Disruption of the *alsSD* operon of *Enterococcus faecalis* impairs growth on pyruvate at low pH

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Diacetyl and acetoin are pyruvate-derived metabolites excreted by many micro-organisms, and are important in their physiology. Although generation of these four-carbon (C4) compounds in *Enterococcus faecalis* is a well-documented phenotype, little is known about the gene regulation of their biosynthetic pathway and the physiological role of the pathway in this bacterium. In this work, we identified the genes involved in C4 compound biosynthesis in *Ent. faecalis* and report their transcriptional analysis. These genes are part of the *alsSD* bicistronic operon, which encodes α -acetolactate synthase (AlsS) and α -acetolactate decarboxylase (AlsD). Our studies showed that *alsSD* operon transcription levels are maximal during the exponential phase of growth, decreasing thereafter. Furthermore, we found that this transcription is enhanced upon addition of pyruvate to the growth medium. In order to study the functional role of the *alsSD* operon, an isogenic *alsSD* mutant strain was constructed. This strain lost its capacity to generate C4 compounds, confirming the role of *alsSD* genes in this metabolic pathway. In contrast to the wild-type strain, the *alsSD*-deficient strain was unable to grow in LB medium supplemented with pyruvate at an initial pH of 4.5. This dramatic reduction in growth parameters for the mutant strain was simultaneously accompanied by the inability to alkalinize the internal and external medium under these conditions. In sum, these results suggest that the decarboxylation reactions related to the C4 biosynthetic pathway give enterococcal cells a competitive advantage during pyruvate metabolism at low pH.

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INTRODUCTION

The genus *Enterococcus* belongs to the lactic acid bacteria group, whose members have been traditionally used for the production of fermented foods. However, the employment of *Enterococcus* species in the food industry remains controversial. Enterococci are extremely versatile and well suited for survival under harsh conditions, and they have been associated with a number of human and animal infections as opportunistic pathogens. On the other hand, they are commensals, constituting the healthy microbiota of the gastrointestinal tract of humans, mammals and birds, and under most circumstances they do not cause any harm to the host (Ogier & Serror, 2008). Although this

genus is not considered as generally recognized as safe (GRAS), some enterococcal strains are used as probiotic agents and have beneficial effects on a number of gastrointestinal and systemic diseases (Franz *et al.*, 2003). Moreover, they are of technological significance, since they play an important role in the ripening of cheeses, probably through proteolysis and lipolysis (Foulquié Moreno *et al.*, 2006). Furthermore, their capacity to metabolize citrate and pyruvate is essential in the generation of four-carbon (C4) compounds, such as diacetyl, acetoin and 2,3-butanediol. These compounds are involved in generating the aroma and flavour of dairy products, and contribute to improving the quality of fermented foods (Giraffa, 2003).

Pyruvate is a key catabolic compound in *Enterococcus faecalis*; it is the final product of glycolysis and other catabolic pathways, and may be further metabolized via several enzymes or combinations thereof, depending on environmental conditions (Fig. 1). Given the homolactic behaviour of this micro-organism, when carbohydrates are present in the medium, pyruvate is reduced primarily to

Abbreviations: AL, acetolactate; ALD, α -acetolactate decarboxylase; ALS, α -acetolactate synthase; BDH, butanediol dehydrogenase; CDCFD, carboxy-2',7'-dichlorofluorescein diacetate; DAR, acetoin-diacetyl reductase; MU, Miller units; VP, Voges–Proskauer.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

Fig. 1. Pyruvate metabolism in *Ent. faecalis*. Enzymes involved in the pyruvate fermentation pathway of *Ent. faecalis*: 1, lactate dehydrogenase; 2, pyruvate formate lyase; 3, pyruvate dehydrogenase; 4, phosphotransacetylase; 5, acetate kinase; 6, alcohol dehydrogenase; 7, non-enzymic oxidative decarboxylation. H^+ , proton scalar consumption is indicated for the ALS and ALD catalytic steps.

lactate by lactic dehydrogenase, which is activated by the glycolysis intermediate fructose 1,6-bisphosphate (Leblanc, 2006). In this manner, high levels of NADH produced during glycolysis are reoxidized and redox balance is achieved.

In addition to its role as a key intermediate, pyruvate has been identified as an energy source for *Ent. faecalis* growth (Deibel & Niven, 1964). It has been previously reported that in the presence of this compound higher cell yields, external pH values and C4 compound production are obtained compared with cultures of *Ent. faecalis* grown in the presence of glucose. As a result of pyruvate utilization, besides lactate, significant amounts of acetate and formate are produced. The production of the last two compounds may be the result of pyruvate dehydrogenase (PDH) complex or pyruvate-formate lyase (PFL) activity (Fig. 1), both generating acetyl-CoA from pyruvate and simultaneously transforming the carboxyl group of pyruvate into CO_2 (via PDH) or into formic acid (via PFL). Then, acetyl-CoA is converted to acetate and ATP (via phosphotransacetylase and acetate kinase) or to ethanol and NAD^+ (via alcohol dehydrogenase), according to the energy needs and redox state of the cell (Leblanc, 2006).

