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Regulation of energy metabolism in *Enterococcus faecalis* studied by transcriptome, proteome and metabolome approaches

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Ås, September, 2011

Ibrahim Mehmeti

ABSTRACT

Lactic Acid Bacteria (LAB) are widely used as starter culture in food fermentation. Among LAB also pathogenic bacteria are found particular in enterococci and streptococci. *Enterococcus faecalis* is a gut commensal bacterium but certain isolates have been shown to be pathogenic while others are food-grade bacteria in LAB fermented food commodities. *E. faecalis* ferments sugars through different pathways, resulting in homo- or mixed acid fermentation. In homolactic bacteria glucose is converted to lactate in an ATP producing reaction. In mixed acid fermentation, in addition to lactate production, glucose is also converted to acetate, acetoin, formate, ethanol and CO_2. However, there is limited information regarding to regulation of the central energy metabolism of *E. faecalis*.

The aim of this work was to extend our knowledge with respect to the central energy metabolism of *E. faecalis* by employing metabolite, transcriptome and proteome approaches. High-performance liquid chromatography and gas chromatography were used for metabolite measurements. DNA microarray technology and two dimensional gel electrophoresis combined with mass spectrometry analysis were used in transcription and protein expression analysis, respectively. Combining these approaches has not been performed in metabolic analysis in *E. faecalis* and this should give an in-depth understanding about regulation of the central energy metabolism in *E. faecalis*.

This work showed that in absence of *ldh* (lactate dehydrogenase) gene, *E. faecalis* metabolizes glucose to ethanol, formate and acetoin. The change from homolactic to mixed acid fermentation affected expression of several genes and proteins mostly involved in energy metabolism. These genes play an important role in the regulatory network controlling energy metabolism in *E. faecalis* including acetoin production, and NAD^+/NADH ratio. Additional studies were carried out in order to investigate the mixed acid fermentation of wild-type *E. faecalis* in chemostat during steady state and glucose limiting growth. Growth at three different growth rates demonstrated that the bacterium responded differently depending on the growth rate. At the highest dilution rate ($D=0.4\ h^{-1}$) most of the glucose was converted to lactate while at the lowest dilution rate ($D=0.05\ h^{-1}$) it changed towards mixed acids fermentation. Interestingly, increased growth rate induced the transcription of the *ldh* gene while the amount of Ldh protein was more or less unaffected. The differences in glucose energy metabolism at different growth and pHs between *E. faecalis* and two other LAB (*Streptococcus pyogenes* and *Lactococcus lactis*) and their LDH negative mutants were also investigated. Of note, deletion of the *ldh* genes hardly affected the growth rate in chemically defined medium under microaerophilic conditions. Furthermore, deletion of *ldh* affected the ability for utilization of various substrates as a carbon source. The final study explored the effect of ascorbate on growth in the absence of glucose and showed that *E. faecalis* can grow on ascorbate.

In summary, the work presented in this thesis gave new insights in regulation and strengthens our knowledge regarding the metabolic pathways of glucose fermentation through the metabolite analysis, regulation of transcription and protein expression.

SAMMENDRAG (NORWEGIAN ABSTRACT)

Melkesyrebakterier brukes som startkulturer i en rekke ulike gjæringsreaksjoner i forbindelse med produksjon av mat. Enkelte melkesyrebakterier har også evnen til å forårsake sykdom, og dette gjelder spesielt for enterokokker og streptokokker. *Enterococcus faecalis* er en kommensal tarmbakterie. Likevel finner man innenfor denne arten både patogene isolater såvel som stammer benyttet i fermentering av matvarer. *E. faecalis* bryter ned sukker gjennom flere ulike veier, med enten melkesyre (homolaktisk gjæring) eller en blanding av syrer (blandet syregjæring) som endeprodukt. Homolaktiske bakterier bryter ned glukose til melkesyre i en reaksjonskjede som produserer ATP. Ved blandet syregjæring av glukose produseres det i tillegg til melkesyre også eddiksyre, acetoin, maursyre, etanol og CO_2. Det er imidlertid lite informasjon om reguleringen av energimetabolismen i *E. faecalis* tilgjengenlig.

Målet med arbeidet bak denne avhandlingen har derfor vært å tilegne oss kunnskap om den sentrale energimetabolismen i *E. faecalis* ved hjelp av ulike metoder for å studere metabolitter, transkriptomet og proteomet. Væskekromatografi og gasskromatografi ble brukt til metabolittmålinger, mens DNA mikromatriseteknologi og to-dimensjonal gelelektroforese kombinert med massespektroskopi ble brukt til henholdsvis transkripsjon- og proteinanalyser. Kombinasjonen av disse metodene har ikke tidligere blitt brukt i metabolske studier av *E. faecalis*, og vil derfor forhåpentligvis gi en dypere forståelse av overgangen mellom homolaktisk- og blandet syregjæring.

Våre studier viser at i fravær av *ldh* genet, som koder for laktatdehydrogenase, blir glukose brutt med til etanol, maursyre og acetoin. Denne overgangen fra homolaktisk til blandet syregjøring påvirker uttrykket av en rekke gener og proteiner involvert i energimetabolismen. Genene innehar viktige roller i det regulatoriske nettverket som kontrollerer energimetabolismen i *E. faecalis*, og inkluderer gener involvert i produksjon av acetoin og balansen mellom NAD^+/NADH. Videre studier ble også gjort for å undersøke blandet syregjæring i villtype *E. faecalis* i kjemostat ved likevektstilstand og glukose-begrenset vekst. Vekst ved tre forskjellige veksthastigheter viste av bakterien responderer forskjellig avhengig av veksthastighet. Ved den høyeste fortynningshastigheten ($D=0.4\ h^{-1}$) ble det meste av glukosen omdannet til melkesyre, mens en endring i retning av blandet syrefermentering ble observert ved den laveste fortynningshastigheten ($D=0.05\ h^{-1}$). Interessant nok så førte økt veksthastighet til økt transkripsjon av *ldh*-genet, men mengden Ldh-protein var tilnærmet uendret. Forskjellene i nedbrytning av glukose ved forskjellige veksthastigheter og ved forskjellig pH mellom *E. faecalis* og to andre melkesyrebakterier (*Streptococcus pyogenes* and *Lactococcus lactis*) ble også undersøkt. Det er her verdt å merke seg at inaktivering av *ldh* genene hadde liten innvirkning på veksthastigheten til de ulike bakteriene i kjemisk definert medium under mikroaerofile vekstforhold. Inaktiveringen av *ldh* påvirket også bakterienes evne til å utnytte andre substrater enn glukose som karbonkilde. I det siste arbeidet i avhandlingen ble det vist at *E. faecalis* i fravær av glukose er istand til å vokse på askorbinsyre.

Sett under ett har arbeidet som er presentert i denne avhandlingen, gjennom analyser av metabolitter, transkripsjonregulering og proteinuttrykk, gitt økt innsikt i reguleringen av og styrket vår kjennskap til veiene for nedbrytning av glukose.

LIST OF PAPERS

Paper I.

Ibrahim Mehmeti, Maria Jönsson, Ellen M. Faergestad, Geir Mathiesen, Ingolf F. Nes and Helge Holo. 2011. Transcriptome, proteome and metabolite analysis of a lactate dehydrogenase negative mutant of *Enterococcus faecalis* V583. *Applied and Environmental Microbiology*. 77:2406-2413.

Paper II.

Ibrahim Mehmeti, Ellen M. Faergestad, Martijn Bekker, Lars Snipen, Ingolf F. Nes and Helge Holo. Growth rate dependent control in *Enterococcus faecalis*: effects on the transcriptome, proteome and strong regulation of lactate dehydrogenase. Accepted with minor revisions, *Applied and Environmental Microbiology*.

Paper III.

Tomas Fiedler, Martijn Bekker, Maria Jönsson, **Ibrahim Mehmeti,** Anja Pritzschke, Nikolai Siemens, Ingolf F. Nes, Jeroen Hugenholtz and Bernd Kreikemeyer. 2011. Characterization of three lactic acid bacteria and their isogenic ldh deletion mutants shows optimization for YATP (cell mass produced per mole of ATP) at their physiological pHs. *Applied and Environmental Microbiology*. **77:**612-7.

Paper IV.

Ibrahim Mehmeti, Ingolf F. Nes and Helge Holo. *Enterococcus faecalis* grows on ascorbic acid. Submitted.

1. INTRODUCTION

1.1 Lactic acid bacteria

The term Lactic Acid Bacteria (LAB) comprises a group of bacteria that produce lactic acid as the major end-product of glucose fermentation (4, 21). LAB are gram positive anaerobic, non-sporulating and acid tolerant bacteria. LAB embraces four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus* (Lactic *Streptococci*) (4, 21, 32, 79). LAB in food fermentation goes probably back to the early time when start to preserve food and today LAB is actively used in food industries as a starter cultures to produce a great variety of fermented food products (103, 128, 137, 171).

Enterococcus faecalis, *Lactococcus lactis,* and *Streptococcus pyogenes* belong to the *Lactococcus* group (Table 1). *L. lactis* is mainly used as a start cultures in dairy technologies (113, 155). *E. faecalis* is considered a major LAB in the human intestinal microbiota (93, 181), a fecal contaminant in food and water (50, 139, 157, 175) and in recent years has also emerged as a hospital pathogen (51, 58, 92). *S. pyogenes* is a significant human pathogen (30, 85). LAB inhibit growth of many gram-positive pathogenic and food-spoilage bacteria by producing not only organic acids such as lactic acid but also antimicrobial agents as bacteriocins (36, 119, 121).

Table 1. Differentiation of *E. faecalis*, *L. lactis* and *S. pyogenes*.

Growth characteristics	*E. faecalis*	*L. lactis*	*S. pyogenes*
10^0C	+	+	-
45^0C	+	-	+
NaCl 6.5%	+	-	-
pH 4.4	+	±	-
pH 9.6 (in broth)	+	-	-

+ Growth; - No growth; ± varies among strains. Adapted from Carr et al (21).

1.1.1 The *Enterococcus*

The first description of *Enterococcus* group was made by Thiercelin in 1899 (176). Seven years later Andrewes and Horder (1906) isolated the *Enterococcus* from the human intestine with properties very similar to the strain described by Thiercelin (154). In 1933 Lancefield proposed the name *Streptococcus faecalis,* and in 1937 Sherman in his review article of the genus *Streptococcus* used the term "*Enterococcus*" to describe the group D streptococci (154). At that time, the genus *Streptococcus* included four species (*Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis* and *Streptococcus equines*). In 1984, the genus *Enterococcus* was again reintroduced based on the DNA hybridization of 16S rDNA sequencing (151).

Presently, the genus *Enterococcus* includes at least 40 species, *E. faecalis* and *E. faecium* being the two dominating ones, especially in food and fecal material (44, 59). An overview of phylogenetic tree of *Enterococcus* species is shown in Figure 1.

In general, enterococci are gram positive cocci, catalase and oxidase negative that occur single, in pairs or in short chain (67). They are facultative anaerobes with an optimum growth temperature of 35°C. The grow between 10° and 45°C and can survive at 60°C for 30 minutes (49, 112). The *Enterococcus* genus can tolerate up to 6.5% NaCl, and pH up to 9.6 (154).

The enterococci, like other lactic acid bacteria have the ability to ferment various carbohydrates to produce lactate, as well as a number of minor metabolites such as acetate, acetoin, formate, ethanol and CO_2 depending on the type and amount of carbohydrates and growth conditions (77). In sugar fermentation enterococci can utilize different pathways, resulting in homo- and mixed acid fermentation. Many members of the genus *Enterococcus* produce antimicrobial substances including bacteriocins (42, 74, 120) and enterococci are even used as probiotics (8, 38, 52).

The enterococci are widely distributed in the environment in foods such as milk, dairy product, meat, vegetables (48, 59, 60, 88, 106) and is also a part of the microflora of humans and animals (75, 81, 86).

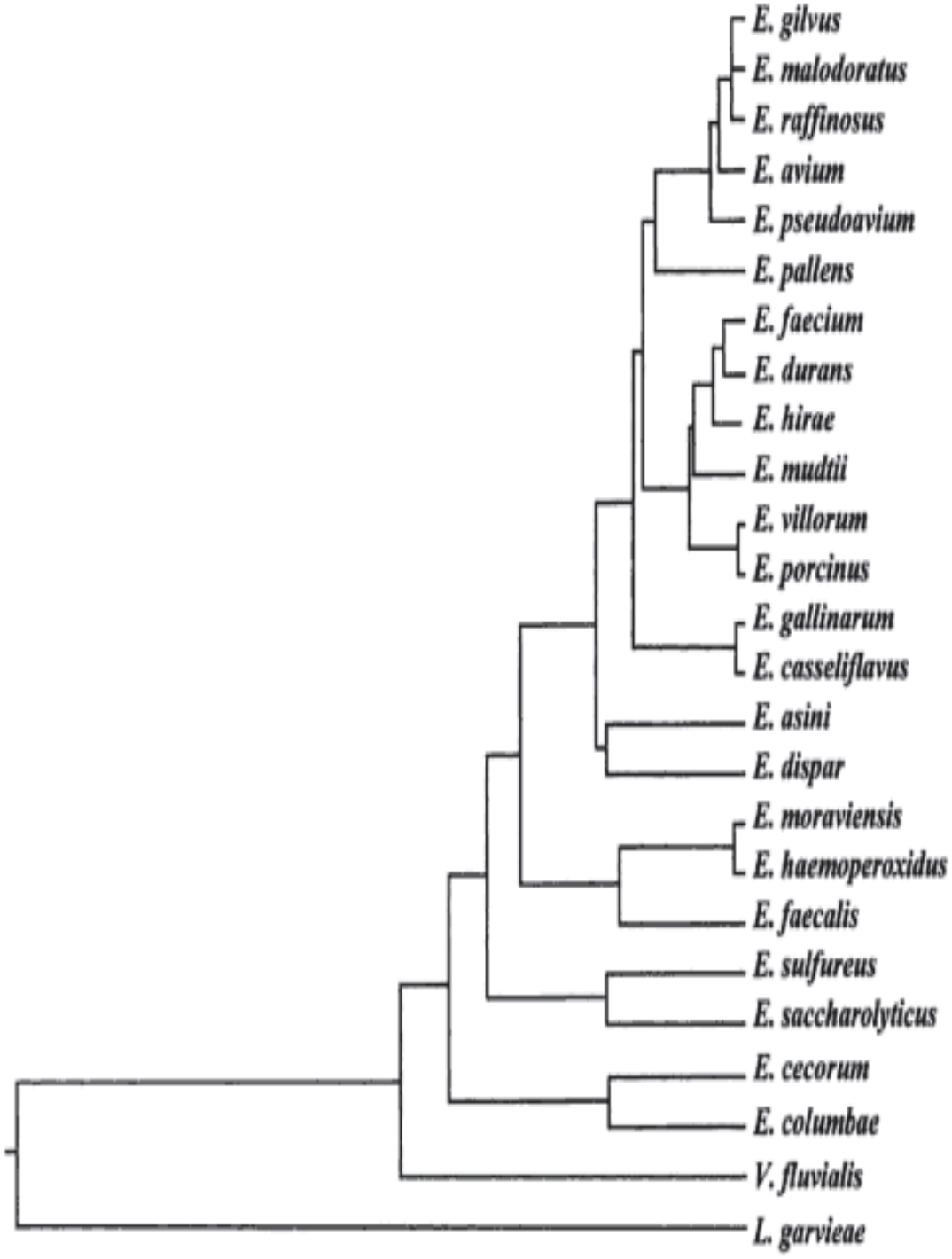

Figure 1. Phylogenetic relationship between *Enterococcus species* based on 16s rRNA sequence analysis. Adapted from Facklam et al (45).

1.1.1.1 *Enterococcus faecalis*

The *Enterococcus faecalis* was initially called *Streptococcus faecalis*. The name *faecalis* is used to indicate fecal origin of the originally identify one (190). Based on the origin, two species within this genus have been named *E. faecalis* and *E. faecium* that differ in sugar fermentation (151). It has been shown that *E. faecalis* is resistant to a number of antibiotics and they are most commonly found in clinical isolates and more frequent than antibiotic resistant *E. faecium* (190). However, nowadays the number antibiotic resistant enterococci have increased in favor of *E. faecium* (80, 115, 179). In the gastrointestinal tract the number of *E. faecalis* range from 10^5 to 10^7 CFU/g feces compared to *E. faecium,* which is lower and variety from 10^4 to 10^5 (23, 122). Both species are found in the intestine and faces of humans and animals (69, 114, 167, 186). Isolates have been used in food fermentation as a starter cultures (29, 51, 133, 180). Enterococci are more frequently found in artisan fermented food than in industrial fermented products (73).

In the present study *E. faecalis* V583 was used because this was the only genome sequenced isolate within this species at the start of this work (138). *E. faecalis* V583 has been isolated from a patient suffering from a persistent bloodstream infection and was the first strain reported in USA as a clinical isolate which was resistant to vancomycine (147, 148) and it is also resistant to number of other antibiotics. It was reported that the strain V583 genome contains 3337 predicted protein-encoding open reading frames (ORFs) including three plasmids (pTEF 1-3). Approximately 25% of the genes identified in V583 are defined as DNA mobile elements include genes that encode drug resistance factors, integrated phage regions and virulence factors. The circular representation genome atlas of *E. faecalis* V583 is shown in Figure 2. Presently, several more *E. faecalis* genomes have been sequenced (12, 16, 38, 130).

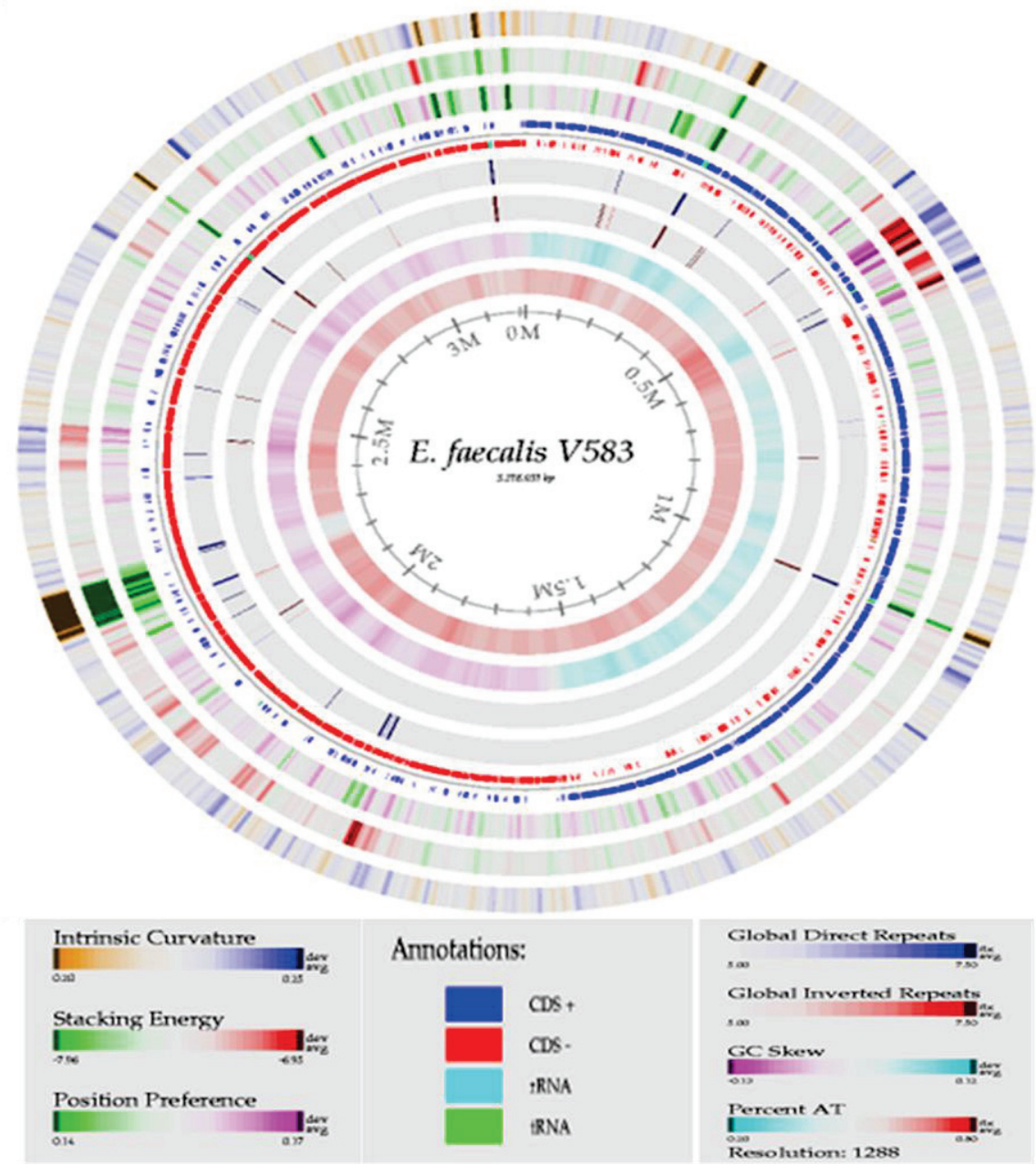

Figure 2. Circular representation of the *E. faecails* strain V583. Adapted from http://www.cbs.dtu.dk/services/GenomeAtlas-3.0

1.2 Energy metabolism in lactic acid bacteria and its regulation

The primary function of energy metabolism is to generate adenosine-triphosphate (ATP) needed for cell growth and cell maintenance (4, 28). During the process of the LAB fermentation the carbon sources are mainly transferred to lactate in addition to a number of other metabolites like acetate, acetoin, formate, ethanol, and CO_2 as a end products which is depending on type of LAB and available energy source (77, 96, 131). LAB have the ability to different growth conditions and to change their metabolism accordingly between homolactic and mixed acid fermentation (76, 107, 131, 163).

1.2.1 Glycolysis

The glycolysis is the central pathway for transforming the glucose into two pyruvate molecules. This process can take place both during aerobic and anaerobic growth. The process of glycolysis is not only taking place in the presence of glucose, but also with numerous sugars such as mannose, galactose, fructose, maltose and lactose (76, 131, 178).

In glycolysis there are two alternative metabolic pathways, which are homolactic fermentation- Embeden-Meyerhof-Parnas pathway (glycolysis) and heterolactic fermentation- the phosphoketolase pathway (Figure 3). Figure 3 shows that Embeden-Meyerhof-Parnas pathway is made up of ten biochemical reactions where five are involved in energy investment and the other five are involved in energy generation. The first phase (energy investment) starts with glucose containing six molecule of carbon which is transferred into two molecules of the pyruvate, more details of this phase is describe below. First, the glucose molecule is phosphorylated immediately by the phosphotransferase system (PTS) which is a transporter and by a glycokinase that produces glucose 6-phosphate with consumption of one molecule of ATP. The phosphoglucoisomerase converts glucose 6-phosphate to fructose 6-phosphatase (F-6-P). F-6-P is further catalyzed to fructose-1,6-diphosphate (F-1,6-P) by phosphofructokinase and this reaction consumes also one molecule of ATP. Fructose-1,6-diphosphate aldolase split F-1,6-P into two triose sugars glyceraldehyde-3phosphate (GAP) and dihydroxyacetone phosphate. In generation phase GAP is converted by a

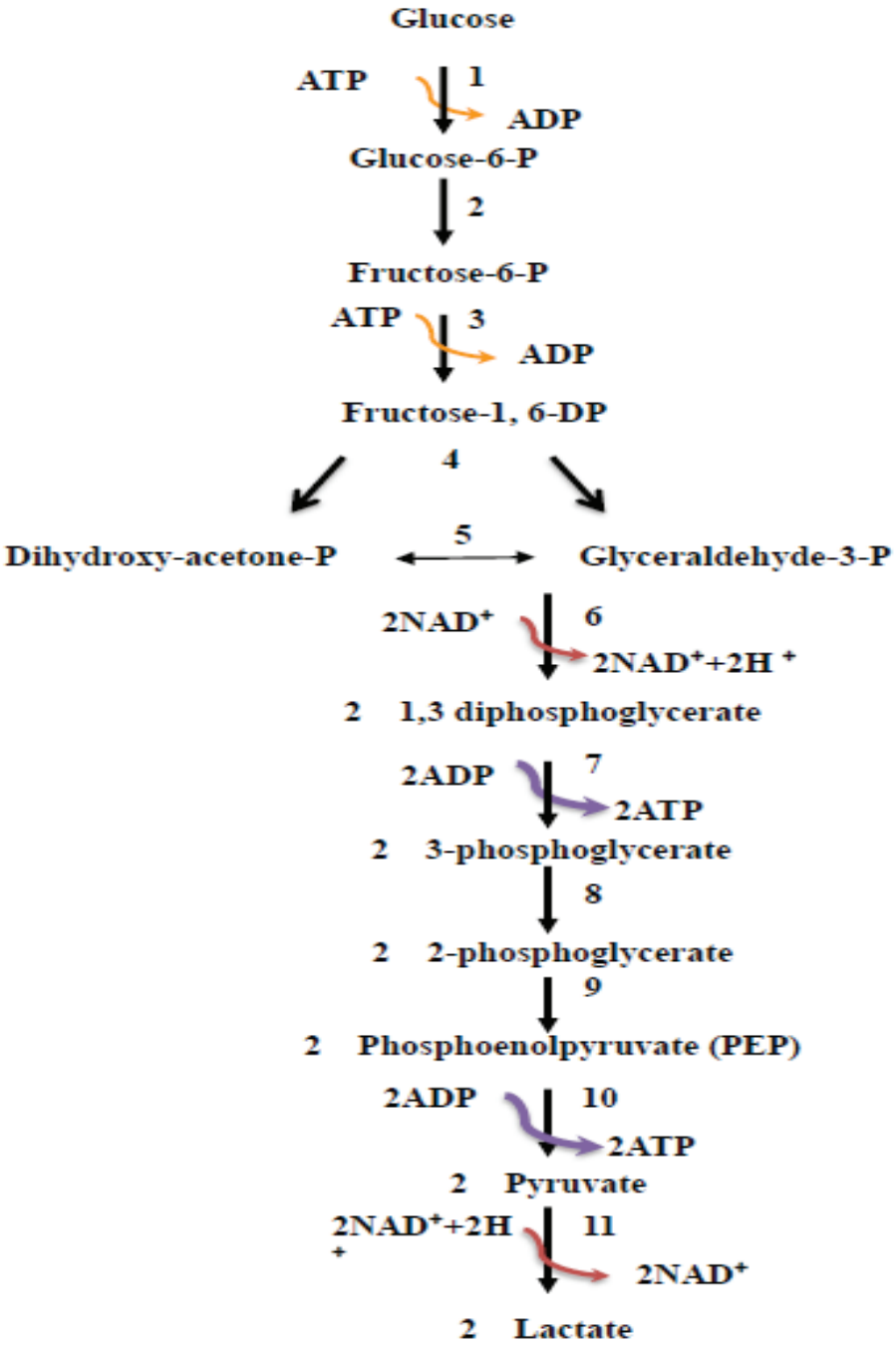

Figure 3. Homolactic fermentation of glucose –glycolysis (Embeden-Meyerhof-Parnas pathways). The enzymes: 1.Glycokinase or PTS; 2. Phosphoglucoisomerase; 3. Phosphofructokinase; 4. Fructose-1,6- biphosphate aldolase; 5. Triosephosphate isomerase; 6. Glyceraldehyde-3-phosphate dehydrogenase; 7.Phosphoglycerate kinase; 8. Phosphoglycerate mutase; 9. Enolase; 10. Pyruvate kinase; and 11. Lactate dehydrogenase. Adapted from Axelsoon L. (4).

glyceraldehyde-3-phosphate dehydrogenase to 1,3-diphosphoglycerate which is catalyzed by phosphoglycerate kinases to one molecule ATP (from ADP) and 3-phosphoglycerate followed by transformation into 2-phosphoglycerate by enzyme phosphoglycerate mutase. Enolase catalyzes 2-phosphoglycerate to phosphoenylpyruvate. Phosphoenylpyruvate is catalyzed to pyruvate by pyruvate kinase to yield one ATP. This glycolytic reaction produces two molecules each of ATP, NADH and pyruvate. In a homolactic fermentation the pyruvates will be converted to the lactate by lactate dehydrogenase by regeneration of nicotinamide adenine dinucleotide (NAD^+) to its reduced form (NADH) to balance the redox potential which it will be describe below in more details (4, 28).

The heterolactic fermentation utilizes the phosphoketolase pathway (Figure 4). Instead of the metabolizing most of the carbon sources into lactate (as seen in a homofermentative LAB fermentation), other end products will also be produced. This pathway begins with converting the glucose to glucose 6-phosphate (G-6P) by the enzyme glycokinase. G-6P then is transfer to 6-phospho-gluconate (6-P-G) and NADH is generated. Ribulose-5 phosphate (R-5-P) is produced by decarboxylation of the 6-P-G and further into the xylose-5 phosphate (X-5-P) by ribulose-5-phosphate 3-isomerase followed by the splitting into two GAP and acetylphosphate by phosphoketolase. The end products following this pathway are 1 mole each of lactate, ethanol, CO_2 and ATP per one mole of glucose (4, 28).

