spectra were processed with FlexAnalysis version 3.0 (Bruker Daltonik). The PMF and LC-ESI-MS/MS data were searched with the local Mascot version 2.2 (Matrix Science) against the in-house database of the published ORF set of *L. rhamnosus* GG, which contains 2944 protein entries (Kankainen *et al.*, 2009), using the Biotools 3.0 (Bruker Daltonik) and ProteinPilot 2.0.1 (Applied Biosystem) interfaces respectively. The search criteria for both Mascot searches were as follows: trypsin digestion with one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification. For the PMF spectra the maximum peptide mass tolerance was  $\pm 50$  ppm. The protonated molecule ion 'MH<sup>+</sup>' and 'monoisotopic' were defined for the peak mass data input. For the LC-ESI-MS/MS spectra, both the maximum precursor ion mass tolerance and the MS/MS fragment ion mass tolerance were 0.2 Da, and a peptide charge state of +1, +2 or +3 was used. A successful identification was reported when a significant match ( $P \leq 0.05$ ) was obtained.

In addition, to consider the LC-ESI-MS/MS identification reliable, a minimum of two peptides with an ions score of at least 40 was required.

## Acknowledgements

Elina Ahola-Iivarinen, Hanna Jefremoff, Saija Laakso and Eeva-Marja Turkki are acknowledged for their technical assistance. This work was supported financially by the Academy of Finland (Grants 210740 and 117746), the Finnish Funding Agency for Technology and Innovations (Grant 201/08) and by an ABS Graduate School scholarship to K.K.

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### Supporting information

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

**Fig. S1.** K-means clustering of transcriptomic expression profiles. Significant changes in gene expression are represented as log<sub>2</sub> intensity ratio values between paired time points. Genes were grouped into 16 clusters using a Euclidean distance metric, and the analysis included 636 genes in

total. The purple expression profile in each cluster represents the cluster median.

**Fig. S2.** Representative 2-D DIGE images of proteins extracted from different growth phases: (A) 4 h and 20 h, (B) 12 h and 20 h and (C) 20 h and 28 h. The total amount of protein used for CyDye labelling was 105 µg. The numbered protein spots were cut from silver-stained 2-D DIGE gels and subjected to MS or MS/MS identification. Protein spots that were more abundant at time point 20 h are shown in red and those more abundant at time points 4 h, 12 h or 28 h are shown in green. The protein spots with no difference in abundance between the treatments are yellow.

**Table S1.** Log<sub>2</sub> ratio values of significantly ( $P \leq 0.01$ ) differentially expressed genes with at least a twofold change in the expression ratio between paired time points. Clusters refer to Fig. S1.

**Table S2.** Set-up of the DIGE experiments. Letters A–D refer to biological replicate samples. Gels 1–4, 5–8 and 9–12 each form a separate DIGE experiment.

**Table S3.** Proteins with differing abundance between the mid-exponential (4 h) and early stationary (20 h) growth phases.

**Table S4.** Proteins with differing abundance between the late exponential (12 h) and early stationary (20 h) growth phases.

**Table S5.** Proteins with differing abundance between the early stationary (20 h) and late stationary (28 h) growth phases.

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