Another enzyme described in *Ent. faecalis* as being involved in pyruvate metabolism is α -acetolactate synthase (ALS), which catalyses the thiamine pyrophosphate (TPP)-dependent condensation of two moles of pyruvate to give one mole of α -acetolactate (AL). ALS constitutes the first enzyme of the C4 biosynthetic pathway (Xiao & Xu, 2007). The apparent K_m for pyruvate of ALS in cell-free extracts of *Ent. faecalis* has been found to be 40 mM (Snoep *et al.*, 1992a), which is consistent with a K_m value of 50 mM and a pH optimum of 6.0, as determined *in vitro* for the purified enzyme of *Lactococcus lactis* (Snoep *et al.*, 1992b). As compared with the other enzymes mentioned above, ALS has a very low affinity for pyruvate, which indicates that this enzymic reaction is favoured under conditions of intracellular accumulation of pyruvate and acidic pH. These conditions are indeed those empirically determined to be optimal for the production of C4 compounds during

L. lactis fermentation: low external pH due to acidic by-product generation and co-metabolism of sugars and secondary carbon sources, such as citrate, which increase internal pyruvate concentration (Hugenholtz & Starrenburg, 1992).

Once AL is formed, it could be converted to acetoin, mediated by α -acetolactate decarboxylase (ALD), or to diacetyl by a non-enzymic oxidative decarboxylation step (Fig. 1). Many of the micro-organisms that express a functional C4 pathway also possess acetoin-diacetyl reductase (DAR) and butanediol dehydrogenase (BDH) enzymes. The former is involved in the irreversible reduction of diacetyl to acetoin and then to 2,3-butanediol, while the latter is capable of regenerating acetoin from 2,3-butanediol, requiring NAD^+ as cofactor (Xiao & Xu, 2007).

It has been reported that C4 metabolism can prevent overacidification of the intracellular environment and culture medium during bacterial fermentative metabolism. This may be achieved by the conversion of pyruvate into C4 compounds and their subsequent excretion (Tsau *et al.*, 1992; Yoon & Mekalanos, 2006). In *L. lactis*, we have previously demonstrated that the genes encoding the C4 pathway are specifically induced at low pH at the transcriptional level, indicating that excess pyruvate is channelled towards the production of neutral compounds. The synthesis of these metabolites is relevant in maintaining internal pH homeostasis at low pH (García-Quintáns *et al.*, 2008).

Given the ability of *Ent. faecalis* to utilize pyruvate as a carbon and energy source as well as to produce acetoin and diacetyl from this compound (Deibel & Niven, 1964), it was important for us to obtain further insight into the regulation and physiological role of the C4 pathway in *Ent. faecalis*. In this work, we identified the genes encoding ALS (*alsS*) and ALD (*alsD*) in the *Ent. faecalis* genome. Our transcriptional analysis demonstrated that these genes form a single operon and that its expression in the exponential phase is enhanced by the addition of pyruvate. Finally, a strain with a disruption of the *alsSD* operon showed susceptibility to increasing pyruvate concentrations under

acidic conditions, confirming the connection between this metabolic route and the mechanisms of pH resistance.

METHODS

Bacterial strains and growth conditions. Cultures of *Ent. faecalis* strain JH2-2 were routinely grown at 37 °C without shaking in 100 ml sealed bottles containing 20 ml Luria–Bertani (LB) broth (Sambrook *et al.*, 1989), supplemented with 25 mM glucose (LBG). Antibiotics were added when appropriate at the following concentrations: 1 mg kanamycin ml⁻¹, 5 µg tetracycline ml⁻¹. Overnight cultures prepared in this way were used to inoculate fresh LB adjusted to different initial pH values, as indicated. The inocula were diluted to an initial OD₆₆₀ of 0.08. Cultures were supplemented with glucose or pyruvate (LBP) at the specified concentrations. Alternatively, *Ent. faecalis* was grown in a pH-controlled fermenter (BioFlo 110, New Brunswick Scientific) in 60 ml LB broth containing 150 mM pyruvate at pH 5.0 or 6.5, which was stirred slowly with a magnetic bar. The fermenter was inoculated with an overnight batch culture, as described above. Growth medium pH was monitored continuously, and it was maintained at a constant level by two pumps controlled by a pH meter and connected to flasks containing 1 M NaOH and 1 M HCl. Growth was monitored by measuring OD₆₆₀ in a Beckman DU640 spectrophotometer.

In order to compare the growth parameters (biomass and growth rate) of wild-type and *alsSD* mutant strain, *Ent. faecalis* was also cultivated in sterile 96-well microplates (Cellstar) in a total volume of 200 µl at 37 °C. Exponentially growing cultures were diluted to an initial OD₆₆₀ of 0.15 in LB broth (at initial pH values of 7.0, 5.5 or 4.5) containing the indicated substrates (glucose or pyruvate). Once the cultures had been set up, OD₆₆₀ was registered automatically every 15 min in a PowerWave XS (BioTek) microplate reader. Data presented are the mean values of three independent experiments in which growth curves were assayed in duplicate.