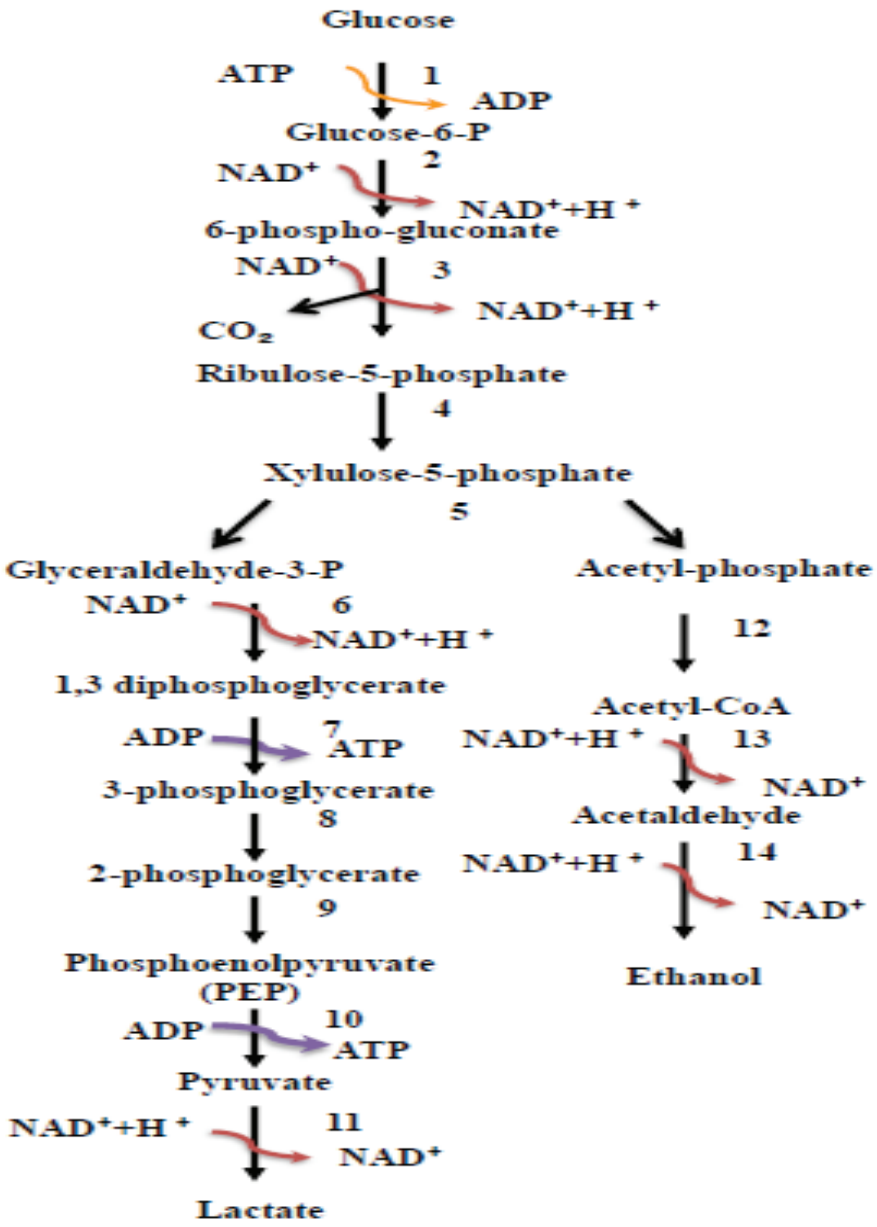

Figure 4. Heterolactic fermentation of glucose-6-phosphogluconate/phosphoketolase pathways. The enzymes: 1. Glycokinase or PTS; 2. Glucose 6-phosphate dehydrogenase; 3. Phosphogluconate dehydrogenase; 4. Ribulose-5-phosphate 3-epimerase; 5. Phosphoketolase; 6. Glyceraldehyde-3-phosphate dehydrogenase; 7. Phosphoglycerate kinase; 8. Phosphoglycerate mutase; 9. Enolase; 10. Pyruvate kinase; 11. Lactate dehydrogenase; 12. Phosphate acetyltransferases; 13. Acetaldehyde dehydrogenase; and 14. Alcohol dehydrogenase. Adapted from Axelsson L. (4).

1.2.2 Pyruvate metabolism

Pyruvate metabolism follows glycolysis process and completes the carbohydrates metabolism (Figure 5). In homolactic acid bacteria most of the carbon sources from glycolysis is transferred into lactate by the enzyme lactate dehydrogenase (LDH) (Figure 5). The genome of *E. faecalis* V583 contains two *ldh* genes (*ldh*-1; EF0255 and *ldh*-2; EF0641) but only one (*ldh*-1) has been reported to play a role in lactate production (12, 77, 138). During the transformation of pyruvate to lactate, LDH regenerates NAD^+ from NADH. Under certain growth conditions such as stress, it has been shown that LAB can shift the metabolism from homolactic to mixed acid fermentation (Figure 5) (56, 107).

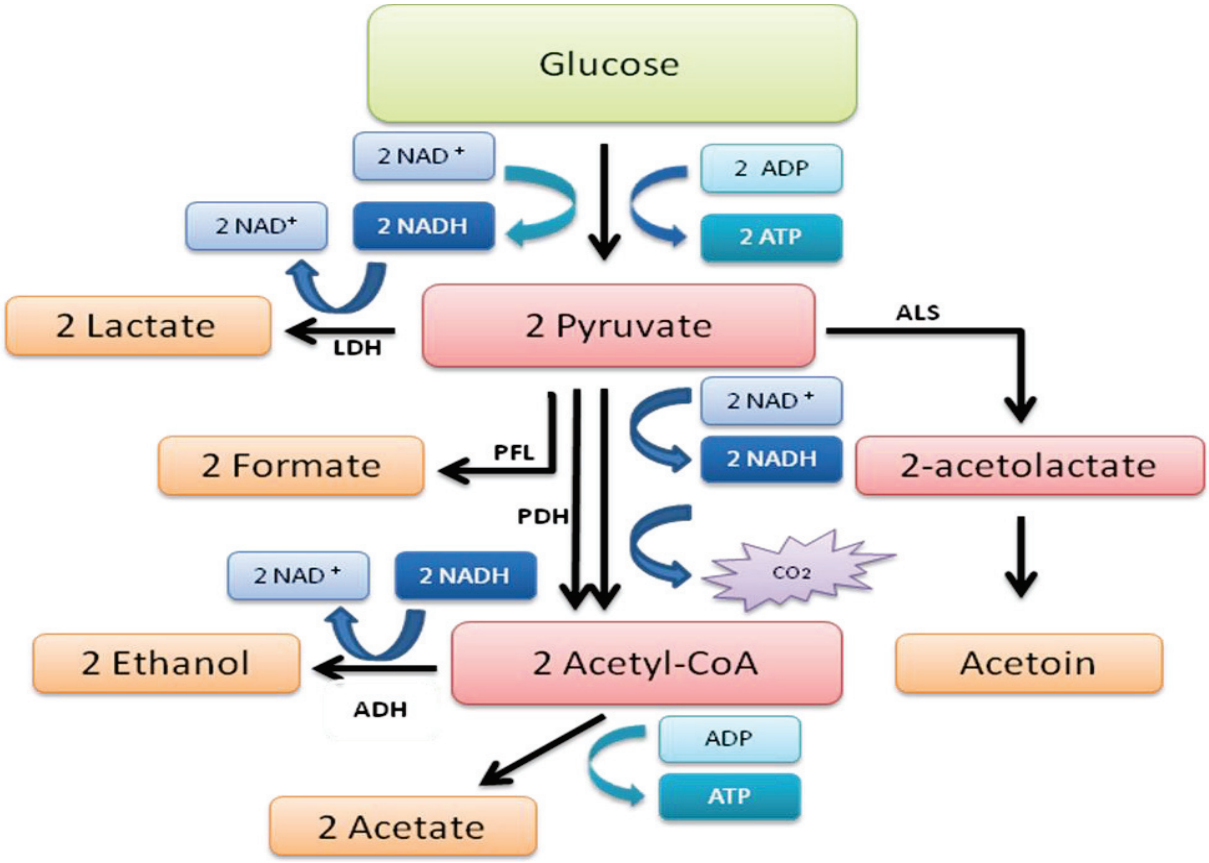

Figure 5. Pyruvate pathway in LAB. LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PFL: pyruvate formate lyase; ALS: acetolactate synthethase; ADH: alcohol dehydrogenase; ADP: adenosine-diphosphate; ATP: adenosine-triphosphate; NAD^+: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide. Adapted from Cocaign-Bousquet et al (28).

In the mixed acid fermentation the bacteria produce metabolites such as acetate, acetoin, ethanol, formate and CO_2 from pyruvate (77). The metabolite composition of the mixed acid fermentation varies and depends on several factors such us oxygen tension, variation in pH or availability of nutrients (131, 162).

Pyruvate dehydrogenase (PDH) and pyruvate formate lyase (PFL) are the two main enzymes that are involved in converting the pyruvate to acetyl-coenzyme A (acetyl-CoA). Aetyl-CoA is converted to ethanol providing an alternative pathway for regeneration of NAD^+ from NADH to re-establish redox balance (28, 33, 165) (Figure 5). Furthermore, expression of the *adh*E gene encoding the alcohol dehydrogenase enzyme, is known to be reduced by presence of oxygen (3). Such a shift involves a modification of pyruvate metabolism with a decrease activity of LDH and increase in PFL (anaerobic conditions) or PDH (aerobic conditions) activity. PFL plays also a role in formate production and is known to be extremely oxygen-sensitive (1, 28, 170). In *E. faecalis*, no enzymatic PDH activity has been disclosed *in vitro* so far (164), however, the corresponding transcription of *pdh* has been shown to be regulated (162). Limitation of sugar may induce shift from homo- to mixed acid fermentation. As mentioned earlier such shifts may involve the activities of LDH, PFL and/or PDH. It has been shown that, inhibition of the PFL by glyceraldehydes-3-phosphate (GAP) and dihydroxiacetone-phosphate (DHAP) is associated with activation of LDH by fructose 1,6-biphosphate (FBP). In addition, all metabolite are shown to be present at higher concentrations during homolactic metabolism than mixed acid fermentation and this has been suggested to control the shift in fermentation (56, 177, 178).

It has been shown that growth at low dilution rate, *L. lactis* changes from homolactic to mixed acid fermentation by activating the PDH and leads to increased production of ethanol and acetate (123).

1.2.3 Amino acids metabolism

Amino acids play a crucial role in metabolic processes with special reference to protein synthesis. Some of the amino acids are synthesized by bacteria and some not. Based on that amino acids may separated into essential and non essential. Amino acids enter into the

metabolic processes via several processes from where they generate energy (ATP, NAD, FAD-flavin adenine dinucleotide and CoA) (134, 135, 185).

Lactic acid bacteria have been reported to metabolize the amino acids (90, 94, 172). For most of the amino acids we do not know their catabolic processes in detail. The best study metabolic processes of the amino acids are known in *Escherichia coli* and *Bacillus subtilis* (2, 174). It has been shown that amino acids stimulate growth rate significantly in *E. faecalis* (89). It has been reported that serine serve as energy source in *L. lactis* in the absence of glucose (40, 124). The amino acid arginine, aspartate, alanine and histidine are known to generate ATP (25, 188).

1.2.4 Carbohydrate uptake and its regulation

1.2.4.1 Sugar uptake and the phosphotransferase systems (PTSs)

Transport of molecules into the cell is the first step in many metabolic processes. In many gram positive and negative bacteria the phosphoenlypyruvate (PEP): carbohydrate specific PTS is the common mechanisms for sugar uptake. The system transport the carbohydrates across the cytoplasmatic membrane with the simultaneous phosphorylation of the sugars (Figure 6) like mannose, fructose and cellobiose, and metabolise them (35, 141). A PTSs consist of several entities. The cytoplasmic components are: EI (enzyme I) and HPr (histidine-containing phosphocarrier protein) and carbohydrate specific enzyme EII. The EII contain two cytoplasmic domains (IIA and IIB) and additional membrane protein IIC which can be also bound with IID. In the mannose PTS family both membrane protein (IIC and IID) are active but for other families only IIC is sufficient (141). The mechanism of carbon uptake starts by the EI enzyme which is phosphorylated by PEP into the P~EI. P~EI is transferred to the His-15 residue in HPr (P~His~HPr) followed by phosphorylation of the EI enzyme by PEP in process of the activation (82, 140). IIB~P transfers the phosphoryl group to the sugar that is translocated via the substrate-specific IIC protein/domain. IIA, IIB and IIC can be separate proteins domains in a single polypeptide or linked as pairs in any possible combination (141, 149). The phosphorylated carbohydrates feed into glycolysis at the sugar-6 phosphatease level (Figure 6) (35, 82) .

Carbohydrate such us glucose will be phosphorylated as it passes through the cytoplasmic membrane, forming glucose-6-phosphate that will not leak out the cell, providing a one-way concentration gradient of glucose. *E. faecalis* grows in different sugars such as glucose, fructose, galactose, sacarose, manose and lactose but glucose is most efficient energy source for its growth (143, 150). *E. faecalis* contain several PTS system and the major PTS uptake system for glucose in the bacteria has been shown to be a mannose PTS (35, 141).

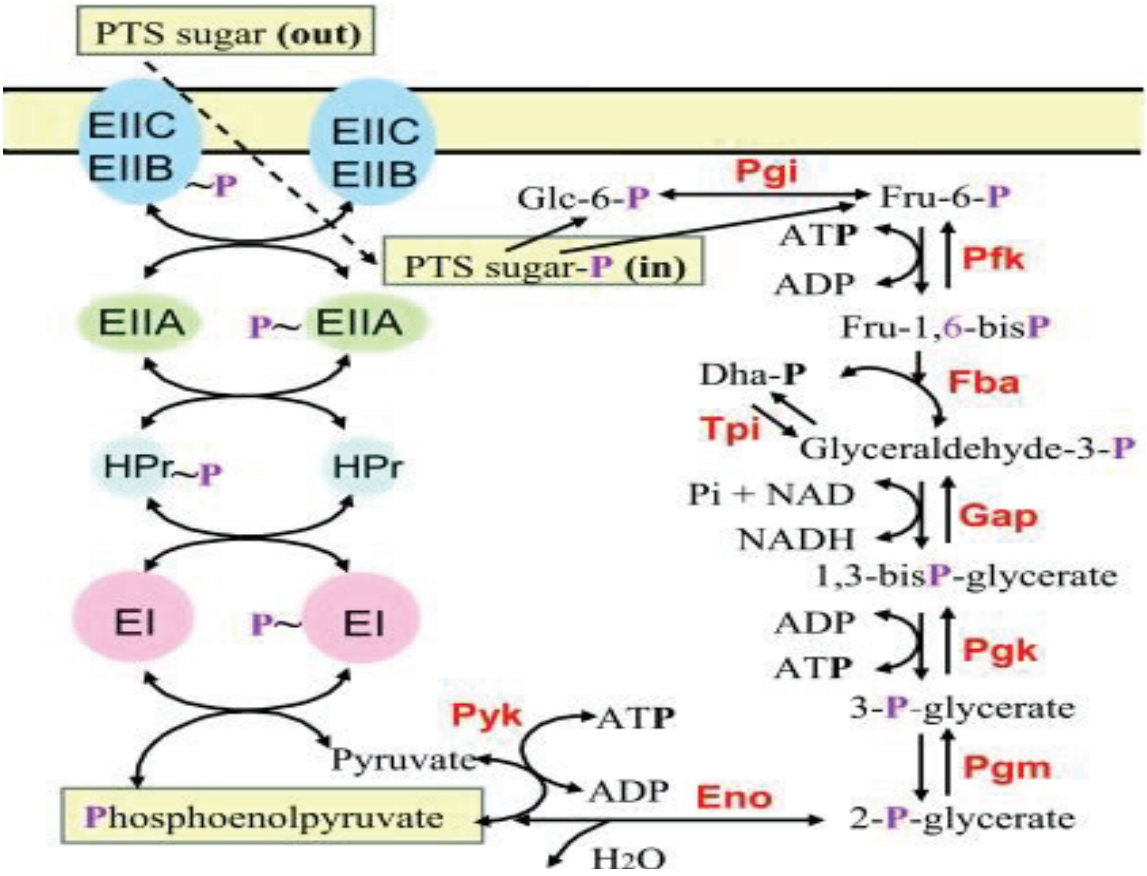

Figure 6. Schematic representation of the PTS system and their coupling to glycolysis. Abbreviations for enzymes (in boldface type) are as follows: Pgi-phosphoglucose isomerase; Pfk-phosphofructokinase; Fba-fructose-1,6-bisphosphate aldolase; Tpi-triose-phosphate isomerase; Gap-glyceraldehyde-3-phosphate dehydrogenase; Pgk-phosphoglycerate kinase; Pgm-phosphoglycerate mutase; Eno-enolase; Pyk-pyruvate kinase. Adapted from Deutscher et al (35).

1.2.4.2 Carbon catabolite control

Carbon Catabolite Control (CCC) is one of the global regulatory mechanisms by which a bacterial cell coordinates carbon and energy uptake and metabolism to maximize its efficiency and regulates other metabolic processes (54, 64, 83). The major goal of CCC system is to ensure that only enzymes necessary for utilizing preferred substrate such as glucose are synthesized as long as the preferred sugar is present (54). In Firmicutes bacteria such as *B. subtilis*, CCC can be divided mainly into CcpA dependent and CcpA independent (54).

The CcpA-dependent CCC system comprises the CcpA protein, HPr (Histidine containing phosphocarrier protein) which is a transcriptional regulatory component with both repressor and activator roles and a conserved DNA target sequence for the CcpA protein and HPr complex, called the catabolite-responsive element (*cre*) (18, 169). The *cre* target sequence proposed for *E. faecalis* is (WTGWAARCGYWWWCW; W is A or T; R is G or A) (127). Activation of HPr by HPr kinase/phosphatase is conserved to serine 46 residue (HPr-Ser-46) (152, 153). The P-Ser-HPr is regulated by the bifunctional enzyme HPr kinase/phosphatase which phosphorylates HPr in response to increased throughput of the two glycolytic metabolites fructose-1,6-bisphosphate (FBP) and glucose-6-phosphate (G6P), what enhance binding of CcpA to CRE. Depending the location of the CRE binding site CcpA-HPr-Ser-46 complex may have either an activation or a repression role on the target genes (54). This termed as carbon catabolite repression (CCR) and carbon catabolite activation (CCA) (18, 169). The CcpA-dependent regulation has been shown to take place in many bacteria such as *B. subtilis*, *E. faecalis*, *Listeria monocytogenes* and *Lactobacillus casei* (101, 127, 184). Studies involving *ccp*A null mutants has revealed the presence of CcpA-independent CCC system in many Firmicutes (54).

There are generally two types of CcpA independent CCC in low gram positive bacteria (54). The first group involves catabolite regulatory protein such as catabolite protein CcpB, CcpC, CcpN as well as the glycolytic genes regulator (CggR). The second group CcpA-indipendent CCC involves the HPr protein of the PTS system (54). The HPr is phosphorylated at His-15 by the PEP-dependent protein kinase which takes place when cell grow in non-preferred or

absence of carbon sources (31, 156). The CcpA-independent regulation has been shown in many bacteria such as *B. subtilis*, *E. faecalis, Streptococcus mutans,* (54, 91, 189, 193).

1.3 Fermentation processes, transcriptomics and proteomics analysis

There are numerous technologies available to study bacterial metabolites, transcriptome and proteome. In this work high-performance liquid chromatography (HPLC) and gas chromatography (GC) were used for metabolite analysis. Transcriptome studies were performed by DNA microarray technology and real-time PCR. The proteome studies were performed 2-dimmensional gel electrophoresis (2DE) combine with mass-spectrometry (MS) analysis. An overview of the methodology used is illustrated in Figure 7.

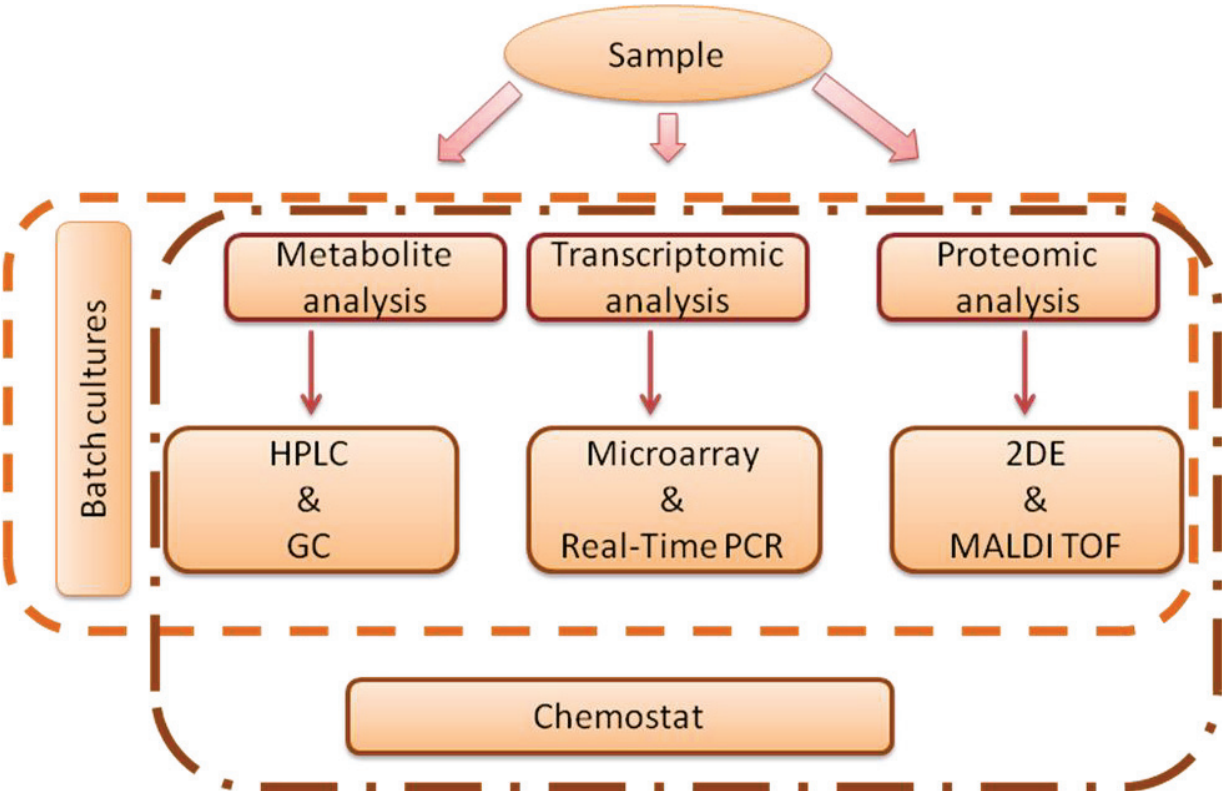

Figure 7. Schematic presentation of methods used in the metabolic analysis of lactic acid bacteria in this work.

1.3.1 Fermentation processes in chemostat

Most of the traditionally designed microbial experiments have been performed by growing the bacteria in "batch" culture. The batch culture approach has limitations when it comes to control of the growth parameters. In 1950 the chemostat was for the first time used to study metabolic processes of microorganisms and has since become an important research tool for microbiologist, biochemists and biotechnologists (125). The chemostat seems to be an ideal tool to study metabolism of microorganisms in a controlled environment (15, 117). The design of a chemostat is shown below in Figure 8. In brief, chemostat consists a bioreactor from where medium continuously flow into the fermentor which at same time is controlled by other growth parameters such as nutrition, temperature, pH, amount of oxygen or nitrogen (70, 72, 78).

The advantages of this technology is to be able to study bacterial metabolism under defined growth (11). A number of chemostat based experiments have been performed to unravel metabolic processes among LAB (20, 99, 158).

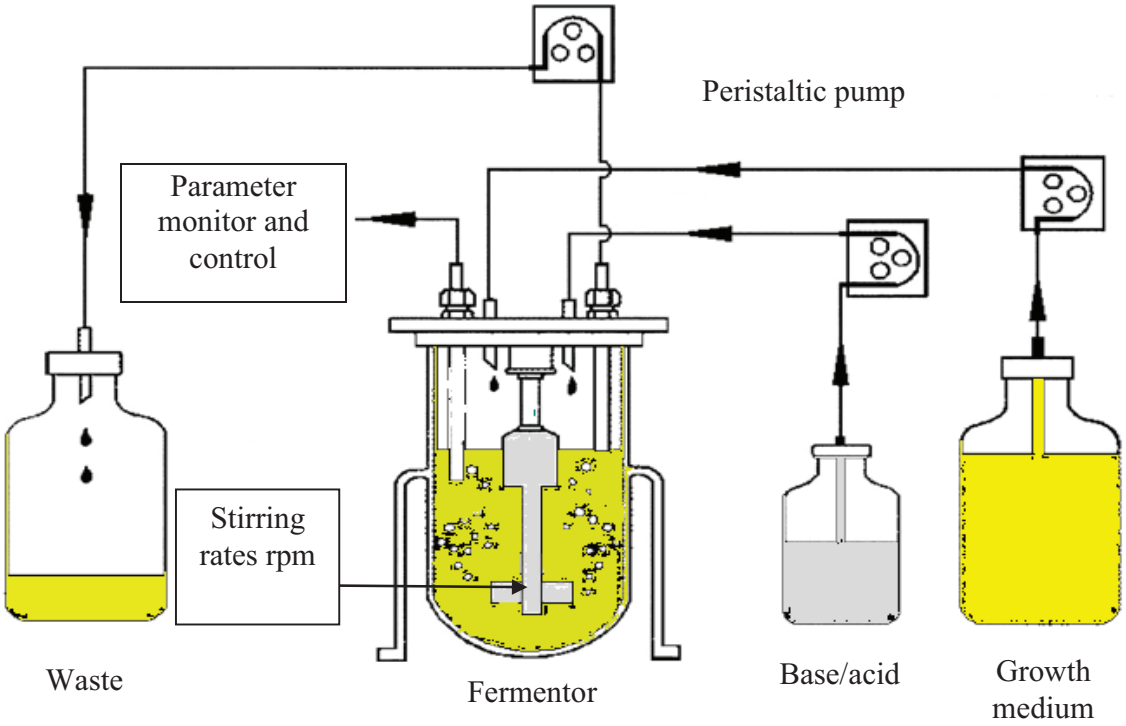

Figure 8. Schematic drawing of a chemostat. Adapted from Malin and Paoletti (102).

1.3.2 Transcriptional analysis

1.3.2.1 Microarray technology and its application

The event of genome sequencing, the complete set of genes of an organism become available and then development of more sophisticated technologies of transcriptome analysis was needed. Instead of analyze the expression of one or a few genes at a time, the DNA microarray technology was developed for global genome gene expression analysis in the 90-ties (111, 191). A DNA microarray is a miniaturized two dimensional matrix of DNA probes spotted on solid phase such as glass which can be used in DNA/RNA hybridization experiments. This technology allows the transcription analysis of thousands of genes at the same time (111).

In DNA microarray experiments analysis can be performed by using DNA or reverse-transcribed RNA (cDNA). DNA microarray is design on glass slides or membrane which contains small gen-probe sequences (68). This technology is used in comparative genome hybridization (CGH) analyses (5, 41) to study the presence or absence of gene between isolates of same species (34, 166, 167, 194). Global transcription profiles of organisms are used to get a "snap-shot" of which genes are expressed in an organism in a given situation under defined conditions. In transcriptional study cDNA is labeled with a fluorophores agent usually with Cy3 and Cy5 dyes (e.g. wild-type versus mutant) (95). The labeled cDNA is hybridzed to the DNA microarray chip, washed and then scanned at suitable wavelengths. Each spot represents a single gene. The ratio of the fluorescence shows if genes are differentially expressed or not.

Several methods and software are used to analyze and interpret the microarray data. In these work the R software (www.r-project.org) was used. The array data needs to be pre-processed (159-161) followed by test for different intensity and calculation of gene expression using linear mixed model as described by Smyth (159). A mixed-model approach is chosen to adequately describe "between-array variation" and to utilize probe-replicates (3 replicates of each probe in each array). Sometimes reference samples are used in microarray.

DNA microarray has been shown to be a powerful tool to study gene expression in bacteria (110, 145). In addition, microarray technology has found application in gene discovery and mapping (22, 24, 26), gene regulation (17, 87, 144, 195) and for medicine purposes (14, 100).

1.3.2.2 Quantitative real-time reverse transcriptase PCR (qRT-PCR)

The limitation of the conventional PCR for quantifying nucleic acids was surmounted by the development of the real-time (RT-PCR) PCR by Higuchi et al (1992). The process includes two steps. In quantitative real-time reverse transcriptase PCR, mRNA is first transcribed into its cDNA (complementary DNA) by reverse transcriptase followed by the second step which amplifies the cDNA by traditional PCR or RT-PCR. The technique is based on continuously monitoring the emission of fluorescent signals, caused by the binding of fluorescence reporters to the amplifications products. The results are presented by threshold cycles of C_T values. The C_T-values shows the number of cycles required to reach a threshold value and based on a standard curve this parameter can be used to estimate the initial amount of target DNA (cDNA). Higher C_T represents the lower expression profiles (low concentration of cDNA). RT-PCR calculation needs C_T-value of a reference gene which in most of studies is 16S or 23S RNA (182, 183). Also qRT-PCR is used to validate expression profiles of microarray data (9, 127), as seen in the present work. The qRT-PCR assay is also applied in rapid quantification of bacteria, thereby providing an estimation of the starting concentration of LAB in a studied sample (55, 118).