Escherichia coli strains were routinely grown aerobically at 37 °C in LB and transformed as described by Sambrook *et al.* (1989). Growth was monitored by measuring OD₆₀₀ in a Beckman DU640

spectrophotometer. Aerobic growth was achieved by gyratory shaking at 250 r.p.m. The appropriate antibiotics (100 µg ampicillin ml⁻¹, 5 µg tetracycline ml⁻¹ or 30 µg kanamycin ml⁻¹) were included in the medium in order to select cells harbouring the different plasmids. Plates supplemented with 20 µg X-Gal ml⁻¹ and 0.15 mM IPTG were used to identify recombinant plasmids with DNA insertions that impaired β-galactosidase activity in a DH5α strain.

Construction of a Pals–*lacZ* transcriptional fusion. A 459 bp fragment corresponding to the 5' upstream region of the *alsS* gene was amplified by PCR using oligonucleotides P1 (5'-GTTTTCGCGAATTCCCAACAACCTGC-3') and P2 (5'-GTCAACGAACGAA-TTCATCAAAAACC-3') (underlined sequences indicate restriction sites). The amplicon was cloned into the vector pGEM-T Easy (Promega), giving plasmid pGEMals (Table 1). Plasmid pGEMals was digested with *EcoRI* and the released fragment was ligated into the corresponding site of the pTCV-lac vector (Poyart & Trieu-Cuot, 1997), giving plasmid pTCV-S (Table 1). The precise orientation of the fragment was determined by restriction analysis. This plasmid was introduced into *E. coli* DH5α, isolated from this host and then electroporated into *Ent. faecalis* strain JH2-2, as described elsewhere (Friesenegger *et al.*, 1991). The cloned fragments were checked by the sequencing service provided by the DNA Sequencing Facility, University of Maine.

β-Galactosidase assays. Cells carrying the Pals–*lacZ* transcriptional fusion were grown in LBG. Overnight cultures were diluted in LB to OD₆₆₀ 0.08, and then cells were harvested at different times until the culture reached the stationary phase. Different carbon sources were added to the growth medium at defined concentrations, when indicated. β-Galactosidase activity was determined as described by Israelsen *et al.* (1995), except that cells were initially permeabilized by treatment with 20 U mutanolysin ml⁻¹ (Sigma) for 10 min at 37 °C.

RNA isolation and analysis. For Northern blot and primer extension analysis, total RNA from *Ent. faecalis* JH2-2 cells grown in LBG was isolated by a method described previously (Martin *et al.*, 2004). RNA was checked for rRNA integrity and yield. rRNA patterns were similar in all preparations. Total RNA concentration was

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source
<i>Ent. faecalis</i> strains		
JH2-2	Fus ^r Rif ^r , plasmid-free wild-type strain	Jacob & Hobbs (1974)
JHGR2	JH2-2 isogenic derivative <i>alsSD</i> mutant	This study
<i>E. coli</i> strains		
DH5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i> , used as an intermediate host for cloning	Hanahan (1983)
EC101	Kan ^r , <i>supE thi</i> (lacproAB) (F' <i>traD36 proAB lacI^r</i> ZAM15) <i>repA</i> , used as host for pGh8 constructs	Law <i>et al.</i> (1995)
Plasmids		
pGEM-T Easy		Promega
pGhost8	Thermosensitive plasmid used for insertion disruption mutagenesis in enterococci, Tet ^r	Maguin <i>et al.</i> (1996)
pmalsS	pGh8 derivative carrying 500 bp <i>alsS</i> internal fragment	This study
pGEMals	pGEM-T derivative carrying Pals promoter region	This study
pTCV-lac	Promoterless vector which allows <i>lacZ</i> fusion construction	Poyart & Trieu-Cuot (1997)
pTCV-S	pTCV-lac derivative carrying Pals promoter region	This study

*Fus^r, fusidic acid resistance; Rif^r, rifampicin resistance; Tet^r, tetracycline resistance.

determined by UV spectrophotometry and gel quantification with a Gel Doc 1000 gel documentation system (Bio-Rad).

Primer extension analysis was performed as previously described (Martín *et al.*, 2004). The primer used for detection of the start site for *alsS* was PrialS (5'-CCCCAAAATATAGGGCTC-3'). A 1 pmol quantity of primer was annealed to 15 µg total RNA. Primer extension reactions were performed by incubation of the annealing mixture with 200 U Moloney murine leukaemia virus reverse transcriptase (Promega), at 42 °C for 1 h. Determination of reaction product size was carried out in 6% (w/v) polyacrylamide gels containing 8 M urea by comparison with non-related sequencing ladders (Promega fmol DNA Cycle Sequencing System). Extension products were detected with a GE Healthcare Life Sciences Phosphorimager.

For Northern blot analysis, samples containing 15 µg total RNA were separated in a 1.2% (w/v) agarose gel. Nucleic acid transfer to nylon membranes and hybridization with radioactive probes were performed as previously described (Martín *et al.*, 2004). The

alsS-hybridizing probe (probe I, Fig. 2a) was synthesized by employing the insert used for the construction of the pmalsS plasmid as template. Probes were labelled by incorporation of [α - ^{32} P]dATP using the Prime-a-Gene Labeling System (Promega). mRNA molecular sizes were estimated by using a 0.3–6.6 kb RNA ladder (Promega).

Construction of an *Ent. faecalis alsSD*-defective strain. An *alsSD*-deficient strain was constructed by a single recombination event using the thermosensitive vector pGhost8 (Maguin *et al.*, 1996). A 500 bp internal fragment of the *alsS* gene was amplified by PCR using chromosomal DNA of *Ent. faecalis* JH2-2 as template and oligonucleotides alsS1 and alsS2. The forward primer alsS1 (5'-GCCA-AAGCTTACCGTATCGCC-3') introduced an *Eco*RI site, while the reverse primer alsS2 (5'-CCAATTAGCTCGAATTCAGGTTGC-3') introduced a *Hind*III site. Underlined sequences indicate restriction sites. The PCR product was digested with the above-mentioned enzymes and ligated into the corresponding sites of the pGhost8 vector. The resulting plasmid, named pmalsS (Table 1), was introduced into the *E. coli* EC101 strain, isolated from this host and then electroporated

Fig. 2. Transcriptional analysis of *alsSD* genes in *Ent. faecalis*. (a) Schematic representation of the *alsSD* operon. Pals, promoter region. Secondary structure, T, represents a putative Rho-independent transcriptional terminator. The nucleotide sequence of the Pals promoter region is shown below. The transcriptional start site is indicated as +1; -10 and -35 regions and the Shine-Dalgarno (SD) sequence are indicated by boxes; the translation start codon is in bold type. The Pals-*lacZ* transcriptional fusion carried by the pTCV-S plasmid is presented above. (b) Northern blot analysis. Cells were grown in LBG, and RNA was extracted at different times (2, 3, 4 and 6 h). Total RNA was hybridized against the specific probe I (see text for details). (c) Primer extension experiments for the determination of the *alsSD* transcriptional start site (lane 1). Lanes A, C, G and T show non-related sequencing ladders. (d) β -Galactosidase activity of the Pals promoter when *Ent. faecalis* strain JH2-2 [pTCV-S] was grown in LBG.

into *Ent. faecalis* strain JH2-2. Plasmid integration was induced as described by Maguin *et al.* (1996). Briefly, the transformant strain was grown overnight at permissive temperature (30 °C) in LBG with the addition of tetracycline for plasmid maintenance. Saturated cultures were diluted 500-fold into fresh medium and incubated at restrictive temperature (37 °C) for inhibition of plasmid replication. When cultures reached OD₆₆₀ 0.5, serial dilutions were plated on LBG with antibiotic at 37 °C. Chromosomal DNA was extracted from colonies thus obtained, and the interruption of the *alsS* gene was confirmed by PCR and Southern blotting. The resulting *alsSD* mutant strain was named JHGR2 (Table 1). It is important to note that the *alsS* gene interruption procedure produces a polar effect on the expression of *alsD*.

Quantification of C4 compounds. C4 compound (diacetyl and acetoin) concentration was determined by an adaptation of the method of Fertally & Facklam (1987). Briefly, strains were grown in LBG or LBP medium for 6 h at 37 °C. Supernatant of each culture (175 µl) was transferred to 96-well microplates and 25 µl of a freshly prepared solution containing 15 % (w/v) α -naphthol and 0.5 % (w/v) creatine in 7.5 M NaOH was added. Once the mix was prepared, the microtitre plate was loaded into the microplate reader and A₅₄₀ was recorded every 2 min until it reached a constant value. The concentration of C4 compounds per well was calculated from the regression equation for a diacetyl standard curve. Results are presented as the mean and SD of assays performed in triplicate.

Loading of cells with the pH-sensitive fluorescent probe. Cells were first grown in batch culture in LBG medium at pH 7.0. The cultures were then harvested by centrifugation after reaching their exponential growth phase at OD₆₆₀ 0.6–0.8 and washed once with 50 mM HEPES buffer (pH 8.0). Harvested cells were then loaded with the pH-sensitive fluorescent probe 5-(and 6-)carboxy-2', 7'-dichlorofluorescein diacetate (CDCFD; Biotium), as described elsewhere (Breeuwer *et al.*, 1996). Briefly, 0.1 mM CDCFD was added to the cell suspension and incubated for 10 min at 30 °C, and the cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0), and finally stored in ice until use.

Internal pH measurements. For each experiment, CDCFD-loaded cells (approximately 10⁹) were suspended in 2 ml 50 mM potassium phosphate buffer, pH 4.5, and introduced into a 3 ml quartz cuvette (1 cm path length) equilibrated at 30 °C. The sample was stirred with a magnetic stirring bar and the fluorescent signal was monitored every second in a fluorescence spectrometer (Perkin Elmer LS 55). Excitation wavelength was 490 nm and fluorescence emission was recorded at 525 nm (slit widths were 5 nm). Internal pH was determined from the fluorescence signal, as described elsewhere (Molenaar *et al.*, 1991). Internal and external pH was equilibrated at the end of each assay by the addition of 1 mM valinomycin, 1 mM nigericin and 2 % (v/v) Triton X-100. Calibration curves were determined in 50 mM potassium phosphate buffer with pH values ranging from 3.0 to 11.0. pH was adjusted with either NaOH or HCl. Experiments were performed in triplicate.