1.3.3 Proteomic analysis

The proteome of the cell is defined as all the proteins present at a particular time. Two techniques are used to analyze the proteome. The first is separation of proteins by 2-DE developed by O'Farell forty years ago (126). The protein spots on the 2-D gel are then analyzed and identified by using matrix-assisted laser desorption/ionization mass-spectrometry (MALDI-TOF) or electrospray ionization (ESI-MS) (46, 71, 173) mass spectrometry (MS). The second technique is based on the liquid chromatography (LC) in one or more dimensional, joined with mass spectrometry. Till now the 2-DE technique is mostly used in proteome analysis.

1.3.3.1 Two-dimensional gel electrophoresis

Before applying samples on 2-DE technology samples needs to be completely solubilized (142). Two-dimensional gel electrophoresis contains two separation steps. In the first step (one dimension) proteins are separated by their electrical charge by use of iso-electric focusing (IEF) and in the second step (second dimension) by use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) when the proteins are separated by their size. Depends on the experimental design and protein composition, one can use strips that have different p*I* range (3-10; 4-7; 4.1-5.1) (63). After isoelectric separationstep, the IEF strips needs to be pH equilibrated, added disulphide reducing agent (dithiothreitol) followed by addition of iodoacetamid which prevent the re-oxidation (61). This step carried out prior to the polymerization process of the gel SDS polyacrylamid gel. After SDS-PAGE, the gel needs to be stained to visualize the proteins.

1.3.3.2 Image analysis and protein identification

Protein amount has to be standardized and results have to be statistically analyzed. Different techniques exist for protein staining such as Coomassie brilliant blue staining (37, 136), silver staining (10, 108) and a number of fluorescence staining methods that include SYPRO Ruby, Flamingo, Krypton and Lava purple (7, 47). Each staining protocol has advantages and disadvantages such as sensitivity, specificity and cost (63).

The samples have to be aligned and checked the intensity of each spot by the software. Several commercial software packages are available to align (27, 105) and data analysis software such as principal component analysis (PCA) and partial least squares (PLS), as well as classical unvaried analysis of variance (ANOVA) are used.

Quantification of the proteins is an important and challenging step in proteome analysis. The important points in quantification processes are to know the amount of the proteins. Traditionally we have relied on several methods such as Bradford assay (13), Lowry assay (98), UV absorbance and other modified protocols as well (187, 192).

In order to identify the protein spots, the protein spot on the gel are picked and digested with e.g., trypsin. The digested sample is analyzed by a mass spectrometer, which separates the peptides into sharp and defined peaks. The resulting data is analyzed by a database of all ORFs digested also silico with the same enzyme. In this work MALDI-TOF has been used for protein identification (71).

The 2DE technology has found application such as in medicine (43, 65, 132), in food industry (6, 104) and also in microbiology (19, 39, 62, 146).

2. OBJECTIVE OF THE THESIS

The overall objective of this thesis was to gain more knowledge on the central energy metabolism of *Enterococcus faecalis* and its regulation under different growth conditions.

This work included the following tasks:

- To investigate the effect of lactate dehydrogenase negative (LDH1.2) mutant of *Enterococcus faecalis* V583 at the transcription, protein and metabolite level.

- To study how growth rate affects transcriptome, proteome and metabolome profiles of *Enterococcus faecalis*.

- To identify metabolic/growth differences between the lactic acid bacteria (*Enterococcus faecalis*, *Lactococcus lactis*, *Streptococcus pyogenes*) at varying growth rates and pH of their wild-type and LDH mutant strains.

- To study growth of *Enterococcus faecalis* in different media in the absence of glucose and to identify the impact on carbohydrate metabolism.

3. MAIN RESULTS AND DISCUSSION

Lactic acid bacteria are widely used in food fermentation and as probiotic bacteria (21, 51, 155). Enterococci are the most abundant LAB in the GI tract but they are also associated to fermented food. However, they are also involved in nosocomial infections as well as becoming carriers of antibiotic resistance genes (66, 155). *E. faecalis* ferments sugars either in homo-, or mixed acid fermentation. In homolactic bacteria glucose is converted to the lactate, while in mixed acid fermentation other pathways producing metabolites such as acetate, acetoin, ethanol, formate and CO_2 are activated (28, 77).

The aim of this thesis was to improve our understanding of how central energy metabolism is regulated in *E. faecalis.* The papers in this thesis are identified with Roman numbers (Papers I-Paper IV).

Paper I is a continuation of previous work (77) in which lactate dehydrogenase (*ldh*-1.2) negative mutants were constructed and studied with respect to metabolite production when glucose was used as energy source. Paper I investigated the gene transcription of the *ldh* mutant, protein expression and a detailed analysis metabolic end products when glucose was utilized. In paper II the growth of *E. faecalis* in chemostat at different growth rate under varying glucose limiting concentration was analyzed. Both the wild-type and the *ldh*-1.2 negative *E. faecalis* V583 mutants were investigated and compared. In paper III both the *ldh*-1.2 negative mutant (Paper I) and wild-type (Paper I and II) were analyzed and compared with wild-type and *ldh* mutants of two other LAB genera (*S. pyogenes* and *L. lactis*) at different growth rates and pHs during batch and chemostat growth (Paper III). In paper I, II and III glucose was used as a major energy sources. In Paper IV growth studies were performed in a defined medium in the absence of glucose but in the presence of ascorbic acid.

In LAB most of the glucose is converted to lactate by ATP production (11, 77). In this process the enzyme lactate dehydrogenase (LDH) catalyses the reaction from pyruvate to lactate in a reversible inter-conversion manner (11, 77). To get a better understanding of the role of LDH, a *ldh* knockout mutant was constructed to investigate its consequence on glucose metabolism. It was known that *E. faecalis* V583 contain two lactate genes (EF0255 (*ldh-1*) and EF0641 (*ldh-2*)) (12, 138) with high similarity to *ldh*A and *ldh*B of *L. lactis*, respectively (11). In

Paper I, the lactate dehydrogenase deletion mutant (*ldh*-1, 2) previously constructed (77), was used to study the effects of this gene at the transcriptome, metabolome and proteome level (Paper I). As expected the *ldh*-1.2 deletion mutant showed different properties compared to the wild-type *E. faecalis*. In the wild-type most of the glucose was converted to lactate in addition to some minor amount of other metabolites while in the LDH-mutant the glucose was used to produce ethanol, formate, and acetoin (Table 2). Our results showed that *ldh*-1.2 mutant in batch cultures produce acetoin in stationary phase and this could be to balance carbon. The production of acetoin is in agreement with a previous study that showed more less similar amount of acetoin production in overnight growth culture (77).

Seeing these differences in metabolite products it was of great interest to know more about expression and regulation of genes and proteins involved. The microarray and 2DE gel/protein MS analysis showed LDH deficiency had profound transcriptional effects of 88 genes and on the amount of 45 proteins (Paper I). Most of the regulated genes found seemed to have a role in the energy metabolism and transport. Comparing the transcriptome and proteome data suggests that, protein expression is regulated beyond the level of transcription.

The rex boxes gene regulatory palindromic DNA sequences (TGTGANNNNNNTCACA) that binds regulatory proteins (Rex protein) which known to be affected by NADH/NAD in their differentially binding to the Rex operators (53). In the present work the differentially regulated genes were examined for the presence of r*ex* boxes based on sequence homology to the established rex boxes of *Staphylococcus aureus* (53). We found 22 putative rex boxes regulated genes/operons within the differently expressed gene. We reported that genes are transcriptionally regulated by the NADH/NAD ratio and this ratio plays an important role in the regulatory network controlling energy metabolism. The data suggest that Rex contributes not only to aerobic growth conditions as published previously (129) but is also involved in gene regulation at anaerobic growth (Paper I).

The transition between homolactic and mixed acid fermentation was also investigated in Paper II and Paper III. In paper II three different dilutions rates were studied ($D=0.05h^{-1}$, $D=0.15h^{-1}$ and $D=0.4h^{-1}$) by metabolite, transcriptomic and proteomic approaches under glucose limitation and steady state growth condition in the absence of oxygen. In paper III the metabolic profile of *E. faecalis* was compared to *S. pyogenes* (a human pathogene) and *L.*

lactis (a dairy starter organism) at two different dilution rates and pHs (Paper II and Paper III) both in wild-type and their *ldh* knockout isogenic strains. The metabolites data from papers (Paper I-IV) are summarized in Table 2. The results show that the fermentation pattern changed with growth rate, from homolactic at high growth rate to mixed acid fermentation in low growth rate (Table 2). At low growth rate metabolic end products are taken over by formate, acetate and ethanol compared to homolactic at high growth rate where lactate is the main end-product. In general, the difference between homolactic and mixed acid fermentation is ATP generation. Mixed acid fermentation generates one more ATP than homolactic fermentation does (131). This was also shown in our experiments. By increasing a growth rate the lactate concentration increased while the ATP level decreased (107).

In this work three members of LAB have been investigated (Paper III). This includes the human pathogen *S. pyogenes* (30), the commensal *E. faecalis* (92) and *L. lactis* which is used in the dairy industry (155). The three LAB are usually found in different environments and one may expect that they behave differently at the same growth conditions (see below). In Paper III the experiments were performed in a chemostat in order to study the shift from homolactic to mixed acid fermentation both three LAB under steady state. This help as to get better understanding differences in energy metabolisms between the three LABs under different growth conditions. Growth in batch cultures were in two different media: i) Chemical Defined Medium CDM-LAB and ii) Todd-Hewitt broth supplemented with 0.5% (wt/vol) yeast extract- THY medium. The results presented in Paper III showed that, the wild-type *E. faecalis* and its *ldh*-1.2 negative mutant reach higher cell density than other two LAB. By comparing growth between the two media all three bacteria reached the highest grow yield in THY medium. None of the three *ldh* deletion strains showed a significant difference with respect to growth rate compared to that of the wild-type, except *ldh*-deletion mutant of *E. faecalis* when grown at pH 7.5. In this condition the mutant reach higher growth rate than wild-type. While from previous study showed another stress factor such as oxygen has a positive effect on the growth of *L. lactis* (11).

Growth of *E. faecalis* in chemostat tended to increase mixed acid fermentation at pH of 6.5 compared to pH of 7.5 which suggested increased ability to produce ATP at lower pH. The effect of pH in transition to mixed acid fermentation in *E. faecalis* is more pronounced than

the effect of dilution rates. This is apparently different in *S. pyogenes* where dilution rates augment the mixed acid fermentation more than pH.

In Paper II the DNA microarray and 2DE technologies have been successfully applied to study gene and protein expression. The microarray and 2DE data shows 223 genes and 56 proteins to be effected by changing the growth rate. Interestingly, transcription of *ldh*-1 showed very strongly upregulation (about 1700 times) at high growth compared to low growth rate while the amount LDH protein was the same at all growth rates. This discrepancy could largely due to post-transcriptional regulation. The discrepancy between trascriptome and proteome data has been shown previously in other bacteria such as *L. lactis* (39), *E. coli* (84).

This study (Paper II) shows that at intermediate growth rate ($D=0.15h^{-1}$) many more genes were significantly regulated than that of lower ($D=0.05h^{-1}$) or higher ($D=0.4h^{-1}$) growth rate. In metabolite by increasing the growth rate *in vivo*, LDH activity has been increased while the PDH between highest and lowest growth rate remained unchanged. Interestingly, at intermediate growth rate, the PFL in vitro activity found to be higher than both the highest and lowest growth rate. The formate flux is maximum at $D=0.15h^{-1}$ which indicates regulation at the enzyme level. It is already shown that K_M of PFL is lower than for LDH (56) for pyruvate which could be explain a higher lactate/formate flux ratio by increasing growth rate (Paper II).

Tables 2. Summary of metabolite data presented in Paper I, II and IV.

Condition	Medium	Strain	Papers	Growth rate (h^{-1})	Optical density OD_{600}	Mean concentration (mM) ± SD					
						Glucose	Ethanol	Acetate[#]	Lactate	Acetoin	Formate
Batch	CDM-LAB	V583	Paper I	ND	0.6	45.3	0.82	0	24.5	1.7	2.8
Batch	CDM-LAB	Δ*ldh* 1.2	Paper I	ND	0.6	46.2	11.3	0	0.78	4.21	10.93
Chemostat	CDM-LAB	V583	Paper II	0.05 h^{-1}	3.0	0	37.6	34.37	27.65	0	68.96
Chemostat	CDM-LAB	V583	Paper II	0.15 h^{-1}	3.1	0	25.23	21.4	64.15	0	44.81
Chemostat	CDM-LAB	V583	Paper II	0.4 h^{-1}	3.3	0	6.16	8.24	97.32	0	16.31
Batch	CDM-LAB*	V583	Paper IV	ND	0.19	0	0.6	4.49	5.01	0	4.02
Batch	CDM-LAB**	V583	Paper IV	ND	0.35	0	0.78	6.21	6.91	0	4.77

Values are averages from three separate experiments. [#] Acetate production. (More detail see paper I, II, and IV). The medium contained: 16 mM acetate. *Medium contained 4.85 mM ascorbic acid no glucose, ** Medium contained 4.85 mM ascorbic acid and 1.25 mM glucose. ND-Not defined

Paper IV describes how *E. faecalis* V583 uses ascorbate as carbon source in absence or presence of glucose. The result shows *E. faecalis* V583 has the potential to grow in the absence of glucose when replaced by ascorbate. Based on our knowledge this is first study that shows a member of gram positive bacteria can grow on ascorbate. The metabolite analysis shows that in the presence of ascorbate, lactate, formate and acetate are produced. Ethanol is not produced when glucose is replaced by ascorbate. A diauxie growth curve was seen in the presence of ascorbate and glucose. Such growth profiles are commonly observed when multiple energy sources are available as seen when *E. coli* is grown in the presence of both glucose and lactose (97). In our study *E. faecalis* will initially consume both of glucose and ascorbate (Paper IV). In Paper II, transcription analysis of *E. faecalis* V583 shows that the gene EF1127 that encodes the ascorbate-specific transporter subunit IIC of the PTS system is upregulated in low growth rate. This result might play a role in regulation processes in mixed acid fermentation which might also explain the ascorbate effect.

Another important aspect of this work was the fate of amino acids under different dilution rates (Paper II). The result shows that, amino acids are consumed differently at different growth conditions. The bacterium clearly showed two different life styles at the various growth rates, one at the slow $0.05h^{-1}$ and $0.15h^{-1}$ and the other at fast $0.4h^{-1}$ growth rate. Arginine, tyrosine and serine were completely metabolized at the high growth rate while at the low growth rates all the amino acids were completely consumed. This shows that at low growth rate amino acids contribute more to the carbon flow. This is in agreement with previous studies in *L. lactis* (40) which shows amino acids contribute to ATP generation. Also both in the presence of amino acids but without glucose it was observed that bacteria could still grow (Paper IV).

4. MAIN CONCLUSION AND FUTURE WORK

The present study investigated the regulation of central energy metabolism of *E. faecalis* by use of transcription, proteome and metabolite analysis. The *E. faecalis* converts as expected most of the glucose into lactate production followed by other minor products (acetate, acetoin, ethanol, formate and CO_2). The composition of end products was dependent on growth conditions and availability of energy source. By study the *ldh*-1 negative mutant at the level of metabolite, transcriptome and proteome it was shown that a new regulatory network was regulated by the Rex. Furthermore, surprisingly large changes in expression of certain genes (*pdh, adh*) did not coincide with changes in their respective protein level while expression of other proteins was more consistent with their transcription level (*pfl*, *gap*-2). Some of the conclusions of this work and future perspectives are mentioned below.

Conclusion:

- NADH/NAD ratio and transcriptional regulation was suggested to play an important role in the regulatory network controlling acetoin, ethanol, formate and pyruvate in *E. faecalis* V583.
- Acetoin synthesis is due to an overflow metabolism in *E. faecalis* V583.
- Regulatory network of glucose fermentation is globally regulated by Rex.
- By decreasing the growth rates, *E. faecalis* V583 gradually switches from homolactic to mixed acid fermentation.
- Two life styles are shown by growing of *E. faecalis* in chemostat. Fast growth is dominated by few amino acids (arginine, tyrosine and serine) degradation while the slow growth rate degrades more amino acid and slow PFL activity.
- The transcription of *ldh*-1 gene is strongly regulated by growth rate while the protein amount is apparently unaffected.
- Deletion of *ldh* genes hardly affected the growth rate and caused higher yields without pH control in batch and in chemostat cultures.
- *E. faecalis* responds differently from *L. lactis* and *S. pyogenes* to pH.
- *E. faecalis* V583 grows on ascorbic acid as energy sources.

Future work:

- The *ldh*-1.2 mutation changes the bacterium from homolactic to mixed acid fermentation. It will be interesting to further make additional mutations of the LDH1.2 mutant in the genes involved in mixed acid fermentation pathways and look at the consequence in growth and metabolism.
- Metabolic studies of the *E. faecalis* when grown in different amino acids should improve our understanding on the role of amino acids in metabolic processes which has not been study extensively in LAB.
- We have shown that bacteria respond differently in metabolic profiles different dilution rates. It will be of interest to extend this investigate by further altering the dilution rates and perform a more detailed analysis of metabolites produced.
- 2DE analyses give an inside on the protein profiles. Further study is needed to fully investigate the relationship between metabolisem involved in post-transcriptional regulation of the enzymes of energy metabolism. This could be performed by the developed phosphoproteomic technology (57, 109, 116, 168).

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PAPER I

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Transcriptome, Proteome, and Metabolite Analyses of a Lactate Dehydrogenase-Negative Mutant of *Enterococcus faecalis* V583▽†

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A constructed lactate dehydrogenase (LDH)-negative mutant of *Enterococcus faecalis* V583 grows at the same rate as the wild type but ferments glucose to ethanol, formate, and acetoin. Microarray analysis showed that LDH deficiency had profound transcriptional effects: 43 genes in the mutant were found to be upregulated, and 45 were found to be downregulated. Most of the upregulated genes encode enzymes of energy metabolism or transport. By two-dimensional (2D) gel analysis, 45 differentially expressed proteins were identified. A comparison of transcriptomic and proteomic data suggested that for several proteins the level of expression is regulated beyond the level of transcription. Pyruvate catabolic genes, including the truncated *ldh* gene, showed highly increased transcription in the mutant. These genes, along with a number of other differentially expressed genes, are preceded by sequences with homology to binding sites for the global redox-sensing repressor, Rex, of *Staphylococcus aureus*. The data indicate that the genes are transcriptionally regulated by the NADH/NAD ratio and that this ratio plays an important role in the regulatory network controlling energy metabolism in *E. faecalis*.

Lactic acid bacteria (LAB) are widely used for production of lactic acid in fermented food. During the fermentation process, pyruvate is converted to lactate in addition to a number of minor metabolites, such as acetic acid, acetaldehyde, ethanol, acetoin, and acetate. However, under certain conditions, these bacteria shift from homolactic to heterolactic (or mixed-acid) fermentation, with formate, acetate, acetoin, ethanol, and CO_2 as end products. In *Lactococcus lactis*, mixed-acid fermentation has been shown to take place at low grow rates under microaerobic conditions (11), under true carbon-limited conditions, and while growing at low pH on carbon sources other than glucose (15, 20).

Mixed-acid fermentation was also seen after removing the lactate dehydrogenase (LDH) activity in *Enterococcus faecalis* V583 (12). This bacterium has two *ldh* genes, but *ldh-1* is the main contributor to lactate production. A mutant with deletions in both *ldh* genes (the Δldh1.2 mutant) was constructed and shown to direct its carbon flow from pyruvate away from lactate toward formate, acetoin, and alcohol production (12). Alternative carbon fluxes in different knockout mutants have also been reported for *Lactococcus lactis* (22).

The mechanism of the shift from homolactic to mixed-acid fermentation is still not fully understood. During transformation of pyruvate to lactate, LDH regenerates NAD^+ from NADH formed during glycolysis. When pyruvate is converted to acetyl-coenzyme A (acetyl-CoA) by either pyruvate formate lyase (PFL) or pyruvate dehydrogenase (PDH), reduction of acetyl-CoA to ethanol regenerates NAD^+ from NADH and is an alternative to lactate formation in redox balancing. The carbon flux is biochemically regulated (4, 5). Fructose-1,6-bisphosphate is an allosteric activator of lactate production, and dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate are strong inhibitors of the pyruvate formate lyase in *Lactococcus lactis* (4).

However, less is known about the regulation of the synthesis of glycolytic enzymes, especially in *E. faecalis*. In *L. lactis*, enzyme levels are regulated in response to growth conditions, and correlations between metabolic and transcriptomic or proteomic data have been established (3, 5). Combining the three approaches in one study provides more information and an improved understanding of the shift in LAB from homolactic to mixed-acid metabolism. Given that lactate production is extremely important for all LAB, including the emerging pathogen *E. faecalis*, we compared the Δldh1.2 mutant and its wild type by metabolic, transcriptomic, and proteomic analyses. Lactate dehydrogenase deficiency affects a large number of genes, and our data provide new insight into the regulation of energy metabolism in *E. faecalis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Enterococcus faecalis* V583 and a mutant lacking lactate dehydrogenase (the Δldh1.2 mutant) (12) were used throughout this study. The bacteria were grown in a chemically defined medium (CDM-LAB) containing 1.1% glucose, 0.1% sodium acetate, 0.06% citrate, 19

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TABLE 1. Genes and primers used for qRT-PCR

ORF	Gene	Primer sequence(5′→3′)		Reference
		Forward	Reverse	
EF0900	*adhE*	TCTGAGCAAGCGGTCCATTGTGG	AGTCGAATTAGAAGGTGCAGGTCCAG	This study
EF1612	*pflA*	CCAGGTGTCCGTTTTATCGTATTTAC	GGCATTCATAACAACCTTAGATACG	This study
EF0082		GCTTGCACGACTTTTCATGGGGAAAC	GGGCCATTTATTGGGATGTTATTG	This study
EF1964	*gap-2*	TAATGACAACTATCCACGCTTACAGG	CTTTTGTTTGAGTTGCATCGAATGAACC	This study
EF0255	*ldh-1*	CGCAGGGAATAAAGATCACCA	GCAATCGTCATAAGTAGCAGCA	This study
23S rRNA		CCTATCGGCCTCGGCTTAG	AGCGAAAGACAGGTGAGAATCC	26

amino acids, and growth factors at 37°C (12, 16). For all analyses, the cells were grown anaerobically in tightly capped, filled 50-ml screw-cap tubes with a starting pH of 7.4 to an optical density at 600 nm (OD_{600}) of 0.6. The cells were then harvested by centrifugation at 4°C for 10 min at 6,000 × *g*, and pellets were either flash frozen in liquid nitrogen or treated according to the protein extraction protocol (see below). Supernatants were frozen at −20°C until metabolite analyses. All experiments were run in triplicate.

Metabolic characterization. After removal of bacterial cells by centrifugation (10 min, 6,000 × *g*), metabolites in the cultures were analyzed by high-performance liquid chromatography (HPLC) (17). Ethanol and acetoin were analyzed by headspace gas chromatography (14). Lactate and glucose were also measured by using Megazyme enzymatic kits (Wicklow, Ireland).

RNA isolation, cDNA synthesis, fluorescence labeling, and hybridization. Flash-frozen pellets were stored at −80°C until RNA isolation. Total RNA was isolated by use of FastPrep (Bio101/Savant) and an RNeasy minikit (Qiagen) as previously described (33). The RNA concentration was determined with a NanoDrop spectrophotometer (NanoDrop Technologies), and the quality was tested by using an RNA 600 Nano LabChip kit and a Bioanalyzer 2100 instrument (Agilent Technologies). cDNA synthesis, labeling, and hybridization were performed as described previously (18). The microarray used was described by Solheim et al. (32). It contained 3,219 70-mer probes representing 3,219 open reading frames (ORFs) of the genome of *E. faecalis* V583. Three replicate hybridizations with mRNAs were obtained with three separate growth experiments. The Cy3 and Cy5 dyes (Amersham) used during cDNA synthesis were swapped in two of the three replicate hybridizations. Hybridized arrays were scanned with a Tecan LS scanner (Tecan). Fluorescence intensities and spot morphologies were analyzed using GenePix Pro 6.0 (Molecular Devices), and spots were excluded based on slide or morphology abnormalities.

Microarray data analysis. Analysis of microarray data was done by the LIMMA package (www.bioconductor.org) in the R computing environment (www.r-project.org). Preprocessing and normalization were done according to the methods of Smyth and Speed (29). A linear mixed model (27) was used in tests for differential gene expression. A mixed-model approach was used to describe variation between arrays as previously described (33). Empirical Bayes smoothing of gene-wise variances was conducted according to the method of Smyth et al. (28).

Real-time qPCR analysis. To verify the microarray results, the following genes were selected for analysis by real-time quantitative reverse transcription-PCR (qRT-PCR): EF0900 (*adhE*; bifunctional acetaldehyde-CoA/alcohol dehydrogenase gene), EF1612 (*pflA*; pyruvate formate lyase activating enzyme gene), EF0082 (major facilitator family transporter gene), EF1964 (*gap-2*; glyceraldehyde-3-phosphate dehydrogenase gene), and EF0255 (*ldh*; L-lactate dehydrogenase gene). 23S rRNA was used to normalize the data (Table 1). Real-time quantitative PCR (qPCR) was performed using a Rotor-Gene 6000 centrifugal amplification system (Corbett Research) and a 20-μl final reaction volume containing 2.5 μl 100×-diluted cDNA, 7.5 μM (each) forward and reverse primers (Sigma), and 12.5 μl Higher Power SYBR green PCR master mix (Roche). The transformation to cDNA was performed as described above. The PCR included an initial denaturation cycle at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 15 s, and elongation at 72°C for 30 s. Relative gene expression was calculated by the ΔC_T method, using the 23S rRNA gene as the endogenous reference gene.

Protein extraction. Proteins from bacterial cultures were isolated by alkaline lysis at 4°C. In brief, 50 ml of bacterial culture was centrifuged at 6,000 × *g* at 4°C. Bacterial pellets were suspended in 0.5 ml 0.9% (wt/vol) NaCl, washed three times, and resuspended in 400 μl of rehydration buffer containing 8 M urea, 2 M thiourea, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.1% IPG buffer, 10 mM dithiothreitol (DTT), and a trace of bromophenol blue. Cells were then broken by use of FastPrep (Bio101/Savant) at 6 m/s three times for 45 s each at 4°C, with 60-s pauses between. Unbroken cells were removed by centrifugation at 8,000 × *g* for 10 min at 4°C. The samples were stored at −80°C until further analysis. The total protein concentration for each sample was measured using the colorimetric assay RC DC protein assay reagent (Bio-Rad), using bovine serum albumin (BSA) as a standard.

Two-dimensional gel electrophoresis, in-gel digestion, MALDI-TOF analysis, and protein identification. Protein separation, gel analysis, trypsin treatment, and extraction of proteins of interest were performed as described previously (1). The gels were scanned and analyzed by Delta2D software (Decodon, Greifswald, Germany) and by a pixel-based analysis of multiple images for the identification of proteome patterns of two-dimensional (2D) gel electrophoresis images (6). Extracted peptides were desalted with C_{18} Stage tips (24). The peptides were eluted with 1 μl 70% (vol/vol) acetonitrile (ACN), and then 0.5 μl of each sample was mixed with 0.5 μl of the matrix mixed with 15 mg/ml alpha-cyano-4-hydroxycinnamic acid and applied to a matrix-assisted laser desorption ionization (MALDI) target plate (Bruker Daltonics, Billerica, MA). Peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) were performed on Ultra Flex MALDI-tandem time of flight (MALDI-TOF/TOF) (Bruker Daltonics) instruments. The mass range for MALDI-TOF/MS was 800 to 4,000 Da, with a mass accuracy of 50 ppm. Protein identification was carried out using Mascot (Matrix Science Inc., Boston, MA) software and searches under "other *Firmicutes*" in the NCBI database.

Microarray data accession number. The microarray data obtained in this study have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-472.

RESULTS

Growth and metabolite analysis. *E. faecalis* V583 and its lactate dehydrogenase-negative mutant (the Δldh1.2 mutant) were grown under anaerobic conditions at 37°C to an OD_{600} of 0.6. As shown in Table 2, lactic acid was the major metabolic end product in the wild type, while the mutant produced in-

TABLE 2. Metabolites of *Enterococcus faecalis* V583 and Δldh1.2 mutant grown in batch cultures harvested at an OD of 0.6[a]

E. faecalis strain	Mean concn (mM) ± SD							Concn of glucose consumed (mM)	% Carbon balance
	Citrate	Lactate	Formate	Ethanol	Acetate	Pyruvate	Acetoin		
V583	2.1 ± 0.01	24.5 ± 0.60	2.80 ± 0.01	0.82 ± 0.03	15.19 ± 0.02	0.05 ± 0.00	1.7 ± 0.20	11.7 ± 0.42	115
Δldh1.2 mutant	1.13 ± 0.01	0.78 ± 0.31	10.93 ± 0.03	11.3 ± 0.61	15.9 ± 0.04	0.40 ± 0.03	4.21 ± 0.36	10.80 ± 0.31	89.90

[a] The medium contained 57.0 mM glucose, 2.17 mM citrate, and 16.01 mM acetate.

TABLE 3. Significantly upregulated genes in the mutant, as identified by microarray

ORF	Gene	Putative function	Functional category	Amt of upregulation in mutant (log_2 value)
EF0255[a]	*ldh-1*	L-Lactate dehydrogenase	Energy metabolism	3.24
EF0552		PTS system, IIC component	Energy metabolism	1.21
EF0677		Phosphoglucomutase/phosphomannomutase family protein	Energy metabolism	1.67
EF0806		Amino acid ABC transporter, permease protein	Transport and binding	1.09
EF0900	*adhE*	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	Energy metabolism	3.57
EF0949	*eutD*	Phosphotransacetylase	Energy metabolism	1.22
EF1017		PTS system, IIB component	Signal transduction	1.42
EF1018		PTS system, IIA component	Signal transduction	1.30
EF1019		PTS system, IIC component	Signal transduction	1.84
EF1213	*alsS*	Acetolactate synthase	Energy metabolism	1.51
EF1214	*budA*	Alpha-acetolactate decarboxylase	Energy metabolism	1.92
EF1343		Sugar ABC transporter, permease protein	Transport and binding	1.50
EF1353	*pdhA*	Pyruvate dehydrogenase complex E1 component, alpha subunit	Energy metabolism	2.65
EF1354	*pdhB*	Pyruvate dehydrogenase complex, E1 component, beta subunit	Energy metabolism	2.45
EF1355	*aceF*	Dihydrolipoamide acetyltransferase	Energy metabolism	2.29
EF1356	*lpdA*	Dihydrolipoamide dehydrogenase	Energy metabolism	2.51
EF1612	*pflA*	Pyruvate formate lyase activating enzyme	Energy metabolism	1.22
EF1613	*pflB*	Formate acetyltransferase	Energy metabolism	1.44
EF1712	*pyrE*	Orotate phosphoribosyltransferase	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	4.07
EF1713	*pyrF*	Orotidine 5′-phosphate decarboxylase	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	4.19
EF1714	*pyrD2*	Dihydroorotate dehydrogenase	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	2.10
EF1718	*pyrC*	Dihydroorotase	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	4.73
EF1719	*pyrB*	Aspartate carbamoyltransferase catalytic subunit	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	3.50
EF1720		Uracil permease	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	1.90
EF2213		PTS system, IIBC components	Energy metabolism	1.64
EF3014		Cation transporter E1-E2 family ATPase	Transport and binding	1.56
EF3199		ABC transporter, permease protein	Transport and binding	3.17
EF3200		ABC transporter, ATP-binding protein	Unknown function	2.61
EF3315		Triphosphoribosyl-dephospho-CoA synthase	Unknown function	1.34
EF3318	*citX*	2′-(5″-Triphosphoribosyl)-3′-dephospho-CoA:apo-citrate lyase	Energy metabolism	1.59
EF3319	*citF*	Citrate lyase, alpha subunit	Energy metabolism	0.92
EF3320	*citE*	Citrate lyase, beta subunit	Energy metabolism	1.66
EF3321	*citD*	Citrate lyase, gamma subunit	Energy metabolism	1.74
EF3322	*citC*	Citrate lyase ligase	Energy metabolism	1.24
EF3324		Sodium ion-translocating decarboxylase, beta subunit	Energy metabolism	2.47
EF3325		Sodium ion-translocating decarboxylase/biotin carboxyl carrier protein subunit	Energy metabolism	2.17
EF3327		Citrate transporter	Transport and binding	2.09
EFB0038		Conserved hypothetical protein	Hypothetical protein	1.03
EFB0042		Hypothetical protein	Hypothetical protein	6.08
EFB0043	*ssb-6*	Single-strand-binding protein	DNA metabolism	6.31
EFB0044		Hypothetical protein	Hypothetical protein	5.84
EFB0045	*nuc-2*	Thermonuclease precursors	DNA metabolism	6.16
EFB0046		Conserved domain protein	Hypothetical protein	6.12

[a] The gene is truncated in the mutant.

creased amounts of acetoin, formate, and ethanol and some pyruvate, but no lactic acid. Neither strain produced acetate as a metabolic end product.

Transcriptome analysis. The differences in expression profiles of the wild type and the mutant were assessed by the expression ratio between each gene in the mutant and the respective wild-type gene. The results presented in Table 3 and in Table S1 in the supplemental material are the means for three independent biological replicates. Altogether, 88 genes were found to be expressed differentially (>2-fold); 43 were upregulated, and 45 were downregulated. Many of the genes affected were genes engaged in energy, pyrimidine, and citrate metabolism and in transport functions, but a number of genes of unknown function were also affected.

Enterococcus faecalis has four main routes of pyruvate catabolism. In addition to lactate formation, these lead to the production of acetoin, formate plus acetyl-CoA, and CO_2. The acetyl-CoA formed can be reduced to ethanol to maintain redox balance. As shown in Table 3, the genes for all of these pathways (EF0900 [bifunctional acetaldehyde-CoA/alcohol dehydrogenase gene],

EF1213 [acetolactate synthetase gene], EF1353 and EF1354 [pyruvate dehydrogenase complex genes], EF1612 [pyruvate formate lyase activating enzyme gene], and EF1613 [formate acetyltransferase gene]) were upregulated in the mutant. The gene encoding the main lactate dehydrogenase, *ldh-1* (EF0255), was truncated in the mutant, but the sequence recognized by the hybridizing probe was present and showed about 10-fold enhanced transcription (Table 3).

Interestingly, most of the genes involved in pyrimidine biosynthesis (EF1712 to EF1720) were significantly upregulated in the Δldh1.2 mutant (Table 3), but the transcriptional data for the EF1714, EF1715, and EF1716 genes were more doubtful due to poor *P* values. Also, the EF0677 gene, encoding phosphoglucomutase, which converts glucose-6-phosphate to glucose-1-phosphate, was significantly upregulated in the Δldh1.2 mutant. This enzyme is also important in the production of uracil-glucose, since glucose-1-phosphate is used as a substrate in UDP-glucose production. The EF1721 (*pyrR*) gene encodes a bifunctional pyrimidine regulatory protein that exerts the uracil phosphoribosyltransferase catalysis that is crucial for UDP-glucose production, and it might be upregulated ($\log_2$ value = 2.1), but a poor *P* value (0.27) precluded it from being included among the upregulated genes. Also, EF1720, the uracil permease gene, is probably upregulated ($\log_2$ value = 1.9), though the *P* value (0.34) kept it from being considered upregulated. In summary, our transcription results suggest that the Δldh1.2 mutant triggers an increased production of UDP-glucose that could be used both in cell wall biosynthesis and in polysaccharide production. However, indications of increased polysaccharide production, such as altered colony appearance or culture viscosity, were not observed.

Unlike the wild type, the mutant consumed some of the citrate present in the growth medium, causing increased acetoin production. In line with this, most of the genes for citrate metabolism (EF3315 to EF3327) were found to be upregulated, indicating that both *cit* operons are affected by the *ldh* deletion.

Table S1 in the supplemental material summarizes the genes downregulated in the mutant. A majority of the genes are hypothetical (17 of 35 chromosomal genes), and eight are located on plasmid pTEF2. Several of the downregulated genes encode cell envelope-associated proteins. The gene showing the strongest reduction of transcription encodes a major facilitator family transporter (EF0082) ($\log_2$ value = −3.7). In a gene cluster involved in the biosynthesis of aromatic amino acids (EF1561 to EF1568), four genes were found to be downregulated significantly (EF1562, EF1564, EF1565, and EF1566).

Among the three plasmids of the V583 strain, only pTEF2 carries genes that were significantly affected in transcription in the deletion mutant. Of the 62 genes annotated for pTEF2, 14 genes were transcriptionally affected. The plasmid-carried genes EFB0038 and EFB0042 to EFB0046 were among the most affected and were upregulated up to a $\log_2$ value of 6.3, while the adjacent gene clusters EFB0048 to EFB0051 and EFB0053 to EFB0056 were strongly downregulated (up to a $\log_2$ value of 2.8).

To verify the quality of the microarray results, the relative amounts of mRNAs of five genes were analyzed by qPCR. As shown in Table S2 in the supplemental material, the qPCR results were in agreement with the data obtained by the microarrays.

Proteomic analysis. The proteomes of the two strains were compared by 2D gel electrophoresis. About 400 gel spots were distinguished. Differentially expressed proteins were isolated and identified by MALDI-TOF/TOF-MS analysis. Altogether, 45 differentially expressed proteins ($P < 0.05$) were identified (Table 4), of which 24 were upregulated and 21 were downregulated. LDH (EF0255) was absent in the mutant, while the cell division protein DivIVA (EF1002) (23) was not found on the gel of the wild type. Among other proteins identified was a bifunctional acetaldehyde-CoA/alcohol dehydrogenase (EF0900). This protein was present in equal amounts in both strains. By sorting the identified proteins according to metabolic function, we found that most of the differences in expression were among proteins engaged in energy metabolism (nine proteins), followed by seven proteins related to fatty acid metabolism, phospholipid metabolism, and amino acid biosynthesis. A single protein (EF3293) involved in purine metabolism was expressed less in the mutant.

Most of the genes encoding the differentially expressed proteins were not represented by statistically significant data in the transcriptomic data. However, the expression of four proteins correlated well with the transcriptomic data, including the pyruvate dehydrogenase complex E1 component beta subunit (EF1354), pyruvate formate lyase activating protein (EF1612), and two hypothetical proteins (EF3313 and EF1617). Discrepancies between proteomic and transcriptomic data were also seen. The transcription of the bifunctional acetaldehyde-CoA/alcohol dehydrogenase (EF0900) gene was upregulated >10-fold in the mutant, but the protein was present in equal amounts in the two strains. The *ldh* mutant also appeared to contain reduced levels of a plasmid-encoded single-strand-binding protein (EFB0043), though its transcription was highly upregulated compared to that in the wild type.

Moreover, the mutant contained more glyceraldehyde-3-phosphate dehydrogenase (EF1964) protein and triosephosphate isomerase (EF1962) protein than the wild type did. The transcriptomic data for the corresponding genes were of unsatisfactory quality, but the RT-PCR showed that EF1964 was not differentially expressed. Altogether, these results indicate that there are important regulations at the translational level as well.

The increased production of pyruvate and ethanol suggests an elevated NADH/NAD ratio in the mutant (30). The global gene regulator Rex is known to respond to this ratio by differential binding to Rex operators (7). We therefore examined the *E. faecalis* V583 genome sequence for putative Rex boxes and compared them to our transcriptomic and proteomic data. We used the consensus palindromic sequence (TGTGANNN NNNTCACA) established for *Staphylococcus aureus* (7) for the genome-wide search. By allowing for two mismatches, we found the sequence in 151 intergenic regions and upstream of open reading frames annotated as genes (data not shown). Putative Rex boxes were found upstream of 22 genes/operons showing differential expression in our transcriptome or proteome analyses (Table 5), among which 16 were positively regulated and 6 were negatively regulated.

DISCUSSION

The biochemical regulation of carbon flow in energy metabolism of LAB has been well investigated, but only a few studies

TABLE 4. Proteins differentially expressed in the mutant

ORF	Gene	Functional class	Putative function	Mass (kDa)	pI	Change in expression in mutant ($\log_2$ value)[a]
EF0020		Transport and binding protein	PTS system, mannose-specific IIAB components	35.5	5.11	0.82
EF0043	*gltX*	Protein synthesis	Glutamyl-tRNA synthetase	55.3	4.96	−0.74
EF0105		Energy metabolism	Ornithine transcarbamylase	38.1	5.02	0.53
EF0146		Cellular processes	Surface exclusion protein, putative	98.9	5.6	−1.47
EF0200		Protein synthesis	Elongation factor G	76.7	4.8	1.07
EF0233		Transcription	DNA-directed RNA polymerase subunit alpha	35.1	4.88	−2.3
EF0255	*ldh-1*	Energy metabolism	L-Lactate dehydrogenase	35.5	4.77	Np1
EF0282	*fabI*	Fatty acid and phospholipid metabolism	Enoyl-(acyl carrier protein) reductase	26.9	5.29	1.27
EF0283	*fabF1*	Fatty acid and phospholipid metabolism	3-Oxoacyl-(acetyl carrier protein) synthetase II	43.5	5.11	1.02
EF0517		Cellular processes	2-Dehydropantoate 2-reductase	98.9	5.6	−1.85
EF0820	*rplY*	Protein synthesis	50S ribosomal protein L25/general stress protein Ctc	22.2	4.48	−1.85
EF1002		Cellular processes	Cell division protein DivIVA	26.6	4.53	Np2
EF1050	*etaR*	Signal transduction	DNA-binding response regulator	26.4	5.13	0.94
EF1131	*araD*	Energy metabolism	L-Ribulose-5-phosphate 4-epimerase	26.3	5.71	1.22
EF1138		Unknown function	Aldo/keto-reductase family oxidoreductase	31	5.28	−0.30
EF1167	*fba*	Energy metabolism	Fructose-bisphosphate aldolase	31	4.86	−1.64
EF1182	*luxS*	Cellular processes	*S*-Ribosylhomocysteinase	17.2	5.31	0.78
EF1183	*asd*	Amino acid biosynthesis	Aspartate-semialdehyde dehydrogenase	38.9	4.97	−2.40
EF1241		Hypothetical protein	Hypothetical protein	19.5	4.94	0.70
EF1354	*pdhB*	Energy metabolism	Pyruvate dehydrogenase complex E1 component, beta subunit	35.3	4.67	0.53
EF1415	*gdhA*	Amino acid biosynthesis	Glutamate dehydrogenase	49.6	5.42	−1.01
EF1526	*gap-1*	Energy metabolism	Glyceraldehyde-3-phosphate dehydrogenase	36.4	4.87	−0.97
EF1611	*ppaC*	Central intermediary metabolism	Putative manganese-dependent inorganic pyrophosphatase	33.5	4.38	−2.44
EF1612	*pflA*	Energy metabolism	Pyruvate formate lyase activating protein	29.4	5.53	1.40
EF1617		Hypothetical protein	Possible NADP:quinone reductase	4.3	16.64	1.80
EF1860	*panB*	Biosynthesis of cofactors, prosthetic group carriers	3-Methyl-2-oxobutanoate hydroxymethyltransferase	30	5.82	−0.69
EF1900		Transport and binding protein	16S rRNA processing protein RimM	19.8	5.09	−1.32
EF1962	*tpiA*	Energy metabolism	Triosephosphate isomerase	27.1	4.63	0.92
EF1964	*gap-2*	Energy metabolism	Glyceraldehyde-3-phosphate dehydrogenase	35.9	5.03	2.09
EF2151	*glmS*	Central intermediary metabolism	D-Fructose-6-phosphate amidotransferase	65.6	4.93	1.18
EF2193	*epaF*	Cell envelope	dTDP-4-dehydrorhamnose 3,5-epimerase	21.3	5.43	0.66
EF2425		Energy metabolism	Phosphoglucomutase/phosphomannomutase family protein	63.8	4.87	1.05
EF2550	*gylA*	Amino acid biosynthesis	Serine hydroxymethyltransferase	44.5	5.47	−0.94
EF2591		Unknown function	Glyoxalase family protein	31.6	4.85	2.15
EF2881	*fabG*	Fatty acid and phospholipid metabolism	3-Ketoacyl-(acyl carrierprotein) reductase	26	5.92	0.51
EF2882	*fabD*	Fatty acid and phospholipid metabolism	Aldo-carrier-protein *S*-malonyltransferase	33.6	5.05	−2.8
EF2894		Cellular processes	General stress protein 13, putative	13.8	6.90	−0.83
EF2898		Unknown function	Peptidyl-prolyl-transisomerase, cyclophilin type	21.5	4.46	−1.40
EF2903		Transport and binding	ABC transporter, substrate binding protein	47.5	4.79	0.93
EF3037	*pepA*	Protein fate	Glutamyl-aminopeptidase	39.4	5.68	0.78
EF3293	*guaB*	Purine, pyrimidine, nucleoside, and nucleotide biosynthesis	Inositol-5-monophosphate dehydrogenase	52.8	5.70	−1.49
EF3313		Hypothetical protein	Hypothetical protein	4.00	4.49	−1.1
EFA0081		Cell envelope	Hypothetical protein	17.9	4.88	0.92
EFA0081		Hypothetical protein	Hypothetical protein	17.9	4.88	1.19
EFB0043	*ssb-6*	DNA metabolism	Single-strand-binding protein	16.8	5.18	−1.03

[a] Np1, no protein in mutant; Np2, no protein detected in wild type.

have been carried out using the new transcriptomic and proteomic technologies. In this study, we also demonstrated regulation of central carbon metabolism at the level of biosynthesis of the proteins involved.

An *E. faecalis* mutant lacking *ldh* metabolizes sugar by pathways that are used very little, if at all, by the wild type, and this is accompanied by increased transcription of genes engaged in these pathways.

Our metabolite data show that pyruvate was converted to acetyl-CoA by PFL and further reduced to ethanol. This generated excess NADH, which had to be reoxidized for redox balance. This could have been done by acetate production

TABLE 5. Identification of putative Rex binding sites upstream of differentially expressed operons

ORF	Gene	Protein description	Rex binding site		Regulation
			Start site	Sequence	
EF0255	*ldh-1*	L-Lactate dehydrogenase	232355	TGTAAAAAATGTCACG	Up[a]
			232430	TGTGCGTAATTTCACT	Up[a]
EF0900	*adhE*	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	863992	TGTGAAAAATATCACA	Up[a]
			864049	TGTGAAATAGTTAACA	Up[a]
EF1314		Aminotransferase AlaT	1281829	AGTGATTTTTGTCCCA	Down[a]
EF1353	*pdhA*	Pyruvate dehydrogenase complex E1 component, alpha subunit	1326691	TGTGAAAATTATCACT	Up[a]
EF1613	*pflB*	Formate acetyltransferase	1570968	TGTGATTAGTATAACA	Up[a]
EF3200		ABC transporter, ATP-binding protein	3072879	TGTGAAACGATTTAAA	Up[a]
EF3256		Pheromone cAD1 precursor lipoprotein	3133773	TGTGAAATGATGGACA	Down[a]
EF3314		Cell wall surface anchor family protein	3201202	TGTTAAAAAACTCACT	Up[a]
EF3327		Citrate transporter	3211936	TTTGTATATTCTCACA	Up[a]
			3211997	TGTGAAACATTTCTCA	Up[a]
EF0020		PTS system, mannose-specific IIAB components	23010	TCTGATTTTTTTCAAA	Up[b]
EF0200	*fusA*	Elongation factor G	194456	AGTAACGTCTATCACA	Up[b]
EF0282	*fabL*	Enoyl-(acyl carrier protein) reductase	268424	TGTGAGAATGATAACA	Up[b]
EF0283	*fabF*	1,3-Oxoacyl-(acyl carrier protein) synthase II	268424	TGTGAGAATGATAACA	Up[b]
EF0517		2-Dehydropantoate 2-reductase	479400	AGTGAACATTTTCACA	Down[b]
EF1002		Cell division protein DivIVA	959270	TGAGAATGTGTTCATA	Up[b]
EF1167	*fba*	Fructose-bisphosphate aldolase	1138206	TGTGAAAGAATAGACA	Down[b]
EF2151	*glmS*	Glucosamine–fructose-6-phosphate aminotransferase	2053359	AGTGATTTTTGTCTCA	Up[b]
EF2550	*glyA*	Serine hydroxymethyltransferase	2465297	TGTCAGCTTCGTTACA	Down[b]
EF3293	*guaB*	IMP dehydrogenase	3174241	TGTAACAAAAATCACT	Down[b]

[a] Found at transcriptomic level.
[b] Found at proteomic level.

from acetyl-CoA formed by either PFL or PDH. The process involving only PFL for pyruvate metabolism would produce more ATP per glucose molecule consumed than does normal lactic acid fermentation. However, excess NADH was used for acetoin production, in a process that produces the same amount of ATP as the wild type. In the mutant, PDH was upregulated, but pyruvate oxidation did not take place. Ward et al. showed that PDH can be active in *E. faecalis* under anaerobic conditions (34), but the activity is reduced at a high NADH/NAD ratio (31). Snoep et al. showed that *E. faecalis* producing ethanol has an elevated NADH/NAD ratio, and this might explain why PDH was not active in the mutant (30).

The NADH/NAD ratio also regulates the activity of the transcription factor Rex, and putative Rex boxes were found upstream of a number of the differentially expressed genes and operons in *S. aureus* (7, 19). In *Bacillus subtilis*, Rex regulates genes encoding proteins of the respiratory chain (25), and in *S. aureus*, Rex controls transcription involved in the transition from aerobic to anaerobic growth (19). Pagels et al. found 461 putative Rex binding sites in the *S. aureus* genome by using their Rex box consensus sequence and allowing for two mismatches. However, they demonstrated that Rex could bind to some, but not all, of these sites, indicating that additional sequence features are required for Rex-mediated regulation (19). Thus, it is likely that our sequence search overestimates the number of Rex boxes in the *E. faecalis* genome. However, our data suggest that Rex also acts as a repressor under anaerobic conditions. All of the genes involved in the four different pathways of energy metabolism of pyruvate appear to be regulated by Rex and were upregulated in the mutant. Interestingly, the genes encoding the enzymes for NAD regeneration during anaerobic growth, *ldh-1* and *adhE*, showed the strongest upregulation and are both preceded by two Rex boxes. The *ldh* of *S. aureus* is also preceded by two Rex boxes (19). A putative Rex box was also found upstream of *ldh-2* (EF0641), the second *ldh* gene in *E. faecalis*, but the biological significance of *ldh-2* is apparently very low in *E. faecalis* V583 compared to that of *ldh-1* (12).

Rex has been recognized as a repressor, and its DNA binding can be influenced strongly by NADH. NADH causes derepression of genes by binding to the Rex repressor in a complex that diminishes its ability to bind the Rex box (10, 19). This appears to happen in *E. faecalis* operons as well, leading to increased transcription in the mutants. However, we also found putative Rex boxes upstream of genes downregulated in the mutant. This suggests that Rex may activate transcription in the wild type. To our knowledge, a role of Rex in activation of transcription has not previously been reported.

In *E. faecalis* V583, the EF2638 and EF2933 genes both encode putative Rex proteins (21). For EF2933, we found enhanced transcription in the mutant (1.9-fold; $P = 0.04$), and a Rex box upstream of the gene suggests that the gene is autoregulated in *E. faecalis* V583. In *Streptomyces coelicolor*, the Rex gene is also preceded by a Rex binding site, and the protein has been shown to repress its own transcription (2).

Enzymes encoded by the central glycolytic operon (EF1962 and EF1964) were transcriptionally expressed at higher rates in the mutant, and this was also confirmed by the proteomic analysis.

The proteome data revealed several differentially expressed proteins that were not verified by the transcriptome analysis. In most cases, this could probably be attributed to noise/poor statistics for the microarray data or just to a low level of transcription but highly efficient translation, including high stability of the transcripts. However, no changes were found by qPCR analysis of EF1964 transcripts. The operon encompass-

ing EF1962 to EF1965 is probably transcriptionally regulated by the cell's energy status via the CggR regulator (18). The data presented here indicate additional posttranscriptional or translational regulation. Discrepancies between transcriptomic and proteomic data were also noticed for EFB0043 and EF0900, again suggesting regulation beyond the level of transcription. Despite an unaltered protein level, the metabolic data clearly reflect increased activity of the *adhE* gene (EF0900) in the mutant. In a study of *Lactococcus lactis*, it was concluded that translational regulation had a major influence compared to transcriptional regulation of glycolytic enzymes (5).

The genes for another energy-yielding process, citrate metabolism, also appear to be regulated transcriptionally by Rex. Pyrimidine synthesis genes were also upregulated in the mutant. It has been demonstrated in *L. lactis* that the expression of these genes is affected by energy sources and by a disrupted regulation of arginine metabolism (9, 13).

Notably, EF0082 was the most downregulated gene in this study, and a similar result has been found for other mutants, including bacteriocin-resistant mutants (8, 18). The gene encodes a major facilitator family transporter, and its transcription has been suggested to be regulated by Ers (8) and the carbon catabolite protein through an upstream catabolite-responsive element (*cre*) (18). A number of the other differentially expressed genes in our mutant appear to be under catabolite control (18). The major glucose phosphotransferase system (PTS), the mannose-PTS, also appears to be dually regulated. In addition to the sigma54 promoter preceding EF0019, a Rex box found in front of EF0020 and elevated levels of the EF0020 protein suggest that the PTS is regulated by the NADH/NAD ratio. Moreover, LDH appears to be regulated by Rex but is also catabolically activated through *cre* regulation mediated by CcpA (18). These and many of the other proteins described here appear to be regulated by a network involving global regulators and energy and redox sensing aimed at maintaining homeostasis. Central in this regulatory network are the global regulators Rex and CcpA. Their interdependence is illustrated by the presence of a Rex box upstream of *ccpA* (data not shown), indicating that CcpA transcription is also sensitive to NAD/NADH.

The present study evokes the complexity of the central energy metabolism of LAB and suggests revised and complex regulations for how these bacteria cope with their changing access to energy sources. Many new aspects and questions related to the regulation of central energy metabolism have been raised, and a substantial amount of work is needed to scrutinize and confirm the various regulatory pathways that govern these pathways.

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Supplemental table S1. Significantly downregulated genes in the mutant identified by microarray.

ORF	Gene	Functional category	Function (gene name)	mutant/wt log_2
EF0014	*pur*A	Adenylosuccinate synthetase	Purines, pyrimidines, nucleosides, and nucleotides	-1.25
EF0025		Membrane protein putative	Cell envelope	-1.40
EF0076		Short chain dehydrogenase/reductase family oxidoreductase	Unknown function	-1.00
EF0082		Major facilitator family transporter	Transport and binding protein	-3.36
EF0083		Hypothetical protein	Hypothetical proteins	-3.71
EF0288		Hypothetical protein	Hypothetical proteins	-1.42
EF0290	*met*C	Cystathionine gamma-synthase/cystathionine beta-lyase	Amino acid biosynthesis	-1.56
EF0746		Penicillin-binding protein, putative	Cell envelope	-1.09
EF0857		Hypothetical protein	Hypothetical proteins	-1.00
EF0872		Potassium uptake protein	Transport and binding proteins	-1.16
EF1037		Aspartate aminotransferase	Amino acid biosynthesis	-1.49
EF1199		Conserved hypothetical protein	Hypothetical proteins	-1.08
EF1314		Aminotransferase AlaT	Amino acid biosynthesis	-1.20
EF1315		Hypothetical protein	Hypothetical protein	-1.06
EF1517		Hypothetical protein	Hypothetical proteins	-1.49
EF1562		3-deoxy-7-phosphoheptulonate synthase	Amino acid biosynthesis	-1.53
EF1564	*aro*C	Chorismate synthase	Amino acid biosynthesis	-1.17
EF1565		Prephenate dehydrogenase	Amino acid biosynthesis	-1.08
EF1566	*aro*A	3-phosphoshikimate 1-carboxyvinyltransferase	Amino acid biosynthesis	-1.25
EF1946		Hypothetical protein	Hypothetical protein	-1.12
EF2245		Hypothetical protein	Hypothetical proteins	-1.01
EF2300		Streptomycin resistance protein, putative	Cellular process	-1.22
EF2615		Hypothetical protein	Hypothetical proteins	-1.00
EF2703		Transcriptional regulator	Regulatory functions	-1.61
EF2713		Cell wall surface anchor family protein	Cell envelope	-1.59

EF3193	*Irg*B	Antiholin-like protein LrgB	Unknown functions	-1.05
EF3194	*Irg*A	Murein hydrolase regulator LrgA	Unknown functions	-1.20
EF3244		Hypothetical protein	Hypothetical proteins	-1.14
EF3245		Cell-envelope associated acid phosphatase	Unknown functions	-1.41
EF3256		Pheromone cAD1 precursor lipoprotein	Cell envelope	-1.09
EF3287		Hypothetical protein	Hypothetical proteins	-1.22
EF3313		Hypothetical protein	Hypothetical proteins	-1.34
EFB0048		Transcriptional regulator Cro/CI family	Regulatory function	-2.32
EFB0049		Glycosyl transferase group family protein	Cell envelope	-2.00
EFB0050		Toxin ABC transporter ATP-binding/permease protein	Transport and binding protein	-2.83
EFB0051		Membrane protein putative	Cell envelope	-1.93
EFB0053		Conserved domain protein	Hypothetical proteins	-1.53
EFB0054		Conserved hypothetical protein	Hypothetical proteins	-1.31
EFB0055		Lipoprotein putative	Cell envelope	-1.87
EFB0056		Hypothetical protein	Hypothetical proteins	-1.35

Supplemental table S2. qRT-PCR and microarray analysis in mutant compared with wild type[a].