RESULTS

Bioinformatic search for pyruvate-utilization genes in *Ent. faecalis*

The recently available draft genomic sequence of *Ent. faecalis* strain JH2-2 (<http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AEBO1>), also designated TX4000 (Nallapareddy *et al.*, 2002), was searched for putative genes encoding

pyruvate-utilization enzymes (Fig. 1). In this strain, genes homologous to genes encoding a classical NAD-dependent pyruvate dehydrogenase complex with an E1 α (EFT41959), E1 β (EFT41958), E2 (EFT41957) and E3 (EFT41956) subunit composition typical for Gram-positive bacteria were found. Moreover, genes that might encode phospho-transacetylase (EFT41751), acetate kinase (EFT40840), pyruvate-formate lyase (EFT41268) and alcohol dehydrogenase (EFT41701) were detected, as well as the two genes (EFT40759 and EFT42481) reported to encode L-lactate dehydrogenases (Jönsson *et al.*, 2009). Furthermore, we identified *alsS* (EFT41074) and *budA* (EFT41073) genes, which could encode proteins that convert pyruvate into C4 compounds. The *alsS* gene encodes an ALS (AlsS) of 550 amino acid residues with a calculated molecular mass of 60 100. The *budA* gene, which we propose to rename as *alsD*, encodes a putative ALD (AlsD) of 234 amino acid residues with a calculated molecular mass of 25 700. The two ORFs are separated by a segment of only 14 bp (Fig. 2a). Putative ribosome-binding sites for Gram-positive bacteria are located 7 nt upstream of both ORFs. Since no other genes encoding proteins related to the C4 metabolic pathway were annotated in the JH2-2 strain genome, we looked for genes potentially encoding DAR or BDH enzymic activities. This analysis was performed employing the BLASTP tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and using the amino acid sequences of the ButA and ButB proteins of *L. lactis* IL1403 as query (García-Quintáns *et al.*, 2008). The results of this search did not allow us to detect proteins encoded in the *Ent. faecalis* genome with significant homology to those present in *L. lactis*.

Transcriptional analysis of the *alsSD* operon

Initial inspection of the DNA sequence of the *alsS* and *alsD* genes suggested the presence of a single transcriptional unit. Therefore, Northern blot experiments were performed to confirm this hypothesis. Total cellular RNA was prepared from *Ent. faecalis* cultures grown for 2 h in LBG. Next, total RNA was hybridized against an *alsS*-specific 524 bp α ³²P-labelled DNA probe (Fig. 2a, probe I). Transcripts were estimated to be approximately 2.5 kb in size by comparison with RNA molecular markers (Fig. 2b), as described in Methods. This size is in good agreement with an *alsS*–*alsD* operon starting upstream of the *alsS* gene and ending in a putative Rho-independent terminator of transcription located 29 bp downstream from the stop codon of *alsD*. The RNA secondary structure predicted with the mfold bioinformatic software (Mathews *et al.*, 1999; Zuker, 2003) is the following: CCACGCCCC-ATTCAAGTTGACTaactaGGTCAACTTGAATGGGCTTA-TTGCTGTTCGTCGC, with a Gibbs free energy (ΔG°) of $-29.8 \text{ kcal mol}^{-1}$ ($-124.7 \text{ kJ mol}^{-1}$) (underlined letters indicate nucleotides involved in the complementary interaction; lower-case letters represent internal loops). In addition to the 2.5 kb transcript, an mRNA of 1.7 kb was detected with probe I (Fig. 2b, time equal to 2 h), which is consistent with the size of the *alsS* gene alone. This could

be the result of the larger transcript being processed during the early exponential growth phase. Consistent with this, several putative RNA secondary structures were found in the intergenic region between the two genes.

Next, a primer extension assay was performed in order to determine the transcription start site of the *alsSD* operon. Therefore, RNA obtained in the same way as described for the Northern blot experiment was hybridized to an oligonucleotide (Pria1S, see Methods) complementary to the *alsS* mRNA. The 3' end of Pria1S hybridizes to a 49 nt stretch downstream of the putative translational start. Comparison of the cDNA band synthesized from this primer with non-related DNA sequencing ladders as standards showed that transcription started at a T residue, 34 nt upstream of the ATG translational start codon of the *alsS* gene (Fig. 2c). The promoter was situated further upstream, with a -35 (TTGAtt) and a -10 extended (aGTGGTATtT) putative box (lower-case letters represent bases that deviate from the *Bacillus subtilis* vegetative sigma A consensus sequence, Jarmer *et al.*, 2001), which are separated by a correct 12 nt spacing for RNA polymerase sigma factor binding and are located at the appropriate distance upstream of the transcriptional start site (Fig. 2a). From the transcriptional analysis, we conclude that the *alsS* and *alsD* genes are part of a bicistronic transcriptional unit that is transcribed from the Pals promoter located just upstream of the *alsS* coding region.

Expression of the *alsSD* operon

In order to further substantiate the transcriptional regulation of the *alsSD* operon, we generated a fusion construct formed by a 459 bp fragment corresponding to the 5' upstream region of the *alsS* gene and the *lacZ* reporter gene (Fig. 2a) present in the promoterless vector pTCV-*lac* (Poyart & Trieu-Cuot, 1997). Thus, the JH2-2 [pTCV-S] strain (Table 1), carrying the Pals-*lacZ* fusion, was grown in LBG at pH 7.0, and β -galactosidase activity was examined during the exponential growth period. As is shown in Fig. 2(d), β -galactosidase activity was maximal after 3 h growth, diminishing after that time. To validate the data observed in β -galactosidase experiments, Northern blot analysis was performed. Total RNA was isolated from *Ent. faecalis* JH2-2 cultures grown for 2, 3, 4 and 6 h in LBG and hybridized against probe I (Fig. 2a). As expected, transcripts of approximately 2.5 kb in size were detected, with maximal RNA expression in cultures grown for 3 h, in agreement with the results obtained by means of the transcriptional fusion to *lacZ* (compare Fig. 2b and d).

In a previous study, our group reported that *L. lactis* CRL264 promoters directing the transcription of genes related to the C4 pathway are induced at low pH (García-Quintáns *et al.*, 2008). An acidic induction of C4 genes has also been described for *Klebsiella terrigena* (Mayer *et al.*, 1995), *Staphylococcus aureus* (Weinrick *et al.*, 2004) and *B. subtilis* (Wilks *et al.*, 2009). These data prompted us to analyse whether the Pals promoter of *Ent. faecalis* JH2-2

was also responding to medium acidification. Therefore, the JH2-2 [pTCV-S] strain was grown in LBG adjusted to initial pH values of 5.5 and 7.0. Although a 24 % reduction in β -galactosidase levels was found at acidic pH [353 ± 13 Miller units (MU) for pH 5.5 versus 467 ± 36 MU for pH 7.0, after 4 h growth], probably due to stress conditions, we observed the same transcriptional pattern as the one observed when cultures were grown at initial neutral pH (data not shown). This experiment indicated that Pals is not induced under acidic conditions.

Next, we decided to analyse whether Pals was responding to the presence of different carbon sources in the medium. Glucose addition was assayed first, considering that in *Bacillus anthracis* (Ahn *et al.*, 2006) and *Vibrio cholerae*, transcriptional activation of the C4 pathway by glucose has been demonstrated. Hence, the strain carrying the transcriptional fusion was grown in the presence of 50 and 150 mM glucose, and then β -galactosidase activity was determined. As a result, we observed that the addition of glucose exerted repression (16 and 28 %, respectively) of Pals transcriptional activity compared with a culture of strain JH2-2 [pTCV-S] grown in LB with no addition (Table 2).

Furthermore, it has been shown for *K. terrigena*, *B. subtilis* and *V. cholerae* that the C4 pathway is transcriptionally activated by the addition of acetate to the growth medium (Kovacikova *et al.*, 2005; Mayer *et al.*, 1995; Turinsky *et al.*, 2000). To investigate whether the *Ent. faecalis alsSD* genes were responding to the same effector, strain JH2-2 [pTCV-S] was cultivated in LB medium supplemented with 50 mM glucose plus 50 mM acetate, as reported for *B. subtilis* (Turinsky *et al.*, 2000). Significant induction by the addition of acetate was not detected (Table 2).

Subsequently, we chose to supplement LB with 50 mM pyruvate (LBP), taking into account its role as a substrate

Table 2. Effect of addition of different carbon sources to the growth medium on the expression of the Pals-*lacZ* fusion carried by *Ent. faecalis* JH2-2

Growth medium*	MU†	I‡
LB	508 ± 68	1.00
LBG 50	426 ± 30	0.84
LBG 150	366 ± 26	0.72
LBG 50 + acetate 50	453 ± 1	0.89
LBP 50	755 ± 17	1.48
LBP 150	995 ± 116	1.96

*Cells were grown for 4 h in LB medium (initial pH 7.0) supplemented with different carbon sources at the concentrations (mM) indicated.

†Results are presented as the mean $\pm$ SD of activity measurements in triplicate. β -Galactosidase activity levels for the strain carrying the empty vector were less than 5 MU.

‡I, ratio of the MU values between a given condition and LB without an added carbon source.

of AlsS. Interestingly, we observed a 1.48-fold induction with respect to the activities obtained for the culture in LB with no addition (Table 2). This increase in activity was dependent on pyruvate concentration, considering that the addition of 150 mM of this compound to the growth medium provoked a 1.96-fold increase of β -galactosidase activity levels when compared with the control culture (Table 2). These experiments showed an enhancement of Pals transcriptional activity upon addition of pyruvate to the medium, which has not been detected for the other micro-organisms studied so far.

C4 compound production in *Ent. faecalis*

To investigate the involvement of the *alsSD* operon in C4 compound production in *Ent. faecalis*, we interrupted this operon by single crossover chromosomal integration of plasmid pmalsS, thus generating an *alsSD* mutant strain (JHGR2; Table 1, Fig. 3a). In this strain, *alsS* is disrupted and the integration of the plasmid provokes a polar effect on the expression of *alsD*. In order to quantify the generation of C4 compounds in *Ent. faecalis*, the Voges–Proskauer (VP) reaction was performed in culture supernatants. The VP test is an important diagnostic assay used to assess bacterial ability to produce C4 compounds. Bacterial cultures producing these compounds turn red in the presence of α -naphthol in basic medium. Thus, *Ent. faecalis* JH2-2 and JHGR2 strains were cultivated in LB medium (pH 5.0) with the addition of 150 mM glucose or pyruvate, and an aliquot of each culture supernatant was taken after 6 h. A cell-free control was included in which LB supplemented with pyruvate was incubated for 6 h at 37 °C with subsequent determination of C4 compound levels. This assay showed that the wild-type strain was capable of producing C4 compounds, whereas the levels obtained for the JHGR2 strain supernatant were in the same range as those for the control (Fig. 3b). This observation implies that the enzymes encoded by the *alsSD* operon are required for C4 compound synthesis under these culture conditions. As expected, we also observed that the wild-type strain synthesized C4 compounds at higher levels when the culture medium was supplemented with pyruvate, reaching concentrations seven times higher than those observed for cultures to which glucose was added (Fig. 3b).