Gene ID	Gene	Microarray Log_2 ratio	qRT-PCR Log_2 ratio
EF0082		-3.36	-3.07
EF0255	*ldh*-1	3.24	3.48
EF0900	*adh*E	3.57	4.03
EF1612	*pfl*	1.22	1.35
EF1964	*gap*-2	0.09	0.02

a) Gene regulation values (log_2) are average results of three biological replicates for microarray experiments and for quantitative RT-PCR

PAPER II

Growth rate dependent control in *Enterococcus faecalis*: effects on the transcriptome, proteome and strong regulation of lactate dehydrogenase

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Running title: Effect of growth rate on *E. faecalis* physiology

ABSTRACT

Enterococcus faecalis V583 was grown in a glucose-limited chemostat at three different (0.05 h^{-1}, 0.15 h^{-1} and 0.4h^{-1}) growth rates. The fermentation pattern changed with growth rate, from a mostly homolactic profile at high growth rate to a fermentation dominated by formate, acetate and ethanol production at low growth rate. A number of amino acids were consumed at the lower growth rates but not by fast growing cells. The change in metabolic profile was mainly caused by decreased flux through lactate dehydrogenase. Transcription of *ldh*-1, encoding the principal lactate dehydrogenase, showed very strong growth rate dependence and differed by three orders of magnitude between the highest and the lowest growth rates. Despite the increase in *ldh*-1 transcript, the content of the Ldh-1 protein was the same under all conditions. Using microarrays and qPCR the levels of 227 gene transcript were found to be affected by the growth rate, and 56 differentially expressed proteins were found by proteomic analyses. Few genes or proteins showed a growth rate-dependent increase or decrease in expression over the whole range of conditions, and many showed at maximum or minimum at the middle growth rate (D=0.15h^{-1}). For many gene products a discrepancy between transcriptomic and proteomic data were seen, indicating post-transcriptional regulation of expression.

INTRODUCTION

A number of regulation mechanisms exist to control the various processes in a cell and to maintain homeostasis. During balanced growth the cells maintain a constant composition, implying regulation of cellular processes tightly coordinated with growth rate. Such regulation is essential at all growth rates, but the cell's composition, size and metabolism may respond to changes in growth rate. The effects of growth rate on cellular processes have been best studied in yeast (3, 25), were hundreds of genes are expressed in a growth rate-dependent fashion (17). In bacteria, growth rate-dependent control has been shown to involve regulation at the levels of transcription and translation, and an increase in growth rate is associated with an increase in cell size and number of ribosomes (11, 16, 24). In *Lactococcus lactis* increases in growth rate have been reported to correlate with altered transcription of 30 % of the genes (6). Moreover, a shift to less efficient energy metabolism is frequently observed at higher growth rates (20). For lactic acid bacteria a reduction in growth rate is specifically associated with a change from homolactic to mixed acid fermentation (9, 12, 19, 31). The metabolic and genetic control of glycolysis has been thoroughly studied (7, 8), but the mechanisms underlying the shift in metabolism are not completely understood (7, 23). Previously, we used a mutant unable to produce lactic acid to show that a change in metabolism was associated with large effects on the *Enterococcus faecalis* transcriptome and proteome profiles (18). In this work we have studied the effects of growth rate on the trancriptome, metabolome and proteome of *E. faecalis* grown under energy limitation in chemostat culture to obtain a better understanding of the mechanisms regulating the cell's metabolic processes.

MATERIALS AND METHODS

Growth condition and analytical procedures.

Enterococcus faecalis V583 (26) was grown anaerobically at 37°C in the chemically defined medium named CDM-LAB. This contained per liter: 1 g K_2HPO_4 , 5 g KH_2PO4, 0.6 g ammonium citrate, 1 g sodium acetate, 0.25 g tyrosine, 0.24 g alanine, 0.125 g arginine, 0.42 g aspartic acid, 0.13 g cysteine, 0.5 g glutamic acid, 0.15 g histidine, 0.21 g isoleucine, 0.475 g leucine, 0.44 g lysine, 0.275 phenylalanine, 0.675 g proline, 0.34 g serine, 0.225 g threonine, 0.05 g tryptophan, 0.325 g valine, 0.175 g glycine, 0.125 g methionine, 0.1 g asparagine, 0.2 g glutamine, 10 g glucose, 0.5 g L-ascorbic acid, 35 mg adenine sulphate, 27 mg guanine, 22 mg uracil, 50 mg cysteine, 50 mg xanthine, 2.5 mg D-biotin, 1 mg vitamin B12, 1 mg riboflavin, 5 mg pyridoxamine-HCl, 10 µg p-aminobenzoëic acid, 1 mg pantothenate, 5 mg inosine, 1 mg nicotinic acid, 5 mg orotic acid, 2 mg pyridoxine, 1 mg thiamine, 2.5 mg lipoic acid, 5 mg thymidine, 200 mg $MgCl_2$, 50 mg $CaCl_2$, 16 mg $MnCl_2$, 3 mg $FeCl_3$, 5 mg $FeCl_2$, 5 mg $ZnSO_4$, 2.5 mg $CoSO_4$, 2.5 mg $CuSO_4$, 2.5mg $(NH_4)_6Mo_7O_{24}$ (9, 13).

Chemostat cultures were grown in BIOSTAT®Bplus fermentor (Sartorius Stedim Biotech) with a working volume of 750ml at dilution rates of $0.05h^{-1}$, $0.15h^{-1}$ and $0.4h^{-1}$. The pH was kept constant at pH 6.4 by automatic addition of 4M NaOH. Cultivation was carried out under anaerobic condition (60ml/min N_2) with a stirring speed of 250rpm. The cultures were considered in steady state when there was no detectable glucose in the culture supernatants and optical density, cell dry weight, and product concentrations of the cultures were constant in samples taken on two consecutive days. Samples used for metabolite, transcriptomic and

proteomic analyses were taken from cultures grown for six generations after the sample confirming steady state had been taken. All experiments were performed in triplicates.

Culture samples of 20 – 50 mL were centrifuged at 4°C at 6000×g for 10 minutes, and pellets were either flash frozen in liquid nitrogen or treated according to the protocols for measuring the dry weight as previously described (1). Supernatants were frozen at -20^{0}C until metabolite analysis.

Metabolite analyses

Bacterial dry weight was measured as previously described (1). Glucose, pyruvate, lactate, formate, acetate, and ethanol were determined by high-pressure liquid chromatography (HPLC; LKB) with a REZEX organic acid analysis column (Phenomenex) at a temperature of 45ºC with 7.2 mM H_2SO_4 as the eluent, using a RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration (9). Lactate and glucose were also measured by using Megazyme enzymatic kits (Wicklow-Ireland).

Amino acid analysis was performed using the Waters AccQ·Tag Chemistry Package. The analyses were performed on an Agilent 1200 series HPLC equipped with a Hitachi fluorimetric detector, operating at an excitation wavelength of 250 nm and detection of emission at 395 nm. The samples were separated on a 3.9 * x 150 mm AccQ·Tag column at a temperature of 37°C. The sample volume was 5 µl. Amino acid derivatives were obtained and separated according to the standard procedure of the Waters Company. The mobile phase was composed of (A) aqueous buffer containing 1 to 10 parts Waters AccQ·Tag Eluent A solution

and (B) 60% acetonitrile/water solution. Conditions of the gradient elution are described in the AccQ·Tag Chemistry Package. The elution flow rate was 1 ml/min.

RNA isolation, cDNA synthesis and transcriptional analyses

RNA was isolated from flash frozen cell pellets stored at -80 °C as previously described (18). cDNA synthesis, labeling and hybridization was performed according to Opsata et al (22), and transcriptome analyses were done by microarrays as described by Solheim et al (29) with some modification. In brief, on each array the dilution rate 0.15 ($D_{0.15}$) was used as the reference and the two treatments, dilution rate 0.05 ($D_{0.05}$) and dilution rate 0.4 ($D_{0.4}$), were analyzed as direct comparisons to dilution rate 0.15. We also made an indirect comparison between the two treatments by considering the contrast $\log_2 (D_{0.4}/D_{0.15}) - \log_2(D_{0.05}/D_{0.15})$. In all cases an empirical Bayes smoothing of gene-wise variances was conducted according to Smyth et al (28). For each gene, the p-value was adjusted to control the false discovery rate; hence all p-values displayed are FDR-adjusted.

A selection of transcripts were quantified by real-time quantitative PCR (RT-QPCR) analyses as previously described (18). The expression level of 23S RNA was used as an internal control.

Microarray data accession number

The microarray data has been deposited in the ArrayExpression database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-725.

Two-dimensional gel electrophoresis and proteomic analyses

Proteins were isolated from flash frozen pellets stored at -80^{0}C. Protein isolation, two-dimensional gel analyses, quantification and protein identification were performed as previously described (18) .

RESULTS AND DISCUSSION

Growth of *E. faecalis* V583 at three different growth rates

Metabolite analysis

To study the effects of growth rate on the physiology of *E. faecalis* V583 we grew the cells in continous, glucose limited cultures (chemostats) allowing precise regulation of growth rate by the pump speed. The cells were grown at a fast (0.4 h^{-1}), slow (0.05h^{-1}) and intermediate (0.15h^{-1}) growth rate under anaerobic conditions. Metabolite analyses from the experiments are shown in Table 1. Glucose was undetectable in all of the culture supernatants, demonstrating that glucose was the growth limiting factor at all growth rates. Furthermore, the pattern of metabolites formed changed with growth rate. At the lowest dilution rate most of the glucose was converted to formate, ethanol and acetate. The contribution of this pathway decreased with increasing growth rate and homolactic fermentation became dominating.

Homolactic fermentation gives 2 ATP per molecule of glucose, whilst mixed acid fermentation producing ethanol, formate and acetate results in a net production of 3 ATP per glucose. Despite a gradual increase in ATP produced per molecule glucose consumed, as seen in the change from a homofermentative to mixed acid fermentation (Table 1) growth yield declined by lowering the growth rate (Table 1). The apparent increase in ATP yield could not compensate for the relatively higher maintenance demands by lowering the growth rate. Some strains of *L .lactis* make a more abrupt shift towards the mixed acid pathway, and unlike *E. faecalis* V583 they can even display an increase in growth yield in response to growth rate reduction (31).

In batch culture with no glucose limitation the homolactic fermentation was even more dominating than in cultures growing at D=0.4h^{-1} (Table 1), and consequently ATP yield was lower. In a previous work we constructed a mutant with no LDH activity (13). The strain secreted large amounts of formate and ethanol, but produced acetoin instead of acetate in batch cultures and thus no more ATP per glucose than the wild type producing lactate (13). However, in glucose-limited chemostats this mutant produced acetate and no acetoin at low growth rates. High acetoin production was only seen in the mutant, and only when it was grown at high growth rates (data not shown). Apparently, acetoin synthesis represents overflow metabolism in *E. faecalis*. As shown in Table 2 the main contributor to the change in metabolic profile in these experiments was the lactate flux which showed a strong increase with increasing growth rate. Thus he specific *in vivo* LDH activity (lactate flux) in cells growing at the highest growth rate were 20 times higher than in cells growing at the lowest growth rate. The metabolite flux through PFL was much less sensitive to changes in growth rate and varied less than twofold. The specific *in vivo* activity of PFL (flux) was highest in cells grown at D=0.15h^{-1} and almost the same at D=0.4h^{-1} and D=0.05h^{-1}. The lower K_M of PFL than LDH for pyruvate (10) could explain a higher lactate/formate flux ratio by increasing growth rate, but the formate flux maximum at the middle growth rate indicates regulation of enzyme activity (see below).

The changes in catabolism were not restricted to carbohydrate metabolism. As shown in Table 3 number amino acids were broken down at different rates depending on the growth rate. Under all conditions tested more than 90-95 % of serine and arginine were consumed. Genes for degradation of these two amino acids are regulated by carbon catabolite repression (CCR) in *E. faecalis* (2, 15, 22), and the results show as expected that the cells are relieved of CCR when grown under glucose limiting conditions. Arginine degradation provides the cells

with one molecule of ATP per arginine molecule consumed and the amino acid is thus an additional energy source. Serine enters the pyruvate pool after enzymatic conversion by L-serine dehydratase. In *L. lactis* grown in glucose limited chemostats about 10 % of the carbon flow through pyruvate could be derived from serine (21). Serine breakdown could also provide additional ATP, as has been shown in *Staphylococcus epidermidis* (27) and suggested in *L. lactis* (30).

Apart from arginine and serine, tyrosine was the only amino acid degraded to large extent at high growth rate. At lower growth rate breakdown of other amino acids made a significant contribution to carbon flow and their degradation rates were close to proportional to the growth rate (Table 3). Amino acids other than arginine and serine can act as energy sources for *E. faecalis* (32). The differences between the middle and the high growth rates reflect a transition from one lifestyle to another. The two lifestyles are characterized by a great difference in catabolism of amino acids, possibly governed by differences in energy supply.

Transcriptome profiling of cells growing at different rates

To assess the contribution of hierarchical regulation on the flux distribution we undertook expression analysis at both transcriptome and proteome level. Values from cells grown at $D=0.15h^{-1}$ were used as the reference in the transcriptome analyses. A tabulated review of the transcriptional results is presented in Supplemental Table S1. As shown in Figure 1 expression of genes of all functional categories were affected by the changes in growth rate. We found 223 gene transcripts to be altered more than two-fold by changing the growth rate from $0.15h^{-1}$ ($p<0.05$). At the growth rate of $0.4h^{-1}$, 88 genes were found to be upregulated and 84 were downregulated. At the lowest growth rate 62 genes were upregulated and and 82

were downregulated. Of the 77 genes represented by significant differences for both growth rate changes, most showed a maximum or minimum at the middle growth rate (Figure 2). We complemented the microarray analyses by transcription analyses of a selection of genes by RT-QPCR, partly to confirm microarray data but also to investigate transcription of genes for which the microarray data were of inadequate quality. This analysis included transcription of genes encoding pyruvate formate-lyase activating enzyme (*pfl*A, EF1612), a major facilitator-family transporter (EF0082), glyceraldehyde-3-phosphate dehydrogenase (EF1964), bifunctional acetaldehyde-CoA/alcohol dehydrogenase (EF0900), and the principal lactate dehydrogenase (*ldh*-1, EF0255). As shown in Table 4, good agreement was found between the results obtained with microarray and RT-QPCR analyses for EF0900.

Only ten genes showed statistically significant difference in transcription between the lowest and the highest growth rates. These genes encode a putative membrane protein (EF0025), a major facilitator family transporter (EF0082), L-lactate dehydrogenase *ldh*-1 (EF0255), putative bacteriocin immunity protein PlnM, (EF0439), conserved hypothetical protein (EF1541), pyruvate formate-lyase activating enzyme (EF1612), glyceraldehyde-3-phosphate dehydrogenase (EF1964), Cro/CI family transcriptional regulator (EF2291), hypothetical protein (EF2518), and resolvase family site-specific recombinase (EF2283).

The most remarkable result obtained by the transcription analysis was the dramatic effect seen with *ldh*-1 encoding the principal lactate dehydrogenase in *E. faecalis* (13). As shown in Table 4 the *ldh-1* transcript increased with growth rate, and fast growing cells contained about 1700 times more *ldh*-1 transcript than cells growing at the lowest rate. No other transcript showed a comparable response. To our knowledge, a difference in *ldh*-1 transcription of this magnitude has not been previously reported.

Previous work has shown that *ldh*-1 transcription can be regulated by the global regulators redox-sensing regulator (Rex) and the catabolite control protein A (CcpA) (18, 22). However, other genes found to be controlled by these regulators did not show a strong response to changes in growth rate. Additional mechanisms, specifically regulating the level of *ldh*-1 transcripts over a wide dynamic range, appear to be involved in the growth rate response. Whether this involves regulation of transcription, mRNA breakdown or both, is not known.

Effects of growth rate on the proteome and comparison with transcriptional effects

The proteomes of cells grown at the three growth rates were analyzed by 2-D gel electrophoresis. By using 2-D gel software, we were able to detect more than 400 spots on silver stained gels. The analyses identified 56 differentially expressed proteins ($p<0.05$) (Table 5). The proteins most affected by the changes in growth rate were the general stress protein (EF1744) and the tRNA modification GTPase TrmE encoded by EF3312. Both showed their lowest expression at $D=0.15\ h^{-1}$. Altogether 24 proteins showed their lowest abundance at this growth rate. Even the ribosomal proteins 30S ribosomal protein S3 (EF0212) and 50S ribosomal protein L6 (EF0221), showed this behavior. Six proteins showed reduced abundance and 13 showed an increase with growth rate over the range tested. In general, the effects on the protein level were smaller than the transcriptional effect. Of the 56 differentially expressed proteins, the quantity of 21 varied more than twofold between the middle and the high or low growth rates (Table 5). Enzymes involved in energy metabolism were the dominant category (16 proteins).

Surprisingly, the content of the major lactate dehydrogenase, Ldh-1 (EF0255), was the same at the three growth rates (results not shown) despite the huge differences in transcript levels. Thus the synthesis of Ldh-1 appears to be controlled by a post-transcriptional mechanism. The amount of bifunctional acetaldehyde-CoA/alcohol dehydrogenase (EF0900) was also constant (data not shown), although the transcript levels varied. Other discrepancies between data from transcriptional and proteomic analyses were seen for enzymes such as glyceraldehyde 3-phosphate dehydrogenase (EF1964) and PflA, pyruvate formate-lyase activating enzyme (EF1612). The downregulation of PflA expression is in line with the reduction of formate flux when growth rate was increased from $0.15h^{-1}$ and $0.4h^{-1}$ (Table 2). Evidence for post-transcriptional regulation of enzymes in energy metabolism has previously been observed in *L. lactis* (5), *Escherichia coli* (14), and in our study of the Ldh mutant of *E. faecalis (18)*.

In conclusion the expression of a number of genes and proteins varied with growth rate. Many showed a maximum or minimum of expression at the intermediate growth rate, coinciding with a shift in energy metabolism. The cells shifted towards a more energy efficient catabolism upon transition from the highest to the intermediate growth rate, possibly triggered by lowered energy status in the cells. The best studied mechanism for transcription regulation in response to energy status in *Firmicutes* is CCR (4), but as already mentioned, even the fast growing cultures in our experiments were relieved of CCR due to glucose limitation. Thus the differences in metabolism, transcriptome and proteome seen in our experiments reflect the involvement of other regulatory mechanisms. Regulation of expression beyond the stage of transcription appears to be common, most strikingly seen with *ldh*-1 expression. High LDH activity is the hallmark of a lactic acid bacterium and is a prerequisite for its competitiveness in natural habitats. Its activity is regulated by several mechanisms, including biochemical

regulation of enzyme activity and genetic regulation. In this work we have demonstrated the presence of a new transcriptional and a post-transcriptional mechanism with the potential to exert a strong control on LDH activity. Under the conditions used in these experiments these mechanisms counteract each other to maintain a constant enzyme level, but high transcript levels of *ldh*-1 might be an important part of the strategy of a lactic acid bacterium to succeed in a (rapidly) changing environment.

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Table 1. Metabolite production of *E. faecalis* V583 grown under different conditions.

Condition	Dilution rate (h^{-1})	Dry weight g/L	Concn (mM) ± SD					ATP yield
			Glucose[b]	Ethanol	Acetate	Lactate	Formate	
Chemostat	0.05	1.45	0	32.8 ± 2.8	36.3 ± 2.1	27.7 ± 2.2	67.4 ± 2.1	2,5 ± 0,1
Chemostat	0.15	1.55	0	26.2 ± 5.1	20.3 ± 8.0	64.2 ± 1.1	46.3 ± 7.3	2,3±0,4
Chemostat	0.4	1.78	0	6.5 ± 0. 9	8.2 ± 2.1	97.3 ± 1.4	16.3 ± 0.5	2,1 ± 0,1
Batch culture[a]		1.1	25.5	3.2	0	60.1	6.5	1.9

[a]Data from (12). [b]Glucose left in supernatant. Growth medium contained 55.5mM glucose and 16 mM acetate.

Table 2. Carbon flux in *Enterococcus faecalis* V583 at different growth rates.

	Flux (mmol/g dw/h^{-1}) ± SD		
Metabolite	$0.05h^{-1}$	$0.15h^{-1}$	$0.4h^{-1}$
Glucose	2.0 ± 0.1	5.6 ± 0.1	12.5± 0.1
Ethanol	1.3 ± 0.1	2.5 ± 0.5	1.5 ± 0.2
Acetate	1.2 ± 0.2	2.1 ± 0.1	1.8 ± 0.5
Lactate	1.0 ± 0.1	6.4 ± 0.1	21.6 ± 0.4
Formate	2.4 ± 0.1	4.5 ± 0.7	3.6 ± 0.1

Table 3. Amino acid flux and rate ratio in *Enterococcus faecalis* V583 grown in different growth rates.

	Flux mmol(g dw)$^{-1}$ h^{-1}			Flux/growth rate mmol(g dw)$^{-1}$		
Dilution rate	0.05 h^{-1}	0.15 h^{-1}	0.4 h^{-1}	Flux/0.05 h^{-1}	Flux/0.15 h^{-1}	Flux/0.4 h^{-1}
Asparagine	0.031±0.002	0.093±0.033	0.028±0.035	0.62±0.05	0.62±0.31	0.07±0.01
Arginine	0.013±0.002	0.037±0.003	0.081±0.012	0.26±0.05	0.25±0.02	0.20±0.04
Alanine	0.003±0.001	0.006±0.009	0.002±0.001	0.06±0.02	0.04±0.08	0.00±0.01
Glutamine	0.041±0.004	0.112±0.044	0.004±0.025	0.82±0.11	0.75±0.41	0.01±0.08
Glycine	0.074±0.005	0.184±0.022	0.114±0.039	1.48±0.14	1.27±0.20	0.28±0.13
Histidine	0.021±0.001	0.055±0.011	0.018±0.025	0.42±0.02	0.37±0.10	0.04±0.08
Isoleucine	0.022±0.001	0.065±0.010	0.054±0.019	0.44±0.02	0.44±0.10	0.13±0.06
Leucine	0.047±0.002	0.141±0.024	0.127±0.052	0.94±0.05	0.94±0.22	0.31±0.18
Lysine	0.015±0.003	0.042±0.022	0.000±0.021	0.3±0.08	0.28±0.20	0±0.07
Proline	0.058±0.036	0.133±0.075	0.000±0.03	1.16±1.01	0.89±0.71	0±0.10
Phenylalanine	0.025±0.002	0.073±0.011	0.057±0.011	0.5±0.05	0.49±0.10	0.14±0.03
Serine	0.090±0.001	0.248±0.010	0.499±0.135	1.8±0.02	1.66±0.10	1.24±0.47
Threonine	0.005±0.001	0.021±0.014	0.001±0.016	0.1±0.02	0.14±0.13	0.00±0.05
Tyrosine	0.026±0.008	0.055±0.001	0.096±0.056	0.52±0.02	0.37±0.01	0.24±0.19
Valine	0.042±0.003	0.132±0.029	0.017±0.005	0.84±0.08	0.88±0.27	0.04±0.01

Table 4. Effect of growth rate on transcription analyzed by qRT-PCR.

ORF	Gene	Gene	0.05/0.15 $\log_2$	0.4/0.15 $\log_2$
EF0255	*ldh*-1	Lactate dehydrogenase	-3.88±0.14	6.88±0.32
EF1612	*pfl*A	Pyruvate formate-lyase activating enzyme	-0.89±0.03	0.14±0.09
EF0082		Major facilitator family transporter	-2.80±0.13	-0.16±0.06
EF1964	*gap*-2	Glyceraldehyde-3-phosphate dehydrogenase	0.17±0.04	2.01±0.10
EF0900	*adh*E	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	-1.06±0.19	-1.5±0.13

Figure 1. Genes differently expressed by changes in growth rate (0.05 to 0.15h $^{-1}$ 0.4 to 0.15h $^{-1}$). The genes are organized by functional category.

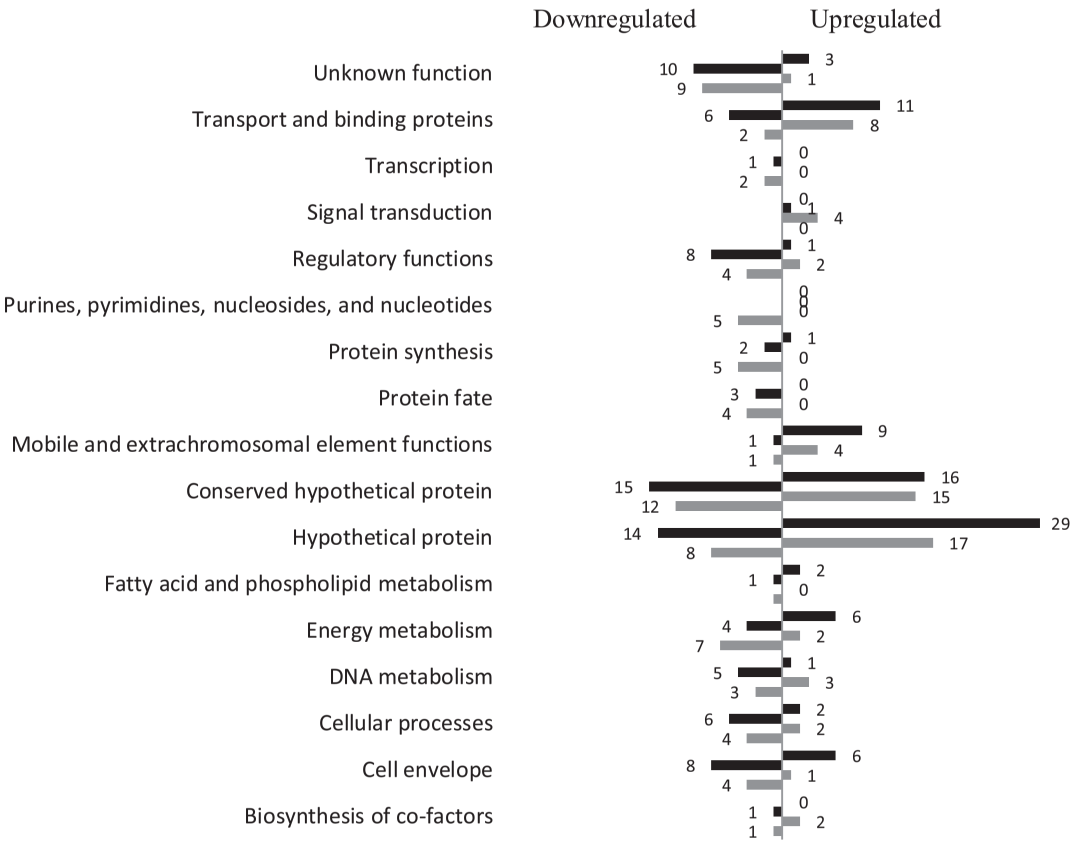

Figure 2. Average expression of genes differentially expressed at three growth rates. (A) Genes repressed by statistically significant data at all growth rates. (B). Genes represented by statistically significant differences between two growth rates only. Gene transcripts showing increasing (⋯⋯) or decreasing (– –) abundance at the middle growth rate. Gene transcripts increasing (——) or decreasing (——) with growth rate. * Genes analysed by qRT-PCR.

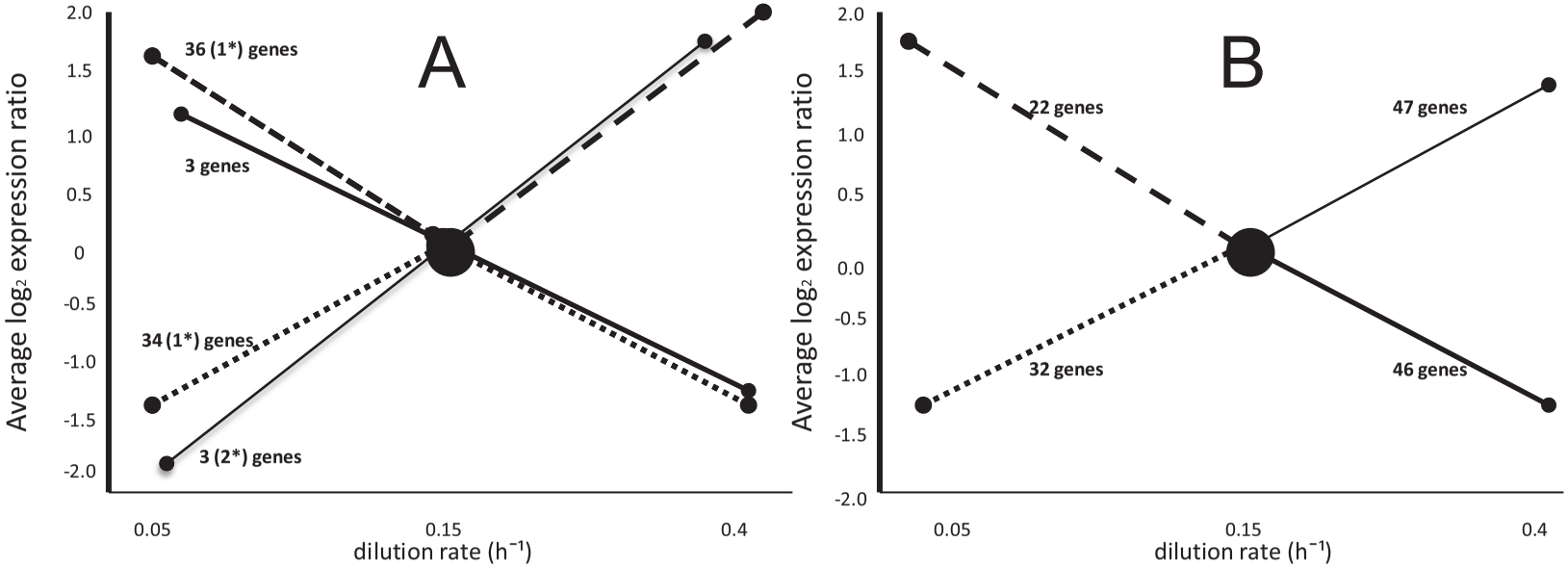

Table 5. Proteins differentially expressed by change in growth rates.