The *alsSD* operon is required for growth on pyruvate at low pH

We analysed the physiological role of the *alsSD* operon during pyruvate fermentation at different pH values. Therefore, growth of the *Ent. faecalis* wild-type and *alsSD* mutant strains was compared when they were grown in LB supplemented with 50 and 150 mM glucose (as a control condition) or pyruvate at initial pH values of 7.0, 5.5 or 4.5. Cultures were performed in microplates over 10 h (see Methods) and growth parameters (μ , maximal growth rate; optical density, maximal biomass) were determined for

Fig. 3. Analysis of C4 compound production in *Ent. faecalis* wild-type and *alsSD*-deficient strains. (a) Schematic representation of *alsS* inactivation by insertion of plasmid pmalsS. *alsS'*, fragment of the *alsS* gene; *Tet^r*, tetracycline-resistance cassette; *oriTs*, thermosensitive replication origin of pGhost-series plasmids (Maguin *et al.*, 1996). (b) C4 compound titration performed on supernatants of LBG- and LBP-grown cultures of wild-type (JH2-2) and *alsSD* mutant (JHGR2) strains after 6 h growth. A cell-free control (C) was included (see text for details).

each condition. No significant differences in growth were observed between the two strains when glucose was added to the medium at any of the tested pH values (see Supplementary Table S1), even though a general decrease in growth parameters for both strains was detected when medium was adjusted to more acidic pH values. In the same way, the effect on the growth of both strains of the addition of pyruvate to the medium was evaluated. At an initial pH of 7.0, growth parameters for strain JH2-2 were similar to those for strain JHGR2 (for 150 mM pyruvate, $\mu=0.83$ h⁻¹ versus 0.82 h⁻¹ and OD₆₆₀ 1.42 versus 1.38, respectively; see Supplementary Table S2). When the pH of the medium was adjusted to 5.5, the addition of 50 mM pyruvate did not provoke any difference in growth between the two strains (compare Fig. 4a and b). However, when the amount of pyruvate was raised to 150 mM, μ for the wild-type strain increased to 0.75 h⁻¹ ($\mu=0.64$ h⁻¹ in LB), while μ for the *alsSD* mutant decreased to 0.47 h⁻¹ ($\mu=0.63$ h⁻¹ in LB). Furthermore, biomass production for the wild-type strain was maximal under these conditions (OD=1.92), whereas a strong reduction was observed for the *alsSD*-defective strain (OD=0.89) (Fig. 4a, b,

Fig. 4. Growth of *Ent. faecalis* JH2-2 and JHGR2 in LB adjusted to pH 5.5 (a and b, respectively) or 4.5 (c and d, respectively). pH remained uncontrolled during the assay. Strains were grown in LB without any further carbon source addition (●) or were supplemented with 50 (▼) or 150 mM (■) pyruvate.

Supplementary Table S2). Unquestionably, the JHGR2 strain phenotype became evident when the pH of the medium was set at 4.5. Even though both the wild-type and the *alsSD*-deficient strain became inhibited when they were cultivated in LB without additions (Supplementary Table S2), this effect was reversed in the case of the wild-type strain when the medium was supplemented with 50 or 150 mM pyruvate (Fig. 4c), and similar growth parameters were obtained to those observed at pH 5.5 (Fig. 4a). In contrast, these amounts of pyruvate were detrimental to the growth of strain JHGR2, which was completely inhibited (Fig. 4d). To confirm the role of the *alsSD* operon in this growth-deficient phenotype, we proceeded to complement the JHGR2 strain by expressing the AlsS protein *in trans*, using to this end the pBM01 expression vector (Marelli & Magni, 2010). JHGR2 cells carrying the wild-type copy of the *alsS* gene partially

recovered their capacity to grow at pH 4.5 in the presence of 50 mM pyruvate (Supplementary Fig. S1).

All these observations indicate that the *alsSD* operon fulfils an important role in pyruvate metabolism at low pH.

Comparison of external and internal pH levels between *Ent. faecalis* wild-type and the *alsSD* mutant strain

To determine whether the observed growth impairment of the *alsSD* mutant was related to its inability to counteract acidification, we decided to evaluate changes in the external pH in batch cultures of JH2-2 and JHGR2 cells. Thus, strains were grown in LB medium supplemented with 150 mM pyruvate at initial pH values of 6.5 and 5.0. As shown in Fig. 5(a), at pH 6.5 the wild-type strain did not

Fig. 5. External and internal pH variations during growth of *Ent. faecalis* on pyruvate. Strains JH2-2 (circles) and JHGR2 (triangles) were grown in LB supplemented with 150 mM pyruvate at initial pH values of 6.5 (a) or 5.0 (b). Filled symbols, pH measurements; open symbols, OD₆₆₀ values. Experiments were carried out in triplicate, and data are mean $\pm$ SD. (c) *Ent. faecalis* internal pH variations in response to pyruvate. Cells were loaded with the CDCFD fluorescent probe and suspended in phosphate buffer at pH 4.5. A pulse of 50 mM pyruvate (Pyr) was added at the time indicated by the arrow.