ID	Name	log_2 0.05/0.15	log_2 0.4/0.15
Amino acid biosynthesis			
EF2550	Serine hydroxymethyltransferase	-1.04	-0.79
Biosynthesis of cofactors, prosthetic group, carriers			
EF1860	3-methyl-2-oxobutanoate hydroxy methyltransferase	0.52	0.75
EF0901	isopentenyl diphosphate delta isomerase, putative	0.63	1.1
Cell envelope			
EF2193	dTDP-4-dehydrorhamnose 3,5-epimerase	-0.30	0.79
EF2194	Glucose-1-phosphate thymidyltransferase	-0.26	0.59
EF3183	Cell wall surface anchor family protein	1.00	0.44
Cellular processes			
EF0080	gls24 protein	-0.15	-0.5
EF1744	General stress protein	1.30	1.46
EF1991	Cold shock protein CspC	-0.25	0.42
EF3312	tRNA modification GTPase TrmE	1.01	1.63
DNA metabolism			
EF0002	DNA polymerase III, beta subunit	0.65	0.32
EF0883	Primosomal DnaI	-0.60	1.15
Energy metabolism			
EF0020	PTS system, manose specific IIAB components	-0.15	-1.3
EF0195	Phosphoglycerate mutase 1	-0.33	0.52
EF0949	Phosphotransacetylase	0.30	0.54
EF1125	Putative L-ascorbate 6-phosphate lactonase	0.02	0.61
EF1131	L-ribulose-5-phosphate 4-epimerase	0.39	0.75
EF1167	Fructose-bisphosphate aldolase	0.77	0.24
EF1353	Pyruvate dehydrogenase complex E1 component, alpha subunit	0.30	0.94
EF1354	Pyruvate dehydrogenase complex E1 component, beta subunit	-0.12	0.65
EF1356	Dihydrolipoamide dehydrogenase	-0.53	-1.64
EF1416	Glucose-6-phosphatase isomerase	-0.02	-0.89
EF1526	Glyceraldehyde-3-phosphate dehydrogenase	0.08	-0.95
EF1612	Pyruvate formate-lyase activating enzyme	0.24	-1.18
EF1962	Triosephosphate isomerase	0.04	0.73
EF1963	Phosphoglycetare kinase	-0.37	-0.2
EF1964	Glyceraldehyde-3-phosphate dehydrogenase	0.35	-1.07
EF0283	3-oxoacyl-(acyl-carrier-protein) synthethase II	-0.12	-0.91
Fatty acid and phospholipid metabolism			
EF2875	Acetyl-CoA carboxylase subunit alpha	0.00	0.89
EF2881	3-ketoacyl-(acyl-carrier-protein) reductase	-0.36	1.10
EF2882	Acyl-carrier-protein S-malonyltransferase	0.00	0.54
Hypothetical proteins			
EF1227	Hypothetical protein	-0.99	0.26
EF1241	Hypothetical protein	-0.14	0.11
EF1560	Hypothetical protein	0.47	0.91

Protein fate			
EF2200	Methionine aminopeptidase	-0.48	0.70
F2898	Peptidyl-prolyl cis trans isomerase	0.75	0.74
EF3066	Peptide deformylase	0.81	1.48
Protein synthesis			
EF0212	30S ribosomal protein S3	0.97	1.39
EF0221	50S ribosomal protein L6	0.60	0.72
EF0701	Peptide chain release factor 3	0.36	1.22
Purines, pyrimidines, nucleosides, and nucleotides			
EF0228	Adenylate kinase	0.61	1.59
EF1713	Orotidine 5'-phosphate decarboxylase	0.57	0.91
EF1721	Bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase	-1.16	0.61
EF2549	Uracil phosphoribosyltransferase	0.04	0.60
EF3293	Inositol-5-monophosphate dehydrogenase	0.08	-0.79
Regulatory functions*, Signal transduction#, Transcription" and Transport and binding proteins¤			
EF1741*	Catabolite control protein A	0.25	0.38
EF3289*	DNA-binding response regulator	0.82	1.31
EF1050#	DNA-binding response regulator	0.38	1.04
EF0233"	DNA-direct RNA polymerase subunit alpha	0.50	-0.26
EF0865¤	Glycine betaine/carnitine/choline transporter, ATP-binding protein	-0.24	-0.54
Unknown function			
EF0076	Oxidoreductase short chain dehydrogenase/reductase family	0.31	-1.64
EF0877	Aldo/keto reductase family oxidoreductase	-0.64	0.48
EF1138	Aldo/keto reductase family oxidoreductase	-0.45	0.23
EF2591	Glyoxalase family protein	0.54	1.07
EF2656	Flavoprotein family protein	0.97	1.16
EF2927	HAD superfamily hydrolase	0.92	2.04

Supplemental Table S1. Genes differentially expressed by change in growth rate. Data from microarray analyses. Significantly regulated genes are q<0.05 (bold). "NA" denotes non expressed or excluded genes. *Genes statistically differences in transcription between the lowest and the higher growth rates.

			0.05/0.15		0.4/0.15	
ID	**Gene**	**Name**	**log_2**	**qVal.**	**log_2**	**qVal.**
Biosynthesis of cofactors, prosthetic group, carriers						
EF0051	*isp*E	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	0.43	0.44	**-1.43**	**0.01**
EF1858	*pan*D	Aspartate 1-decarboxylase	**-1.64**	**0.04**	-1.24	0.09
EF2776	*thi*E	Thiamine-phosphate pyrophosphorylase	**2.21**	**0.04**	0.73	0.62
EF2777		Hydroxyethylthiazole kinase	**1.70**	**0.04**	0.43	0.73
Cell envelope						
EF0025*		Membrane protein. putative	**-1.00**	**0.02**	**0.60**	**0.02**
EF0235		Membrane protein. putative	0.24	0.70	**1.11**	**0.04**
EF0370		Septation ring formation regulator EzrA	**-1.31**	**0.01**	**-1.44**	**0.00**
EF0490		LPX-motif cell wall anchor domain protein	**2.07**	**0.00**	**3.05**	**0.00**
EF0516		Transcriptional regulator, putative	0.58	0.49	**1.59**	**0.04**
EF0989	*mra*W	S-adenosyl-methyltransferase MraW	-0.50	0.32	**-1.14**	**0.02**
EF0991	*pbp*C	Penicillin-binding protein	-0.74	0.28	**-1.69**	**0.03**
EF1583		N-acetylmuramoyl-L-alanine amidase	-0.97	0.10	**-1.29**	**0.02**
EF1740		Penicillin-binding protein 1, putative	0.78	0.17	**1.23**	**0.02**
EF1751		Membrane protein. putative	**-1.12**	**0.02**	**-0.54**	**0.22**
EF2367		N-acetylmuramoyl-L-alanine amidase	-0.69	0.13	**-1.29**	**0.01**
EF2891		Glycosyl transferase, group 1	-0.77	0.13	**-1.02**	**0.04**
EFB0051		Membrane protein, putative	0.33	0.58	**1.34**	**0.02**
Cellular processes						
EF0439*		Immunity protein PlnM, putative	**0.50**	**0.02**	**-1.00**	**0.00**
EF0990		Cell division protein	-0.37	0.52	**-1.29**	**0.01**
EF1151		Cell division protein DivIVA	-1.19	0.17	**-1.81**	**0.03**
EF2052		Cell division protein FtsK	**-1.61**	**0.02**	**-2.42**	**0.03**
EF2387		ParA family chromosome partitioning ATPase	**-1.08**	**0.02**	**-1.51**	**0.01**
EF2739	*ahp*C	Alkyl hydroperoxide reductase	**-1.59**	**0.02**	-0.56	0.40
EFA0007		Ribosomal RNA adenine dimethylase family protein	**-0.50**	**0.18**	**-1.52**	**0.00**
EFA0042		Cell wall surface anchor signal protein	**1.16**	**0.05**	**2.33**	**0.00**
EFB0012	*prg*C	Surface protein PrgC	1.03	0.24	**1.45**	**0.03**
Conserved hypothetical protein						
EF0003		Conserved hypothetical protein	**-1.00**	**0.01**	**-0.72**	**0.04**
EF0145		Conserved hypothetical protein	**1.10**	**0.05**	**1.50**	**0.03**
EF0349		Conserved hypothetical protein	**1.63**	**0.02**	**2.40**	**0.01**
EF0350		Conserved hypothetical protein	**1.49**	**0.04**	**2.56**	**0.00**
EF0469		Conserved hypothetical protein	**-1.19**	**0.01**	**-1.16**	**0.00**
EF0486		Conserved hypothetical protein	**2.00**	**0.02**	**2.46**	**0.00**
EF0487		Conserved hypothetical protein	**2.64**	**0.02**	**1.54**	**0.03**
EF0491		Conserved hypothetical protein	**2.17**	**0.00**	**3.42**	**0.00**
EF0500		Conserved hypothetical protein	**2.50**	**0.00**	**3.67**	**0.00**
EF1026		Conserved hypothetical protein	-0.48	0.30	**-1.35**	**0.00**

EF1541*		Conserved hypothetical protein	**-1.27**	**0.02**	**0.84**	**0.23**
EF1682		Conserved hypothetical protein	**-1.39**	**0.05**	**-1.37**	**0.05**
EF1752		Conserved hypothetical protein	**-1.42**	**0.01**	**-1.08**	**0.02**
EF1753		Conserved hypothetical protein	**-1.46**	**0.03**	**-1.3**	**0.04**
EF1861		Conserved hypothetical protein	**-1.51**	**0.04**	**-1.07**	**0.12**
EF2281		Conserved hypothetical protein	-0.02	0.97	**-1.33**	**0.00**
EF2437		Conserved hypothetical protein	**-3.35**	**0.00**	**-2.55**	**0.00**
EF2604		Conserved hypothetical protein	0.62	0.35	**1.67**	**0.01**
EF2606		Conserved hypothetical protein	**-1.06**	**0.02**	**-0.85**	**0.05**
EF2770		Conserved hypothetical protein	**2.19**	**0.01**	1.05	0.27
EF2923		Conserved hypothetical protein	-0.31	0.60	**-1.37**	**0.01**
EF2965		Conserved hypothetical protein	**-1.82**	**0.00**	-0.65	0.18
EF3009		Conserved hypothetical protein	**1.18**	**0.02**	**1.63**	**0.01**
EF3115		Conserved hypothetical protein	-0.53	0.13	**-1.22**	**0.00**
EF3151		Conserved hypothetical protein	-0.58	0.24	**-1.25**	**0.01**
EFA0035		Conserved hypothetical protein	**2.36**	**0.05**	**1.93**	**0.02**
EFA0036		Conserved hypothetical protein	**1.85**	**0.03**	**3.5**	**0**
EFA0037		Conserved hypothetical protein	**2.17**	**0.03**	**1.56**	**0.02**
EFA0048		Conserved hypothetical protein	**1.99**	**0.04**	**1.26**	**0.05**
EFA0065		Conserved hypothetical protein	**-1.32**	**0.02**	**-1.77**	**0.00**
EFA0066		Conserved hypothetical protein	**-0.95**	**0.01**	**-1.53**	**0.00**
EFB0013		Conserved hypothetical protein	**1.97**	**0.02**	**2.38**	**0.01**
EFB0033		Conserved hypothetical protein	**1.82**	**0.02**	**2.69**	**0.00**
DNA metabolism						
EF0004		recF protein	**-1.32**	**0.00**	**-1.02**	**0.01**
EF0005		DNA gyrase, B subunit	**-1.06**	**0.02**	**-0.86**	**0.04**
EF0325		DNA polymerase I, putative	**1.3**	**0.03**	0.67	0.31
EF0499	*ssb*-2	Single-strand binding protein	**1.29**	**0.04**	**1.95**	**0.01**
EF0762	*uvr*B	Excinuclease ABC, subunit B	0.68	0.20	**-1.29**	**0.01**
EF1521	*dna*G	DNA primase	**-0.73**	**0.02**	**-1.68**	**0**
EF2283*		Resolvase family site-specific recombinase	**1.26**	**0.00**	**-0.356**	**0.02**
Energy metabolism						
EF0900	*ald*E	Aldehyde-alcohol dehydrogenase	**-1.30**	**0.00**	**-1.00**	**0.01**
EF0956	*mal*B/*bop*E	Beta-phosphoglucomutase	**1.20**	**0.03**	0.31	0.67
EF1109		Iron-sulfur cluster binding protein	**-2.12**	**0.03**	-1.39	0.14
EF1125		Putative L-ascorbate 6-phosphate lactonase	**-1.45**	**0.10**	**-2.54**	**0.00**
EF1211	*npr*	NADH peroxidase	**-1.10**	**0.01**	-0.65	0.09
EF1356	*lpd*A	Pyruvate dehydrogenase complex. E3 component. Lipoamide dehydrogenase	-0.38	0.53	**-1.22**	**0.02**
EF1568		Prephenate dehydratase	**-1.53**	**0.02**	-0.31	0.68
EF2058		Transport P-binding protein CydC, putative	**-1.00**	**0.03**	-0.20	0.70
EF2059		Transport P-binding protein CydD, putative	**-1.34**	**0.01**	-0.85	0.06
EF2559		Pyruvate flavodoxin/ferredoxin oxidoreductase	0.67	0.30	**-1.19**	**0.05**
EF2863		Beta-N-acetylglucosaminidase, putative	**1.28**	**0.01**	-0.17	0.75
EF3317		Oxaloacetate decarboxylase, alpha subunit	-0.02	0.96	**1.01**	**0.01**
EF3319	*cit*F	Citrate lyase, alpha subunit	-0.17	0.75	**1.00**	**0.03**

EF3321	*cid*D	Citrate lyase, gamma subunit	-0.24	0.54	**1.00**	**0.01**
EF3322	*cit*C	Citrate lyase ligase	-0.05	0.94	**1.13**	**0.02**
EF3324		Sodium ion-translocating decarboxylase, beta subunit	0.18	0.76	**1.92**	**0.00**
EF3325		Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	-0.17	0.83	**1.50**	**0.02**
Fatty acid and phospholipid metabolism						
EF0779		Glycerophosphoryl diester phosphodiesterase family protein	0.44	0.32	**1.10**	**0.01**
EF1608		Cardiolipin synthetase, putative	0.70	0.12	**1.79**	**0.00**
EF2618		Carboxylesterase precursor, putative	**-1.21**	**0.00**	**-0.99**	**0.01**
Hypothetical protein						
EF0083		Hypothetical protein	**-1.56**	**0.03**	**0.54**	**0.43**
EF0128		Hypothetical protein	**-1.05**	**0.04**	**-1.30**	**0.01**
EF0340		Hypothetical protein	1.46	0.10	**2.31**	**0.03**
EF0341		Hypothetical protein	1.49	0.07	**2.04**	**0.04**
EF0477		Hypothetical protein	-0.53	0.24	**-1.59**	**0.00**
EF0532		Hypothetical protein	-0.76	0.25	**-1.34**	**0.04**
EF0566		Hypothetical protein	-0.79	0.10	**-1.40**	**0.02**
EF0573		Hypothetical protein	**1.71**	**0.01**	NA	NA
EF0605		Hypothetical protein	-0.63	0.46	**-2.23**	**0.00**
EF0723		Hypothetical protein	0.41	0.35	**1.09**	**0.01**
EF0769		Hypothetical protein	-0.60	0.19	**-1.12**	**0.01**
EF0778		Hypothetical protein	0.66	0.22	**1.25**	**0.02**
EF0908		Hypothetical protein	0.97	0.06	**1.77**	**0.00**
EF1067		Hypothetical protein	-0.10	0.83	**-1.22**	**0.00**
EF1095		Hypothetical protein	0.57	0.49	**1.73**	**0.02**
EF1107		Hypothetical protein	**-1.76**	**0.00**	0.65	0.14
EF1262		Hypothetical protein	0.68	0.17	**1.10**	**0.02**
EF1263		Hypothetical protein	0.54	0.24	**1.11**	**0.01**
EF1309		Hypothetical protein	**-1.28**	**0.02**	-0.94	0.08
EF1315		Hypothetical protein	**-0.98**	**0.04**	0.32	0.52
EF1365		Hypothetical protein	0.76	0.14	**1.32**	**0.01**
EF1375		Hypothetical protein	**1.06**	**0.01**	**1.32**	**0.00**
EF1418		Hypothetical protein	-1.05	0.10	**-2.48**	**0.00**
EF1774		Hypothetical protein	0.40	0.43	**1.08**	**0.02**
EF1946		Hypothetical protein	-0.23	0.67	**-1.09**	**0.02**
EF1947		Hypothetical protein	-0.50	0.41	**-1.35**	**0.02**
EF2169		Hypothetical protein	0.40	0.67	**1.83**	**0.02**
EF2229		Hypothetical protein	**1.4**	**0.04**	0.63	0.44
EF2231		Hypothetical protein	**2.01**	**0.00**	0.97	0.13
EF2287		Hypothetical protein	**1.58**	**0.03**	1.17	0.16
EF2317		Hypothetical protein	0.45	0.51	**-1.47**	**0.02**
EF2366		Hypothetical protein	-0.64	0.13	**-1.39**	**0.00**
EF2368		Hypothetical protein	**-1.01**	**0.04**	**-1.18**	**0.01**
EF2466		Hypothetical protein	**1.59**	**0.01**	**1.72**	**0.00**
EF2467		Hypothetical protein	**1.23**	**0.02**	-0.43	0.42

EF2490	*cps*F	Hypothetical protein	0.23	0.69	**1.16**	**0.02**
EF2518*		Hypothetical protein	**-0.63**	**0.02**	**1.13**	**0.01**
EF2588		Hypothetical protein	-0.08	0.89	**1.16**	**0.01**
EF2619		Hypothetical protein	**-2.19**	**0.02**	**-2.91**	**0.05**
EF2707		Hypothetical protein	0.79	0.33	**2.22**	**0.02**
EF2768		Hypothetical protein	**2.40**	**0.01**	1.10	0.29
EF2967		Hypothetical protein	**2.13**	**0.04**	**3.06**	**0.01**
EF3087		Hypothetical protein	**1.92**	**0.00**	**2.46**	**0.00**
EF3088		Hypothetical protein	**1.85**	**0.00**	**2.41**	**0.00**
EF3101		Hypothetical protein	**1.15**	**0.05**	**1.61**	**0.02**
EF3247		Hypothetical protein	0.69	0.17	**1.07**	**0.03**
EF3323		Hypothetical protein	**0.5**	**0.04**	**2.32**	**0.00**
EFA0017		Hypothetical protein	**1.02**	**0.02**	**1.21**	**0.00**
EFA0029		Hypothetical protein	1.72	0.08	**1.99**	**0.01**
EFA0050		Hypothetical protein	**1.95**	**0.02**	**1.69**	**0.03**
EFA0051		Hypothetical protein	**1.9**	**0.03**	**2.12**	**0.02**
EFB0015		Hypothetical protein	**1.63**	**0.01**	**2.78**	**0.00**
Mobile and extrachromosomal element functions						
EF0125		IS256 transposase	**1.3**	**0.02**	**1.4**	**0.01**
EF0351		Structural protein	**1.59**	**0.03**	**1.99**	**0.02**
EF0355	*atl*B	Endolysin	**1.83**	**0.03**	**1.98**	**0.02**
EF2145		Integrase, phage family	**-1.5**	**0.00**	**-1.48**	**0.00**
EF2173		ISEf1, transposase	**1.51**	**0.02**	**1.39**	**0.03**
EFA0001	*tra*A-1	Replication-associated protein RepA	1.38	0.10	**1.2**	**0.04**
EFA0002	*tra*B-1	Pheromone shutdown protein TraB	1.10	0.10	**2.15**	**0.00**
EFA0003	*tra*C-1	traC protein	0.56	0.38	**1.59**	**0.01**
EFA0083	*rep*B-1	Replication-associated protein RepB	0.58	0.32	**1.25**	**0.02**
EFB0063	*prg*N	Replication control protein PrgN	1.39	0.09	**1.67**	**0.04**
Protein fate						
EF0302		Aminopeptidase C	-0.59	0.26	**-1.04**	**0.03**
EF0306		Cro/CI family transcriptional regulator	-0.71	0.11	**-1.01**	**0.02**
EF1307	*grp*E	Heat shock protein GrpE	**-1.49**	**0.03**	-0.70	0.30
EF1308	*dna*K	dnak protein	**-1.31**	**0.04**	-1.09	0.07
EF1763	*sec*A	Preprotein translocase, SecA subunit	**-1.73**	**0.04**	**-2.59**	**0.05**
EF2634	*gro*ES	Chaperonin, 10 kDa	**-1.29**	**0.02**	-0.26	0.66
Protein synthesis						
EF0206	*rpl*C	50S ribosomal protein L3	**-1.18**	**0.00**	**-0.87**	**0.02**
EF0633	*try*S-1	Tyrosyl-tRNA synthetase	-0.68	0.17	**1.19**	**0.01**
EF1694	npsP	Ribosomal protein S16	**-1.19**	**0.01**	0.05	0.95
EF2715	*rpl*L	Ribosomal protein L7/L12	**-1.23**	**0.01**	0.15	0.77
EF2716	*rpl*J	50S ribosomal protein L10	**-1.32**	**0.02**	-0.40	0.47
EF2731	*rpm*G-2	Ribosomal protein L33	**-1.08**	**0.01**	**-1.47**	**0.00**
Purines. pyrimidines. nucleosides. and nucleotides						
EF1714	*pyr*D	Dihydroorotate dehydrogenase	**-1.44**	**0.03**	-0.36	0.61
EF1715	*pyr*DII	Dihydroorotate dehydrogenase electron transfer subunit	**-1.63**	**0.02**	-0.61	0.38

EF1716	*car*B	Carbamoyl-phosphate synthase, large subunit	**-1.72**	**0.02**	-0.86	0.19
EF1717	*pyra*A	Carbamoyl-phosphate synthase, small subunit	**-1.64**	**0.04**	-0.85	0.27
EF1720		Uracil permease	**-1.39**	**0.05**	-0.48	0.50
Regulatory functions						
EF0129		Transcription regulator	-0.85	0.08	**-1.36**	**0.01**
EF0869		Cro/CI family transcriptional regulator	-0.67	0.13	**-1.06**	**0.02**
EF1297		PadR family transcriptional regulator	-1.05	0.12	**-1.84**	**0.01**
EF1306	*hrc*A	Heat-inducible transcription repressor HrcA	**-1.64**	**0.01**	-0.40	0.55
EF1357		AraC family transcriptional regulator	-0.28	0.68	**-1.64**	**0.04**
EF1579	*lex*A	LexA repressor	-0.51	0.16	**-1.10**	**0.00**
EF1709		GntR family sugar-binding transcriptional regulator	**-1.31**	**0.01**	**-0.95**	**0.05**
EF2291*		Cro/CI family transcriptional regulator	**1.16**	**0.01**	**-0.86**	**0.03**
EF2966		BglG family transcriptional antiterminator	**-1.74**	**0.00**	-0.22	0.70
EFA0071		PemK family protein	**-0.75**	**0.05**	**-1.00**	**0.01**
EFB0005.1		Transcriptional regulator, Cro/CI family	**0.99**	**0.05**	**2.02**	**0.00**
Signal transduction						
EF0406		PTS system, component	**1.74**	**0.04**	1.28	0.08
EF0958	*mak*T	PTS system component	**1.33**	**0.03**	1.20	0.08
EF1126		PTS system component	-0.64	0.53	**-2.3**	**0.02**
EF2598		PTS system component	**2.59**	**0.02**	1.80	0.06
EFA0067		PTS system. IIABC components	**1.13**	**0.02**	0.06	0.97
Transcription						
EF2617	*vac*B	Ribonuclease R	**-1.09**	**0.02**	-0.80	0.07
EF2729	*nus*G	Transcription antitermination protein NusG	**-2.24**	**0.00**	**-1.81**	**0.00**
Transport and binding proteins						
EF0022		PTS system component	0.67	0.29	**1.42**	**0.02**
EF0246		Amino acid ABC transporter, ATP-binding protein	0.49	0.36	**1.06**	**0.04**
EF0431		TRAP dicarboxylate transporter DctM subunit, putative	**2.13**	**0.02**	**1.67**	**0.04**
EF0475	*feo*A	Ferrous iron transport protein A, putative	-0.45	0.52	**-1.33**	**0.03**
EF0909		Peptide ABC transporter, permease	0.30	0.56	**1.09**	**0.02**
EF0938		Sugar ABC transporter ATP-binding protein	**-1.31**	**0.01**	-0.69	0.11
EF1027		Putative transport protein SgaT protein	NA	NA	**-2.5**	**0.02**
EF1127	*ula*A	PTS system ascorbate-specific transporter subunit IIC	-0.38	0.77	**-2.5**	**0.02**
EF1398		Molybdenum ABC transporter, permease protein	**1.4**	**0.03**	0.09	0.93
EF2232		Sugar ABC transporter, permease	**1.97**	**0.01**	1.44	0.16
EF2233		Sugar ABC transporter, permease	**1.62**	**0.01**	0.54	0.47
EF2394		ABC transporter ATP-binding protein	-0.46	0.21	**-1.00**	**0.01**
EF2650		Spermidine/putrescine ABC transporter, permease	0.35	0.53	**1.05**	**0.04**
EF2720		ABC transporter ATP-binding protein	**-1.81**	**0.00**	**-1.22**	**0.02**
EF2769		ABC transporter ATP-binding protein	**2.51**	**0.00**	0.70	0.44
EF2992		Major facilitator family transporter	**1.14**	**0.02**	0.43	0.46
EF3004		Sulfate permease family protein	0.55	0.20	**1.41**	**0.01**
EF3015		Serine/threonine transporter SstT	**0.98**	**0.05**	**1.51**	**0.01**
EF3099		Transporter accessory protein. putative	-0.13	0.93	**-3.09**	**0.03**

EF3108		Peptide ABC transporter, permease	0.08	0.87	**1.00**	**0.02**
EF3277		Cytosine permease, putative	**1.46**	**0.01**	**2.57**	**0.00**
EFA0014		Drug resistance transporter. putative	0.75	0.27	**1.78**	**0.00**
EFB0050		Toxin ABC transporter. ATP-binding/permease protein	0.02	0.98	**1.17**	**0.03**
Unknown function						
EF0468		lemA protein	**-1.37**	**0.01**	**-1.05**	**0.04**
EF0512		DNA-damage-inducible protein. putative	**-0.79**	**0.03**	**-1.11**	**0.00**
EF0796		Type 2 phosphatidic acid phosphatase family protein	1.20	0.08	**1.57**	**0.02**
EF0856		Aldo/keto reductase family protein	**-1.11**	**0.02**	**-1.00**	**0.03**
EF1025		CBS domain-containing protein	-0.64	0.24	**-1.29**	**0.01**
EF1108		Oxidoreductase, putative	**-1.62**	**0.00**	-0.84	0.07
EF1110		YkgG family protein	**-1.52**	**0.01**	-0.50	0.37
EF2214		Glyoxylase family protein	1.05	0.08	**1.28**	**0.03**
EF2436	*mur*Q	N-acetylmuramic acid-6-phosphate etherase	**-1.56**	**0.04**	**-1.49**	**0.04**
EF2577		Aspartate/ornithine carbamoyltransferase family protein	**1.04**	**0.03**	0.32	0.51
EF2738	*ahp*F	Thioredoxin reductase	**-1.43**	**0.01**	-0.96	0.06
EF2756	*din*P	DNA-damage-inducible protein P	-0.78	0.26	**-1.34**	**0.04**
EF2874		HAD superfamily hydrolase	**-1.22**	**0.04**	**-1.17**	**0.04**
EF2933		Redox-sensing transcriptional repressor Rex	-0.64	0.12	**-0.95**	**0.02**
EF3114		DAK2 domain-containing protein	-0.16	0.68	**-1.00**	**0.01**
EF3311	*gid*A	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	0.83	0.16	**1.34**	**0.02**
EFA0072		PemI family protein	**-0.95**	**0.01**	**-1.48**	**0.00**

PAPER III

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Characterization of Three Lactic Acid Bacteria and Their Isogenic *ldh* Deletion Mutants Shows Optimization for Y_{ATP} (Cell Mass Produced per Mole of ATP) at Their Physiological pHs▽†

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Several lactic acid bacteria use homolactic acid fermentation for generation of ATP. Here we studied the role of the lactate dehydrogenase enzyme on the general physiology of the three homolactic acid bacteria *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*. Of note, deletion of the *ldh* genes hardly affected the growth rate in chemically defined medium under microaerophilic conditions. However, the growth rate was affected in rich medium. Furthermore, deletion of *ldh* affected the ability for utilization of various substrates as a carbon source. A switch to mixed acid fermentation was observed during glucose-limited continuous growth and was dependent on the growth rate for *S. pyogenes* and on the pH for *E. faecalis*. In *S. pyogenes* and *L. lactis*, a change in pH resulted in a clear change in Y_{ATP} (cell mass produced per mole of ATP). The pH that showed the highest Y_{ATP} corresponded to the pH of the natural habitat of the organisms.

Comparative analyses, as demonstrated by comparative genomics and bioinformatics, are extremely powerful for (i) transfer of information from (experimentally) well-studied organisms to other organisms and (ii) when coupled to functional and phenotypic information, insight into the relative importance of components to the observed differences and similarities. The central principle is that important aspects of the functional differences between organisms derive not only from the differences in genetic components (which underlies comparative genomics) but also from the interactions between their components. Although this type of analysis is much discussed, only a very few studies focus on cross-species comparisons.

Here we study three relatively simple and highly related lactic acid bacteria (LAB) which nevertheless exhibit stark and important differences in their functional relationship with humans: these organisms are homofermentative lactic acid bacteria, namely, *Lactococcus lactis*, the major microorganism used in the dairy industry (21); *Enterococcus faecalis*, a major LAB in the human intestinal microbiota and a (fecal) contaminant in food and water as well as a contributor to food fermentation (16); and *Streptococcus pyogenes*, an important human pathogen (9, 15). These organisms have a similar primary metabolism but persist in completely different environments (milk, feces, skin/mucous membranes/blood).

L. lactis is by far the best-studied lactic acid bacterium (3, 13, 14, 23, 24, 30), and a kinetic model for its complete glycolysis, including some branching pathways, has been developed (2, 10). For the other two lactic acid bacteria, less information is readily available.

Generally, observations made with *L. lactis* are quickly translated to other LAB. With this approach we are able to separate organism-specific observations from observations that are general for LAB.

Here three LAB and their isogenic *ldh* deletion strains were characterized with respect to growth rates, catabolic flux distribution, ATP demand, and their ability to utilize different carbon sources. Our data identified important differences in the physiologies of these three LAB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* NZ9000 and the lactate dehydrogenase (LDH)-deficient strain NZ9010 (11, 17), *E. faecalis* V583 and V583 Δ*ldh-1* (12), and *S. pyogenes* M49 591 and M49 591 Δ*ldh* were grown in batch cultures at 37°C in 96-well plates in either Todd-Hewitt broth supplemented with 0.5% (wt/vol) yeast extract (Oxoid) (THY medium) or a chemically defined medium (CDM) specifically designed to support the growth of all three LAB (pH 7.4). The CDM-LAB medium (12) contained the following per liter: 1 g K_2HPO_4, 5 g KH_2PO_4, 0.6 g ammonium citrate, 1 g acetate, 0.25 g tyrosine, 0.24 g alanine, 0.125 g arginine, 0.42 g aspartic acid, 0.13 g cysteine, 0.5 g glutamic acid, 0.15 g histidine, 0.21 g isoleucine, 0.475 g leucine, 0.44 g lysine, 0.275 phenylalanine, 0.675 g proline, 0.34 g serine, 0.225 g threonine, 0.05 g tryptophan, 0.325 g valine, 0.175 g glycine, 0.125 g methionine, 0.1 g asparagine, 0.2 g glutamine, 10 g glucose, 0.5 g L-ascorbic acid, 35 mg adenine sulfate, 27 mg guanine, 22 mg uracil, 50 mg cystine, 50 mg xanthine, 2.5 mg D-biotin, 1 mg vitamin B_{12}, 1 mg riboflavin, 5 mg pyridoxamine-HCl, 10 μg *p*-aminobenzoic acid, 1 mg pantothenate, 5 mg inosine, 1 mg nicotinic acid, 5 mg orotic acid, 2 mg pyridoxine, 1 mg thiamine, 2.5 mg lipoic acid, 5 mg thymidine, 200 mg $MgCl_2$, 50 mg $CaCl_2$, 16 mg $MnCl_2$, 3 mg $FeCl_3$, 5 mg $FeCl_2$, 5 mg $ZnSO_4$, 2.5 mg $CoSO_4$, 2.5 mg $CuSO_4$, and 2.5 mg $(NH_4)_6Mo_7O_{24}$. Both media were buffered with either 100 mM morpholineethanesulfonic acid buffer or 100 mM morpholinepropanesulfonic acid buffer for growth at pH 6.5 and 7.5, respectively. Cultures were

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grown statically to provide anaerobic (or rather low microaerophilic) conditions (7). Group A streptococcal (GAS) mutants harboring recombinant pUC18Erm1 plasmids (4) were maintained in medium containing 5 mg/liter erythromycin or 60 mg/liter spectinomycin. *Escherichia coli* DH5α isolates transformed with pUC18Erm1 derivatives were grown on Luria-Bertani broth supplemented with 300 mg/liter erythromycin and/or 100 mg/liter spectinomycin. All *E. coli* cultures were grown at 37°C under ambient air conditions.

Chemostat cultures. *L. lactis* NZ9000, *E. faecalis* V538, and *S. pyogenes* M49 591 wild-type strains and their *ldh*-negative mutants were grown in anaerobic glucose-limited chemostat cultures in CDM-LAB medium (12). Cultures were grown in a Biostat Bplus fermentor unit with a total volume of 750 ml for *S. pyogenes* at a stirring rate of 100 rpm. *L. lactis* and *E. faecalis* were grown in Applikon-type fermentors at a stirring rate of 400 rpm in a total culture volume of 1,000 ml. The temperature was kept at 37°C for all three organisms.

The pH was maintained at the indicated value by titrating with sterile 2 M NaOH. Growth rates were controlled by the medium dilution rates (D; 0.05 h^{-1} or 0.15 h^{-1}). Culture volume was kept constant by removing culture liquid at the same rate that fresh medium was added. The cultures were considered to be in steady state when no detectable glucose remained in the culture supernatant and the optical densities (ODs), dry weights, and product concentrations of the cultures were constant on two consecutive days. All chemostat results showed a carbon balance of 80% ± 10% on the basis of glucose consumption and organic acid formation. This concurs with previously published data obtained from continuous cultures (13).

Construction of recombinant vectors and GAS strains. For the construction of an *S. pyogenes* M49 591 *ldh*-knockout strain, a 2,977-bp fragment comprising the L-lactate dehydrogenase gene (*ldh*) and 1,000 bp of the upstream and 993 bp of the downstream flanking sequences was PCR amplified from chromosomal DNA of GAS M49 591 using the forward/reverse primer pair 5′-CAC TTG AGC TCT ATT GAC GCC ATA GGG AAA-3′/5′-CCA ACG CAT GCG CAA AGA AGT GGT TCT GAT-3′. The resulting PCR fragment was digested with SacI and SphI and ligated into the equally treated pUC18Erm1 vector (4). The resulting plasmid was used as a template for an outward PCR with primer pair 5′-TAA TCG GAT CCG AGA CTT CGG TCT CTT TTT-3′/5′-AGT GCA GTC GAC TCT AAA CAT CTG CTT AAT-3′ binding to the flanking regions. Thus, the resulting PCR product comprised the whole plasmid, including the upstream and downstream flanking regions of the *ldh* gene but excluding the *ldh* gene itself. After restriction of this fragment with BamHI and SalI, it was ligated with an equally treated PCR fragment (primer pair 5′-GGC GGC GTC GAC TTG ATT TTC GTT CGT GAA TAC ATG-3′/5′-GGC GGC GGA TCC CCA ATT AGA ATG AAT ATT TCC CAA A-3′) comprising the spectinomycin resistance gene *aad9* from plasmid pSF152 (26). The resulting recombinant plasmid, pUCerm-ldh-ko, was transformed into *S. pyogenes* M49 591, and assays for double-crossover events were performed by selection for erythromycin-sensitive but spectinomycin-resistant transformants. The correct replacement of the *ldh* gene by the *aad9* gene in the respective transformants was confirmed by appropriate PCR assays and LDH activity assays. For all PCR amplifications, a Phusion high-fidelity PCR kit (Finnzymes) was used. For *L. lactis* and *E. faecalis*, the *ldh* deletion strains NZ9010 Δ*ldh-1* and V538 Δ*ldh-1* respectively, have been published previously (11, 12).

Analysis of carbon fluxes. Steady-state bacterial dry weight was measured as described previously (1). Glucose, pyruvate, lactate, formate, acetate, succinate, and ethanol were determined by high-pressure liquid chromatography (HPLC; LKB) with a Rezex organic acid analysis column (Phenomenex) at a temperature of 45°C with 7.2 mM H_2SO_4 as the eluent, using a RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration. Discrimination between D- and L-lactate was performed using a D-/L-lactate assay kit (Megazyme).

Aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine, and phenyalanine were determined by HPLC (Agilent) by use of the Waters AccQ Tag method. Fluorescence was analyzed using a Hitachi F-1080 fluorescence detector set to 250 nm excitation, and emission was recorded at 395 nm.

Substrate utilization assays. For substrate utilization assays, bacteria were grown overnight in a chemically defined medium (12), pelleted by centrifugation, washed twice in phosphate-buffered saline (pH 7.4), and suspended in glucose-free CDM-LAB medium. Optical densities were adjusted to 0.05, and 100 μl bacterial suspension was applied to each well of Biolog phenotype microarray plates PM1 and PM2. The microarray plates were incubated for 24 h at 37°C in a 5% CO_2 atmosphere, and the optical densities of each well were measured. The optical densities in well A1 of the arrays containing no carbon source were subtracted from all values. Optical densities in the wells containing α-D-glucose were set equal to 100%, and all other values were related accordingly.

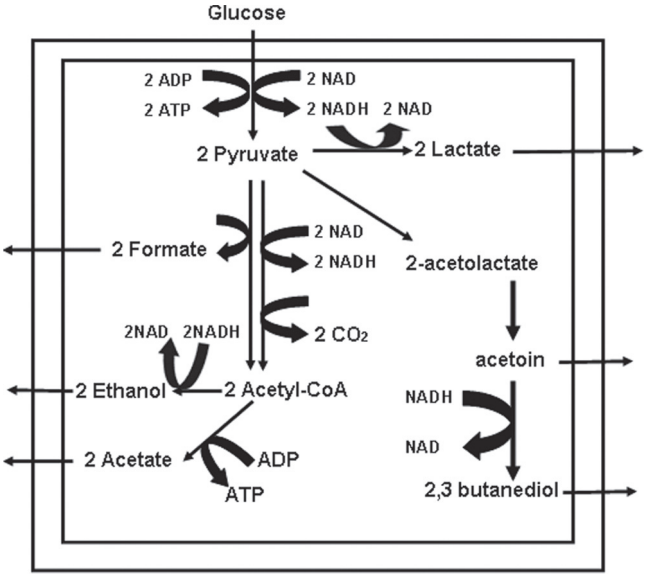

FIG. 1. Schematic representation of the anaerobic pathways of glucose catabolism in LAB.

Calculation of specific ATP synthesis rates. The rate of substrate-level ATP synthesis [$q_{ATP\ (SLP)}$] is stoichiometrically coupled to the rate of lactate, acetate, and ethanol synthesis as follows: 1 glucose + 2ADP + 2P_i → 2 lactate + 2ATP and 1 glucose + 3ADP + 3P_i → 1 acetate + 1 ethanol + 3ATP. The energy required for maintenance ($q\text{ATP}_{\text{maintenance}}$) was estimated by extrapolation of the linear line of D plotted against the total energy ($q\text{ATP}_{\text{total}}$) to D equal to 0 (see Fig. S1 in the supplemental material for an example). The qATP at the maximal specific growth rate ($q\text{ATP}\mu_{\text{max}}$) was estimated by extrapolating the same line to the D at which the specific organism has its maximal specific growth rate. This method is adapted from previously published methods (5, 8, 19, 27). Here we assumed a constant $q\text{ATP}_{\text{maintenance}}$, since (i) $q\text{ATP}_{\text{maintenance}}$ is very small and would show only a small contribution to the qATP at μ_{max} and (ii) no consensus on the calculation of $q\text{ATP}_{\text{maintenance}}$ at μ_{max} exists (27).

RESULTS

Deletion of *ldh* does affect growth rate of lactic acid bacteria in rich medium but not in CDM-LAB medium. *L. lactis*, *E. faecalis*, and *S. pyogenes* are referred to as LAB because of the fact that in the presence of glucose, lactate is produced as the main fermentation product. This metabolic pathway is relatively inefficient, since only two ATP molecules are generated from one glucose molecule (Fig. 1). All three LAB possess the genetic make up for mixed acid fermentation (6, 18, 20, 28), a more effective way of fermentation generating three ATP molecules per molecule of glucose (Fig. 1). All three genomes reveal (at least) two genes encoding an LDH. *S. pyogenes* possesses one (L-LDH) (18), *E. faecalis* two (L-LDH, L-LDH2) (20), and *L. lactis* three (L-LDHA, L-LDHB, L-LDHX) (21) L-lactate dehydrogenases. *E. faecalis* and *S. pyogenes* each encode one additional D-lactate dehydrogenase (D-LDH). In both *L. lactis* and *E. faecalis* it has been shown that the L-LDH is responsible for over 95% of total lactate synthesis (7, 12). This could also be confirmed for *S. pyogenes* in the present study (data not shown).

In all three LAB, the main *ldh* gene (encoding the L-LDH responsible for over 90% of the total lactate flux in the wild-type strains) was removed (11, 12). The resulting *ldh* deletion strains were analyzed in a batch growth setup in CDM-LAB or

TABLE 1. Maximal specific growth rates of the three lactic acid bacteria and their *ldh* deletion mutants[a]

Medium	pH	Maximum specific growth rate (h^{-1}) of strain:					
		L. lactis		*E. faecalis*		*S. pyogenes*	
		NZ9000	NZ9010	V583	V583 Δ*ldh-1*	M49 591	M49 591 Δ*ldh*
CDM-LAB	6.5	0.43 ± 0.06	0.45 ± 0.06	0.74 ± 0.01	0.63 ± 0.01[b]	0.43 ± 0.01	0.39 ± 0.04
	7.5	0.49 ± 0.01	0.49 ± 0.06	0.79 ± 0.07	0.89 ± 0.07	0.39 ± 0.02	0.35 ± 0.01
THY	6.5	0.76 ± 0.05	0.59 ± 0.05[b]	1.14 ± 0.05	0.99 ± 0.05[b]	0.86 ± 0.13	0.69 ± 0.01[b]
	7.5	0.81 ± 0.09	0.72 ± 0.01	1.16 ± 0.02	1.01 ± 0.02[b]	0.57 ± 0.06	0.53 ± 0.03

[a] Strains were grown in 96-well plates at 37°C under low microaerobic conditions. Values indicate the average μ_{max} ± standard deviation.
[b] Significantly different, $P < 0.05$ (two-tailed Mann-Whitney U test).

rich THY medium at pH 6.5 and pH 7.5 under low microaerobic conditions. As expected, all wild-type strains performed complete homolactic acid fermentation under all conditions. None of the three *ldh* deletion strains showed a significant difference with respect to growth rate compared to that of the wild-type counterpart (Table 1) in CDM-LAB medium, except for *E. faecalis* grown at pH 6.5.

In THY medium at both pHs, all three wild-type LAB showed higher growth rates than their isogenic counterparts with *ldh* deletions, although not to the extent observed previously for *L. lactis* in MRS medium (7). Only small pH-dependent differences in the maximal growth rates were observed for wild-type *L. lactis* and *E. faecalis*. *S. pyogenes*, however, showed a significantly lower specific growth rate at pH 7.5 in rich medium but only a slightly lower specific growth rate at pH 7.5 in CDM-LAB medium. All strains with *ldh* deletions grew 10 to 20% slower than the wild-type strains, except for *S. pyogenes* at pH 7.5, where deletion of *ldh* did not result in a significant decrease in growth. In late stationary phase, all three *ldh* deletion strains grew to a higher optical density and the activity of LDH remained below 10% of total fermentation activity for all the three *ldh* deletion strains (data not shown).

To verify whether a similar μ_{max} also signified that in a mixed culture deletion of *ldh* does not represent a disadvantage, the *S. pyogenes* M49 591 wild type and its *ldh* deletion strain were cocultivated in THY medium (pH 7.5). A 52%/48% distribution of wild-type and mutant bacteria was shown after 18 h of cocultivation of both strains in THY medium and subsequent plating of serial dilutions on THY agar plates with and without spectinomycin. This indicates that the lack of an L-LDH represented no significant disadvantage to the organism under the conditions tested.

Effect of deletion of *ldh* in LAB on substrate utilization. The decrease in maximal specific growth rate of the *ldh* deletion in rich medium might be due to differences in the ability to utilize carbon sources other than glucose. To assess the impact of the *ldh* knockout on the ability of LAB to utilize different substrates as a carbon source, Biolog phenotype microarrays were applied. Using these arrays, growth of the strains on 190 different carbon substrates was evaluated. Comparison of the substrate utilization of the three strains and their isogenic *ldh* deletion strains showed that there were 11 carbon sources on which all wild-type strains were able to grow to at least 10% of the optical densities reached by growth on glucose (for complete lists, see Tables S1 to S3 in the supplemental material). These substrates were α-D-glucose, D-mannose, maltose, maltotriose, *N*-acetyl-D-glucosamine, D-fructose, D-trehalose, D-glucosamine, sucrose, salicin, and dextrin. However, *S. pyogenes* showed optimal growth on glucose and sucrose (101.1%) (Table 2). With all the other C sources tested, *S. pyogenes* ended up at lower ODs after 24 h of growth. For *E. faecalis* there was no carbon source that led to an equal or even better growth yield compared to that achieved with glucose. In contrast, the growth yield of *L. lactis* was the same or improved compared to that with glucose on gentiobiose (128.3%) and D-cellobiose (114.5%).

TABLE 2. Substrate utilization of *S. pyogenes* M49 591, *E. faecalis* V583, and *L. lactis* NZ9000 and their *ldh* deletion mutants[a]

Substrate	Final optical densities of strains compared to that with glucose substrate (%)					
	L. lactis		*E. faecalis*		*S. pyogenes*	
	NZ9000	NZ9010	V583	V583 Δ*ldh-1*	M49 591	M49 591 Δ*ldh*
Gentiobiose	128.3 ± 12.3	98.3 ± 12.5[b]	63.3 ± 8.9	70.5 ± 4.1	1.4 ± 5.0	5.7 ± 7.9
D-Cellobiose	114.5 ± 6.3	67.8 ± 13.4[b]	68.4 ± 1.9	72.4 ± 0.9[b]	6.2 ± 6.9	6.9 ± 11.2
D-Trehalose	124.4 ± 26.5	42.9 ± 41.2[b]	68.8 ± 4.5	69.9 ± 0.9	90.4 ± 16.5	38.4 ± 9.8[b]
Sucrose	23.2 ± 35.2	6.5 ± 8.5	45.8 ± 2.6	46.0 ± 2.9	101.1 ± 20.4	38.6 ± 11.3[b]
Maltose	72.5 ± 9.6	35.8 ± 18.9[b]	93.5 ± 2.2	96.7 ± 5.3	52.3 ± 14.1	40.5 ± 15.2
Maltotriose	107.9 ± 21.6	70.3 ± 30.8	85.2 ± 13.0	102.8 ± 3.5[b]	87.4 ± 16.5	78.9 ± 9.0
D-Glucosaminic acid	3.3 ± 8.1	2.7 ± 2.4	12.8 ± 3.1	9.1 ± 1.0[b]	0.6 ± 1.6	1.4 ± 2.5
D-Mannose	117.8 ± 26.3	105.5 ± 5.7	93.8 ± 6.8	101.3 ± 9.1	82.0 ± 17.7	41.3 ± 14.7[b]

[a] Out of the 190 tested carbon sources, only those with significant differences between at least one mutant and wild-type pair are shown. Optical densities of the cultures grown on glucose were set equal to 100% for all strains, and optical densities for growth on all other substrates were related to this value.
[b] Significantly different, $P < 0.05$ (two-tailed Mann-Whitney U test).

TABLE 3. Relative flux distribution in the three lactic acid bacteria at two dilution rates and two pHs during continuous cultivation in glucose-limited CDM-LAB medium[a]

Strain	Dilution rate (h^{-1})	pH	Lactate (mol/mol glucose)	Formate (mol/mol glucose)
L. lactis NZ9000	0.05	6.5	1.0 ± 0.3	0.6 ± 0.3
		7.5	1.1 ± 0.5	0.2 ± 0.2
	0.15	6.5	1.4 ± 0.4	0.4 ± 0.1
		7.5	1.5 ± 0.3	0.2 ± 0.1
E. faecalis V583	0.05	6.5	0.9 ± 0.3	0.4 ± 0.3
		7.5	0.2 ± 0.1	1.2 ± 0.1
	0.15	6.5	0.7 ± 0.1	0.7 ± 0.1
		7.5	0.2 ± 0.1	1.1 ± 0.1
S. pyogenes M49 591	0.05	6.5	0.8 ± 0.4	0.5 ± 0.1
		7.5	0.6 ± 0.3	0.5 ± 0.2
	0.15	6.5	1.4 ± 0.1	0.1 ± 0.1
		7.5	1.1 ± 0.3	0.2 ± 0.2

[a] Values indicate mol product/mol glucose ± standard deviation.

The deletion of the *ldh* gene in *S. pyogenes* resulted in a significantly reduced growth yield of this strain on D-mannose (−49.6%), D-trehalose (−57.5%), and sucrose (−61.8%) as the carbon source in comparison to that of wild-type *S. pyogenes* (Table 2). For *L. lactis* the deletion of the *ldh* gene also resulted in hampered growth yield on D-trehalose (−65.5%), D-cellobiose (−46.8%), maltose (−50.7%), and D-gentiobiose (−23.4%). Almost no significant changes in the substrate utilization of the *E. faecalis ldh* knockout strain were observed, but a small significant reduction of the growth yield on D-glucosaminic acid (−29.2%) was detected, although the wild-type *E. faecalis* strain also showed small growth on this substrate (12.1% compared to that on glucose). The *ldh* knockout led to a small improvement of the growth yield on maltotriose (+20.7%) and D-cellobiose (+5.8%) compared to that of the *E. faecalis* wild-type strain, which is probably the result of increased efficiency of ATP formation from pyruvate in the *ldh* deletion strain. For the other substrates tested, no significant differences were observed.

ATP demand under glucose-limited continuous growth conditions is strongly pH and organism dependent. ATP demand can be estimated by performing growth in continuous cultures and subsequently allows calculation of the $q\mathrm{ATP}_{\mathrm{total}}$ from the formed fermentation products (see Materials and Methods and Fig. S1 in the supplemental material). This allows estimation of the $q\mathrm{ATP}\mu_{\mathrm{max}}$ and $q\mathrm{ATP}_{\mathrm{maintenance}}$. Furthermore, this could give indications on the role of *q*ATP in the pyruvate flux distribution.

In order to determine the pH and growth rate dependency of the flux distribution (i.e., homolactic acid, acetate, ethanol, acetoin, and butanediol formation) and the cell mass (g) produced per mol of ATP generated by substrate catabolism (Y_{ATP}) of the LABs under defined continuous conditions, all three strains were grown as anaerobic glucose limited chemostat cultures in CDM-LAB medium under conditions that varied in growth rate and pH. Glucose limitation was verified by HPLC analysis and by a linear correlation between changes of the glucose concentration in the medium and cell density; i.e., a 2-fold decrease in the glucose concentration resulted in a 2-fold decrease in biomass (data not shown). None of the 17 amino acids except arginine (data not shown) was completely consumed for all three organisms. Arginine was consumed completely. Under these energy-limited growth conditions, this is likely due to use of arginine for ATP formation by formation of ornithine.

TABLE 4. Physiological parameters of LAB grown in C-limited continuous cultures[a]

Strain	pH	$q\mathrm{ATP}_{\mathrm{maintenance}}$	Y_{ATP}	$q\mathrm{ATP}\mu_{\mathrm{max}}$
L. lactis NZ9000	6.5	5.0 ± 1.5	8.4 ± 1.0	33 ± 2.9
	7.5	8.2 ± 3.4	4.4 ± 0.5	94 ± 15
E. faecalis V583	6.5	2.0 ± 1.2	12.8 ± 2.6	49 ± 3.3
	7.5	2.0 ± 0.8	14.2 ± 3.3	47 ± 2.7
S. pyogenes M49 591	6.5	−2.6 ± 2.2	5.2 ± 0.7	88 ± 9.2
	7.5	2.9 ± 2.1	9.4 ± 2.3	38 ± 7.1

[a] $q\mathrm{ATP}_{\mathrm{maintenance}}$ was calculated according to the methods applied by Tempest and Neijssel (27). Y_{ATP} was determined at a *D* of 0.15 since Y_{ATP} at low dilution rates is strongly influenced by $q\mathrm{ATP}_{\mathrm{maintenance}}$. *q*ATP at the maximal specific growth rate ($q\mathrm{ATP}\mu_{\mathrm{max}}$; for data on μ_{max}, see Table 1) was estimated by extrapolation of the slope for $q\mathrm{ATP}_{\mathrm{total}}$ to *D*, similar to μ_{max}.

E. faecalis mainly showed mixed acid fermentation under all conditions, while *L. lactis* and *S. pyogenes* mainly exhibited homolactic acid fermentation (Table 3). Only *S. pyogenes* showed more mixed acid fermentation at lower dilution rates at both pH 6.5 and pH 7.5. *E. faecalis* and *L. lactis* did not show a significant growth rate-dependent change in fermentation pattern at these growth rates. Mixed acid fermentation did show a strong pH dependency for *E. faecalis*, with a more homolactic acid fermentation phenotype occurring at pH 6.5. For *L. lactis* and *S. pyogenes*, no significant pH-dependent differences were observed.

L. lactis NZ9010 with the single *ldh* deletion showed increased activity of alternative LDH proteins, as was observed previously (7) and as was shown by an increase in homolactic acid fermentation during prolonged growth. Deletion of the main *ldh* of *E. faecalis* and *S. pyogenes* resulted in complete mixed acid fermentation under all conditions (data not shown) and the $q\mathrm{ATP}_{\mathrm{total}}$ values were similar to those for the cognate wild-type strains (data not shown). This indicates that deletion of *ldh* does not result in an overall increase in ATP-dissipating reactions.

$q\mathrm{ATP}_{\mathrm{maintenance}}$ and $q\mathrm{ATP}\mu_{\mathrm{max}}$ (Table 4) were calculated as described above (see Fig. S1 in the supplemental material). $q\mathrm{ATP}_{\mathrm{maintenance}}$ did not show large pH- or species-dependent differences for any of the three LAB (Table 4), although in general the $q\mathrm{ATP}_{\mathrm{maintenance}}$ for *L. lactis* was found to be higher than that for the other two LAB. However, large differences were observed with respect to Y_{ATP} (27). The Y_{ATP} for *S. pyogenes* was almost 2-fold higher at pH 7.5 than at pH 6.5, while for *L. lactis* the Y_{ATP} at pH 7.5 was almost 2-fold lower that that at pH 6.5. For *Enterococcus faecalis* no significant pH dependence of Y_{ATP} was observed.

DISCUSSION

Here we have studied the general physiological characteristics of three well-known LAB and their isogenic *ldh* deletion

strains. Previously, Bongers et al. (7) reported that deletion of the main *ldh* in *L. lactis* did affect the μ_{max} under anaerobic but not under aerobic conditions in the rich M17 broth. In accordance with those data, we observed that in the rich THY medium the lack of the LDH enzyme also resulted in a reduced maximal growth rate at pH 6.5 for *L. lactis* and also for *S. pyogenes* and *E. faecalis*. In contrast to that, in a buffered chemically defined medium supplied with glucose as the main carbon source, deletion of *ldh* did not result in significantly lower growth rates for all three LAB under anaerobic/microaerobic conditions.

Thus, the effects of *ldh* deletions on growth rates of lactic acid bacteria apparently depend not only on oxygen availability, as shown by Bongers et al. (7) for *L. lactis*, but also on the medium and pH. A major difference between M17, THY, and CDM-LAB media is the availability of carbon sources. While the complex rich media M17 and THY contain a variety of different potential carbon sources, in CDM-LAB medium, glucose is the only sugar component. It has been shown previously that utilization of sugars like maltose or galactose as the carbon source by LAB led to changes in product formation compared to that from growth on glucose (24, 25, 29). By screening a large variety of carbon sources, we could show that the *ldh* deletion mutants are not able to utilize all carbon sources as efficiently as their cognate wild types. This might contribute to the phenotypic differences observed during growth on rich medium. It seems that for growth on disaccharides, i.e., gentiobiose, D-cellobiose, D-trehalose, maltose, and sucrose, deletion of *ldh* in *L. lactis* results in impaired growth yields, while this is not observed in *E. faecalis* and only to a minimal extent in *S. pyogenes*, i.e., for D-trehalose and sucrose. Of note, all di- or trisaccharides linked by a β1,4-glucoside bond do not result in lower growth rates for *S. pyogenes* with the *ldh* deletion. Sugars that are linked by an α1,1-glucoside or α1,2-glucoside bond do result in strong retardation of growth yield upon deletion of *ldh* in *S. pyogenes*. However, the exact reasons for these differences remain unclear.

E. faecalis clearly showed the highest specific growth rate in both chemically defined medium and rich medium. *L. lactis* and *S. pyogenes* showed roughly similar growth rates under all conditions, with the exception that *S. pyogenes* grew slower in both media at pH 7.5.

The three lactic acid bacteria showed stark differences in growth under glucose-limited continuous conditions. *E. faecalis* showed mixed acid fermentation under almost all conditions but showed clearly more homolactic acid fermentation at low pH. Both *S. pyogenes* and *L. lactis* mainly showed homolactic acid fermentation, as was shown previously (24), under all conditions and showed no pH dependency with respect to mixed acid versus homolactic acid fermentation. This is in contrast to previous observations, caused by the strong differences in the amount of arginine in the medium (28) (data not shown). These data also indicate that the switch to mixed acid fermentation by these organisms is not caused by a decrease of their (relative) growth rate. This is especially exemplified by the clear pH dependence of the fraction of mixed acid fermentation at a D of 0.15 for *E. faecalis*, since the maximal specific growth rate does not show any pH dependency.

E. faecalis did not show a pH dependence of its Y_{ATP}, and the data shown here correlate very well with previously published data that showed a $q\text{ATP}_{maintenance}$ of 2 and a Y_{ATP} of about 13 (22) for glucose-limited continuous cultures grown at pH 7.0. Both *S. pyogenes* and *L. lactis* showed clear pH dependence with respect to their Y_{ATP} values, with each having the highest Y_{ATP} if growth is performed at or near the pH of their physiological environment, i.e., pH 6.5 for *L. lactis* (milk) and pH 7.5 for *S. pyogenes* (blood). This strongly indicates that the growth yields for these organisms are somehow optimized at their natural pH and quickly encounter (ATP-dissipating) difficulties at alternative pHs. The exact reason for or mechanism behind the observed differences in Y_{ATP} are beyond the scope of this study. Interestingly, no major impact for pH on the μ_{max} was observed for these lactic acid bacteria (except for *S. pyogenes* in rich medium). It seems, therefore, that μ_{max} is less dependent on the pH than Y_{ATP}.

Combining the maximal specific growth rate, $q\text{ATP}_{maintenance}$, and Y_{ATP} allows calculation of the qATP at μ_{max} (see Table 4 and Materials and Methods for the formula used). This shows that $q\text{ATP}_{total}$ at μ_{max} is much higher for *L. lactis* and *S. pyogenes* under unfavorable conditions at pH 7.5 and 6.5, respectively. This indicates that growth at their natural pHs, 6.5 and 7.5, respectively, is not limited by ATP formation rates under the conditions tested here. The differences observed between the three wild-type lactic acid bacteria and their isogenic *ldh* deletion strains were strongly strain dependent. This leads to the (obvious) indication that observations made for a single species belonging to the order *Lactobacillales* cannot be translated to the other species in this specific order. The fact that these organisms are so closely related ensures, however, that these types of studies can more easily zoom in on the specific phenotypic differences and the physicochemical causes thereof. It may even help resolve the long-standing question as to what is the basis of the homolactic acid fermentation to mixed acid fermentation switch, since it is expected that a similar mechanism regulates this in all three organisms.

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Supplementary Table 1. List of carbon sources utilizable by *E. faecalis* V583

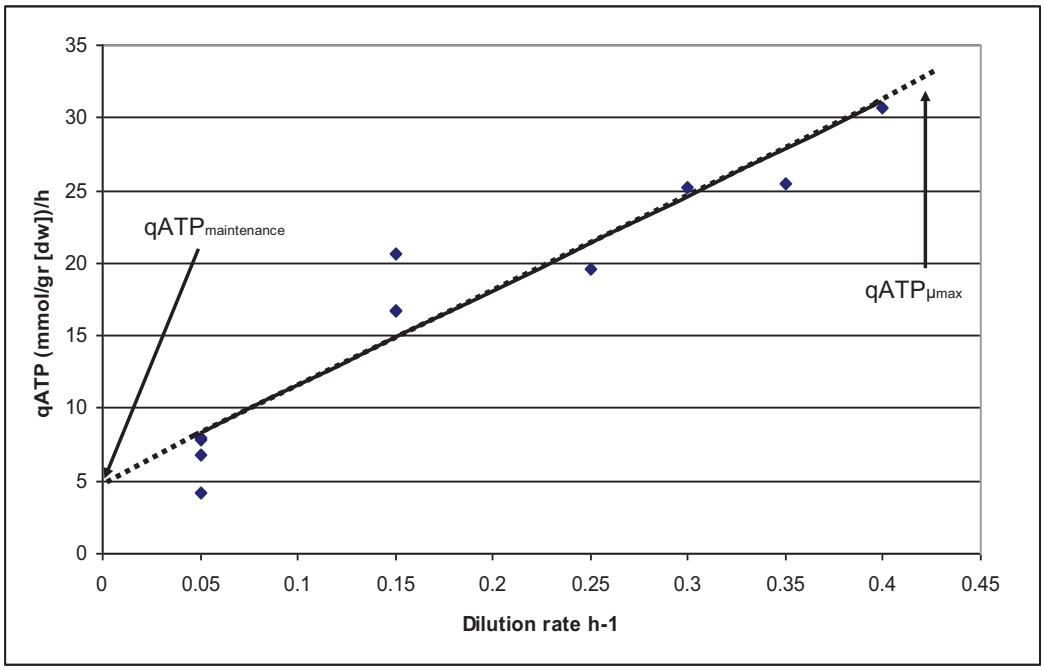
	***E. faecalis* V583 wt**	**standard deviation**	***E. faecalis* V583 *Δldh***	**standard deviation**	**growth change ldh- compared to wt in %**	**significance (two tailed U test)**
a-d-glucose	100.0	0.0	100.0	0.0	0.0	no
d-mannose	93.8	6.8	101.3	9.1	8.1	no
maltose	93.5	2.2	96.7	5.3	3.5	no
maltotriose	85.2	13.0	102.8	3.5	20.7	$P < 0.05$
n-acetyl-d-glucoseamine	83.8	11.4	86.2	2.5	2.9	no
d-fructose	77.8	3.8	79.5	8.8	2.1	no
d-trehalose	68.8	4.5	69.9	0.9	1.6	no
d-cellobiose	68.4	1.9	72.4	0.9	5.8	$P < 0.05$
d-glucoseamine	65.5	14.9	71.7	11.2	9.6	no
gentiobiose	63.3	8.9	70.5	4.1	11.4	no
pectin	54.8	47.2	41.2	25.9	-24.7	no
sucrose	45.8	2.6	46.0	2.9	0.3	no
d-ribose	44.9	3.0	49.5	2.9	10.2	no
tween 20	43.9	76.3	8.6	3.9	-80.5	no
d-tagatose	35.3	14.5	41.5	4.5	17.6	no
salicin	33.4	8.8	37.2	2.0	11.3	no
d-mannitol	29.2	0.9	29.9	3.1	2.1	no
n-acetyl-d-galactoseamnine	26.6	10.5	27.0	7.3	1.7	no
d-melezitose	25.9	16.0	25.1	9.1	-3.3	no
dextrin	21.3	9.3	20.5	5.4	-3.4	no
2-deoxy-d-ribose	21.2	3.7	19.8	5.6	-6.8	no
glycerol	17.5	4.1	16.7	1.7	-4.8	no
ß-methyl-d-glucoside	16.1	2.6	13.9	2.9	-14.0	no
a-cyclodextrin	15.4	7.7	15.2	6.9	-0.9	no
d-sorbitol	15.3	3.6	11.2	1.8	-27.0	no
l-arginine	13.9	29.6	1.9	3.0	-86.1	no
dihydroxy acetone	13.7	9.7	11.9	6.1	-13.4	no
d-galactose	13.3	3.1	13.0	1.0	-2.5	no
d-gluconic acid	13.0	3.3	10.6	1.7	-18.5	no
d-glucosaminic acid	12.8	3.1	9.1	1.0	-29.2	$P < 0.05$
amygdalin	12.7	4.7	15.5	1.8	21.9	no
3-0-ß-d-galactopyranosyl-d-arabinose	12.7	17.1	5.3	1.8	-57.9	no
d-fructose-6-p	11.3	3.9	7.9	1.2	-30.6	no
2-butanone	11.1	8.3	9.4	2.9	-15.3	no
l-methionine	10.7	16.8	7.2	15.1	-32.3	no
uridine	10.3	2.5	7.2	0.6	-30.2	no
chondroitin sulfate c	9.2	9.2	11.3	9.9	22.9	no

Supplementary Table 2. List of carbon sources utilizable by *L. lactis* NZ9000

	***L. lactis* NZ9000**	**standard deviation**	***L. lactis* NZ9010**	**standard deviation**	**growth change ldh- compared to wt in %**	**significance (two tailed U test)**
arbutin	233.6	84.3	143.3	17.2	-38.7	no
gentiobiose	128.3	12.3	98.3	12.5	-23.4	$P < 0.05$
d-trehalose	124.4	26.5	42.9	41.2	-65.5	$P < 0.05$
d-mannose	117.7	26.3	105.5	5.7	-10.4	no
d-cellobiose	114.5	6.3	67.8	53.4	-40.8	$P < 0.05$
maltotriose	107.9	21.6	70.3	30.8	-34.9	no
a-d-glucose	100.0	0.0	100.0	0.0	0.0	no
n-acetyl-d-glucoseamine	84.0	8.1	76.9	5.6	-8.5	no
d-fructose	81.4	5.5	77.9	9.7	-4.4	no
maltose	72.5	9.6	35.8	18.9	-50.7	$P < 0.05$
ß-cyclodextrin	65.7	55.9	68.2	46.5	3.7	no
salicin	64.4	13.3	35.1	12.7	-45.4	no
d-glucoseamine	63.7	6.9	59.3	14.6	-6.9	no
d-galactose	56.6	37.7	36.8	24.5	-34.9	no
amygdalin	47.7	10.6	36.1	28.3	-24.2	no
a-cyclodextrin	46.2	39.1	31.0	18.5	-32.9	no
n-acetyl-ß-d-mannosamine	26.5	2.0	26.1	4.1	-1.4	no
l-methionine	17.8	18.8	4.4	7.4	-75.4	no
2-hydroxy 2-butanone	17.0	10.2	5.5	7.9	-67.4	no
dextrin	16.7	7.7	10.9	1.5	-34.9	no
2-deoxy-d-ribose	16.1	8.2	12.5	4.9	-22.2	no
d-gluconic acid	11.6	8.2	7.4	6.5	-35.6	no

Supplementary Table 3. List of carbon sources utilizable by *S. pyogenes* M49 591

	***S. pyogenes* M49 wt**	standard deviation	***S. pyogenes* M49 *Δldh***	standard deviation	**growth change ldh- compared to wt in %**	**significance (two tailed U test)**
sucrose	101.1	20.4	38.6	11.3	-61.8	$P < 0.05$
a-d-glucose	100.0	0.0	100.0	0.0	0.0	no
d-trehalose	90.4	16.5	38.4	9.8	-57.5	$P < 0.05$
maltotriose	87.4	16.5	78.9	9.0	-9.8	no
d-mannose	82.0	17.7	41.3	14.7	-49.6	$P < 0.05$
n-acetyl-d-glucoseamine	77.5	10.7	90.3	6.8	16.5	no
d-glucoseamine	63.3	7.3	68.2	17.9	7.8	no
ß-methyl-d-glucoside	62.6	17.1	38.7	21.7	-38.1	no
maltose	52.3	14.1	40.5	15.2	-22.5	no
salicin	46.3	11.3	24.1	16.6	-47.9	no
d-fructose	43.0	39.7	26.5	32.0	-38.4	no
a-d-lactose	26.6	24.2	20.9	19.7	-21.6	no
gelatin	25.7	42.0	5.3	2.2	-79.4	no
n-acetyl-ß-d-mannosamine	22.9	17.1	17.7	14.7	-22.7	no
uridine	17.1	24.5	10.5	11.2	-38.4	no
3-0-ß-d-galactopyranosyl-d-arabinose	17.0	26.9	14.0	11.0	-17.7	no
pectin	13.9	18.5	44.0	23.7	215.7	no
dextrin	13.8	9.0	12.3	5.5	-10.8	no
2-hydroxy benzoic acid	7.3	3.7	11.0	6.5	51.4	no
chondroitin sulfate c	6.5	3.9	19.1	11.6	193.1	no



Supplementary Figure 1: Graphical depiction of estimation of $qATP_{maintenance}$ and $qATP\mu_{max}$. $qATP_{maintenance}$ was estimated from the extrapolated qATP at D = 0. $qATP\mu_{max}$ was estimated by extrapolating the $qATP_{total}$ to the D of the μ_{max} measured for that specific organism at the specific pH.

PAPER IV

Enterococcus faecalis grows on ascorbic acid

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ABSTRACT

Ascorbic acid is widespread in nature, but bacterial growth with ascorbate as energy source has not been much studied. We report here that *Enterococcus faecalis* can utilize ascorbic acid as energy source for growth under anaerobic conditions in a chemically defined medium. Ascorbate utilization by *E. faecalis* was not repressed by glucose, but growth on a mixture of glucose and ascorbate was diauxic showing a halt when glucose levels became low. The cells had a much higher demand for amino acids when grown on ascorbate or lactose than when glucose was the energy source, showing that only glucose was able to down-regulate amino acid catabolism. To our knowledge this is the first description of a Gram positive bacterium growing on ascorbate. *Lactococcus lactis* NZ9000 and *Streptococcus pyogenes* M49 591 could not utilize ascorbate as energy source.

INTRODUCTION

Ascorbic acid, or vitamin C, is found in fruits and in humans it plays important roles in a number of processes (Smirnoff, 1996, Szeto*, et al.*, 2002). Ascorbic acid has antimicrobial activities, but can also serve as an energy source for microorganisms. The metabolism of ascorbate in *Escherichia coli* has been thoroughly described (Yew & Gerlt, 2002), but ascorbic acid degradation has not been described in many bacteria. Recent annotation of genome sequences suggests that uptake systems for ascorbic acid exist in other bacteria. A recent re-annotation of the *E. faecalis* V583 genome indicates that this organism can degrade ascorbic acid (www.ncbi.nlm.nih). The genes encode an uptake system and metabolic enzymes similar to what is found in *E. coli*. As ascorbic acid appears to be an accessible in body fluid, would be of interest to study the potential of potentially bacteria for their ability to utilize this compound as an energy sources.

Enterococcus facalis is a versatile lactic acid bacterium (LAB), growing in a number of environments including the gastrointestinal tract (GIT) of humans and animals (Kühn*, et al.*, 2003, Qin*, et al.*, 2009). The species is often associated with food, is in use as a probiotic, but also associated with nosocomial disease causing sepsis, endocarditis and urinary tract infections (Gross*, et al.*, 1976, Tailor*, et al.*, 1993, Bertolami*, et al.*, 1999). Enterococci are characterized as strict fermenters, fermenting carbohydrates to lactic acid alone or in mixture with compound like formate, ethanol and CO_2 (Snoep*, et al.*, 1990, Snoep*, et al.*, 1994, Mehmeti*, et al.*, 2011). Compared to other lactic acid bacteria *E. faecalis* is versatile being able to grow in different environments. This is because of its robustness and possibly because of its large metabolic potential possible. In this paper we show that *E. faecalis* is able to

utilize ascorbic acid, a property important not only for growth on plant material but probably also for growth in animal and human hosts.

MATERIALS AND METHODS

E. faecalis V583 (Sahm*, et al.*, 1989), *Lactococcus lactis* NZ9000 (Linares*, et al.*, 2010) and *Streptococcus pyogenes* M49 591 (Fiedler*, et al.*, 2011) was grown in tightly capped filled 50-ml screw-cap tube with a starting pH 7.4 in 37^0C in anaerobic condition. Growth rate was measured by a spectrophotometer and monitoring every hour. The bacteria were grown in chemical defined medium (CDM-base) with the modification shown below. The CDM-base medium are contained per liter: 1 g K_2HPO_4 , 5 g KH_2PO_4, 0.6 g ammonium citrate, 1 g sodium acetate, 0.25 g tyrosine, 0.24 g alanine, 0.125 g arginine, 0.13 g cysteine, 0.5 g glutamic acid, 0.15 g histidine, 0.21 g isoleucine, 0.475 g leucine, 0.44 g lysine, 0.275 phenylalanine, 0.675 g proline, 0.34 g serine, 0.225 g threonine, 0.05 g tryptophan, 0.325 g valine, 0.175 g glycine, 0.125 g methionine, 0.1 g asparagine, 0.2 g glutamine, 35 mg adenine sulfate, 27 mg guanine, 22 mg uracil, 50 mg cystine, 50 mg xanthine, 2.5 mg D-biotin, 1 mg vitamin B12, 1 mg riboflavin, 5 mg pyridoxamine-HCl, 10 μg p-aminobenzoeic acid, 1 mg pantothenate, 5 mg inosine, 1 mg nicotinic acid, 5 mg orotic acid, 2 mg pyridoxine, 1 mg thiamine, 2.5 mg lipoic acid, 5 mg thymidine, 200 mg $MgCl_2$, 50 mg $CaCl_2$, 16 mg $MnCl_2$, 3 mg $FeCl_3$, 5 mg $FeCl_2$, 5 mg $ZnSO_4$, 2.5 mg $CoSO_4$, 2.5 mg $CuSO_4$, and 2.5mg $(NH_4)_6Mo_7O_{24}$ (Jönsson*, et al.*, 2009, Fiedler*, et al.*, 2011, Mehmeti*, et al.*, 2011). The medium was been supplemented with glucose, sodium ascorbate or lactose as indicated.

Metabolites were analysed by high-performance liquid chromatography (HPLC), ethanol and acetoin by using headspace gas chromatography (GC) (Narvhus, 1990). Lactate and glucose production were also analyzed enzymatically (Megazyme Bray, Ireland). All experiments were run in triplicate.

RESULTS AND DISCUSSION

To study the growth of *E. faecalis* on ascorbate we used a chemically defined growth medium, CDM-base, supplemented with ascorbate as energy source. The composition of CDM-base is identical to CDM-LAB medium (Jönsson*, et al.*, 2009, Fiedler*, et al.*, 2011, Mehmeti*, et al.*, 2011) from which glucose was omitted.

As shown in Figure 1, *E. faecalis* grows on CDM-base supplemented with 5mM ascorbic acid. The cells did not grow in CDM-base alone (result not shown). As shown in Table 1 the dominating end products were acetate, lactate and formate in ascorbate grown cultures, different from when glucose was the energy source when only lactate was formed. Ascorbate is at the same oxidation state as pyruvate. The high levels of formate show that pyruvate was metabolized by pyruvate formate lyase and the acetyl-CoA thus formed was converted to acetate in an adenosine-5'-triphosphate (ATP) yielding process. Lactate formation from ascorbate creates an excess of reducing equivalents and a demand for nicotinamide adenine dinucleotide (NAD) regeneration, which can be met by pyruvate oxidation by pyruvate dehydrogenase (PDH). In the late growth stage of cultures supplemented with 12 mM ascorbate acetate and lactate were formed in equal amounts (Table 1), showing that the metabolism was dominated by Lactate dehydrogenese (LDH) and PDH activities.

In *E. coli* ascorbic acid is taken up and metabolized by a specific phosphotransferase system (PTS) and a series of enzymatic reactions to give D-xylulose-5-phosphate which can enter central metabolism. The genes encoding PTS and the enzymes involved are encoded by in an operon (Yew & Gerlt, 2002). The genes EF1127 through EF1131 in the *E. faecalis* V583

chromosme appear to be organized in an operon and to encode the same functions, indicating that ascorbate is metabolized by the same pathways in *E.coli* and *E.faecalis*.

We also investigated if *Streptococcus pyogenes* M49 591and *Lactococus lactis* NZ9000 could grow on ascorbate. These strains grow well in CDM-LAB with glucose as energy source (Fiedler*, et al.*, 2011). A full set of putative ascorbate metabolic genes highly similar to those of the *E. coli* and *E. faecalis* was found in all the complete *S. pyogenes* genome sequences published at NCBI. However, we were not able to grow *S. pyogenes* M49 591in CDM supplemented with 5 mM ascorbate. Neither did *L.lactis* NZ9000 grow in this medium. An ascorbate gene cluster with homology homologous to those of *E. coli* or *E. faecalis* was not found in any of the published *L. lactis* genome sequences. However, the *L. lactis* NZ9000 gene LLNZ04460 encodes a protein annotated as a putative EIIC component of an ascorbate specific PTS.

The growth yield of *E. faecalis* V583 on ascorbate was much lower than in glucose. In CDM-LAB containing 55 mM glucose OD 1.5 was reached, with the same amount of ascrobate and 1.25 mM glucose the cells grew to OD_{600} 0.36. With 5 mM ascorbate the cells grew to OD_{600} 0.19, and 0.2 with 12.5 mM ascorbate. As shown in Table 1, the cells did not consume all the ascorbate when grown with 12.5 mM ascorbate. The cells did not grow in CDM-base supplemented with 55 mM ascorbate (data not shown).

We compared the growth yields on glucose, ascorbate and lactose. As shown in Figure 1, the same low growth yield was found for lactose as for ascorbate. This suggests that the growth yield was limited by other components than the energy source. We therefore investigated the

effect of adding more amino acids to the growth medium. As shown in Figure 1 increased growth yield was obtained by doubling the amount of all amino acids in the growth medium, showing that yield was indeed limited by amino acids supply when *E. faecalis* was grown on ascorbic acid. We also tried to increase the amounts of amino acids twentyfold, but this was inhibitory and the cells did not grow (data not shown). Glucose grown cells showed a much lower demand for amino acids; lowering the concentration of all amino acids in CDM-base by 95 % still supported growth to OD 0,45 when the cells used glucose as energy source. Thus, when growing on ascorbate the cells had a requirement for amino acids about 30 times higher than when glucose was the energy source.

In the presence of glucose a number of catabolic genes are down-regulated by carbon catabolite control, including genes encoding breakdown of alternative carbohydrates supporting growth at slower rates (Deutscher, 2008, Opsata*, et al.*, 2010). As shown in Figure 1, the growth rate on ascorbate was very similar to that on glucose at the early stages of growth. We grew *E. faecalis* V583 in CDM-base supplemented with a mixture of glucose and ascorbate. As shown in Figure 2, both compounds were used simultaneously. Thus glucose does not repress the genes necessary for ascorbate uptake and metabolism. However, the growth curve for cells growing on a mixture of the two energy sources was diauxic (Figure 2). A halt in growth was observed at the point when virtually all glucose had been consumed. Such a transition state usually reflects a re-programming of metabolic activity in the cells. Our data show that the halt was not due to induction of ascorbate metabolism. Possibly, the shift was associated with the onset of amino acid degradation. In chemostat culture we have shown that amino acid catabolism is regulated by growth rate in the presence of limiting glucose

concentrations (Mehmeti et al, submitted). A shift in energy source appears to have a similar effect.

In this paper we have shown that *E. faecalis* can use ascorbate as energy source for fermentative growth. To our knowledge, this is the first description of a Gram positive bacterium growing on ascorbate. Enterococci can grow in environments were ascorbate is found, such as plant material, and possibly more important in the human body (Szeto*, et al.*, 2002). Typical serum ascorbate levels are in the 0,1mM range, but the concentrations can reach 100 times higher levels in tissues (Vissers*, et al.*, 2011). Moreover, ascorbate is secreted in the urine, and *E. faecalis* is a frequently associated with urinary tract infections. A large number of traits have been suggested as pathogenicity factors in *E faecalis*. The ability to use ascorbate adds to this list.

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Table 1. Extracellular metabolites of *E. faecalis* V583.

			Mean concen (mM) ± SD						
Growth medium	OD600	Dry weight g/L	Lactate	Formate	Ethanol	Acetate	Pyruvate	Ascorbic acid	Glucose
	0.09	0.18	1.74±0.23	1.18±0.03	0.23±0.01	16.01±0.12	0.1±0.01	3.70±0.08	0
	0.19	0.28	2.44±0.28	1.52±0.02	0.29±0.02	16.12±0.21	0.12±0.03	3.24±0.05	0
CDM-base + 4.87 mM ascorbate	0.19	0.3	5.01±0.42	4.02±0.02	0.60±0.02	20.49±0.29	0.25±0.02	0.05±0.02	0
	0.16	0.22	2.46±0.12	1.69±0.07	0.32±0.1	16.03±0.38	0.11±0.02	4.1±0.13	0.45±0.22
CDM-base + 4.87 mM ascorbate + 1.25mM glucose	0.24	0.3	4.26±0.42	1.72±0.11	0.64±0.05	16.52±0.22	0.20±0.02	3.62±0.03	0.21±0.12
CDM-base + 12.5mM ascorbate	0.36	0.34	6.91±0.53	4.77±0.23	0.78±0.08	22.21±0.32	0.25±0.04	0.21±0.15	0
CDM-base+ 11.5mM glucose*	0.2	0.3	5.11±0.52	4.14±0.23	0.61±0.02	22.82±0.45	0.26±0.01	6.53±0.2	0
	0.6		24.5±0.6	2.8±0.01	0.82±0.03	15.19±0.02	0.05±0.0	nm	45.5

Growth medium contained 16mMol acetate. nm-not measured. Values are averages of results from three separate experiments. *Data from Mehmeti *et al,* 2011.

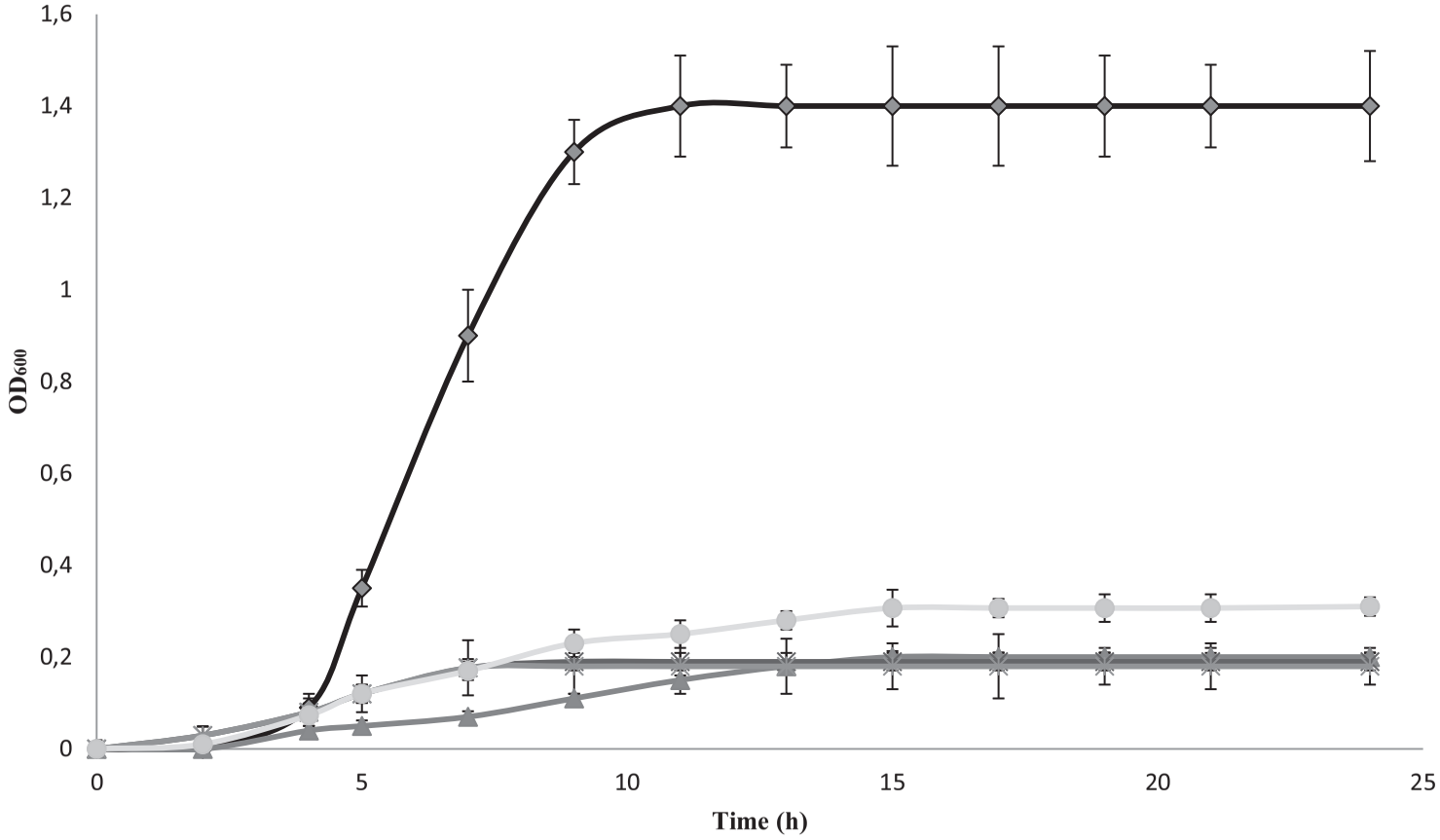

Figure 1. Growth the *Enterococcus faecalis* in different energy sources. Data points represent growth in: CDM-base + 55.5mM glucose (◆), CDM-base + two time increased of cocktail of amino acids and 5mM ascorbate (●), CDM-base +1.25mM glucose (✳), CDM base + 55.5mM ribose (▲) and CDM-base + 5mM ascorbate (✕).

Figure 2. Growth on ascorbic acid and glucose; ( ) glucose, ( ) ascorbate and ( ) growth curve (OD). The medium contained 4.87mM ascorbate and 1.25mM glucose. Values are average of results from three separate experiments.