cause a significant acidification of the growth medium, and the final pH value was 6.3. Under such conditions, the JH2-2 strain grew to OD₆₆₀ 1.5 after 7 h. On the other hand, acidification of the external pH, reaching 5.6 after 5 h, was observed for strain JHGR2, remaining fairly constant thereafter. This means that for the *alsSD*-deficient strain, the external pH fell 0.7 units more than for the wild-type strain. Remarkably, the *alsSD* mutant reached OD₆₆₀ 1.3 after 7 h, which is lower than the corresponding value for the wild-type.

When the initial pH was set to 5.0, the JH2-2 strain produced an increase of 0.4 units in the external pH in the first 4 h, after which growth started, reaching OD₆₆₀ 0.4 (Fig. 5b) after 7 h. In contrast, the JHGR2 strain was unable to alkalinize the medium and this led to growth impairment.

To continue analysing how external pH was affecting *Ent. faecalis* growth on pyruvate, cultures of wild-type and *alsSD* mutant strains were grown at a constant external pH of 6.5 or 5.0 in LB medium containing 150 mM pyruvate. When the pH was set to 6.5, both strains reached OD₆₆₀ 2.6 after 8 h (Supplementary Fig. S2a), in contrast to the results of the experiments in which external pH remained uncontrolled, in which a delay in growth of the *alsSD* mutant was detected after 4 h (Fig. 5a).

In contrast, when the pH was kept constant at 5.0, none of the strains was able to grow in the pyruvate-supplemented medium (Supplementary Fig. S2b). These results indicated that the alkalinization of the external medium observed for the wild-type strain in batch cultures (Fig. 5b), which is

constrained by the fermenter, is vital for cells to start growing.

Interestingly, when after 6 h at constant pH 5.0 the fermenter corresponding to the *alsSD* mutant strain was reset to pH 5.6 (the value at which exponential growth of the wild-type strain in batch cultures started, Fig. 5b), the deficient strain showed an increase in optical density (Supplementary Fig. S2b).

In order to correlate these findings with intracellular pH changes in response to the addition of pyruvate, internal H⁺ levels were monitored by using the pH-sensitive fluorescent probe CDCFD. First, *Ent. faecalis* wild-type and *alsSD* mutant cells were grown in LBG medium adjusted to pH 7.0 without pH control. Then, cells were loaded with the fluorescent probe and suspended in phosphate buffer (pH 4.5). The fluorescence measurements and their conversion to pH units indicated that the resting cells maintained a pH gradient across the membrane of about 1.1 units, more alkaline inside the bacteria, when suspended in this buffer. As shown in Fig. 5(c), addition of 50 mM pyruvate produced an immediate cytoplasmic acidification for both strains, which was followed by a stabilization of the internal pH until the 3 min time point. Remarkably, after that time, the wild-type strain showed an additional increase in the internal pH with respect to that observed for the *alsSD* mutant. This alkalinization allowed the wild-type strain to reach an internal pH value of 6.0, while for the *alsSD*-deficient strain, the pH remained constant at 5.8. All these experiments suggest that by redirecting the accumulated pyruvate to the C4 pathway,

the wild-type strain achieves a further increase in the internal pH, which makes an extra contribution to pH homeostasis.

DISCUSSION

Excretion of C4 compounds, which can be determined by the VP test, has vital implications for the physiology of different micro-organisms because, as described elsewhere, it is used to avoid acidification, participates in the regulation of the NAD:NADH ratio and functions as a carbon-storing strategy (Xiao & Xu, 2007). Although production of these compounds in *Ent. faecalis* has been widely used as a microbial classification marker (Fertally & Facklam, 1987), little is known about the gene regulation of the C4 pathway and its physiological role in this bacterium. In this work, the genes involved in the biosynthesis of acetoin and diacetyl in *Ent. faecalis* were identified: *alsS*, which encodes an ALS, and *alsD*, which encodes an ALD. Accordingly, it was demonstrated that the disruption of *alsSD* genes affects the production of C4 compounds. No other genes encoding enzymes involved in C4 metabolism, such as DAR or BDH, were detected by homology searches. This is in agreement with a recent publication in which the final metabolic products from glucose and citrate metabolism were determined in *Ent. faecalis* V583. Under these growth conditions, production of 2,3-butanediol could not be detected (Jönsson *et al.*, 2009).

We have also determined that the *alsS* and *alsD* genes form a bicistronic transcriptional unit directed by the Pals promoter region. Furthermore, we found that the *alsSD* operon is induced in the middle of the exponential phase and that its expression is enhanced by the addition of pyruvate to the culture medium. However, in *Ent. faecalis* no transcriptional induction of the *alsSD* genes was observed when the medium was supplemented with glucose or acetate or when the external pH was reduced, as has been reported for other micro-organisms (García-Quintáns *et al.*, 2008; Kovacicova *et al.*, 2005; Mayer *et al.*, 1995; Renna *et al.*, 1993; Turinsky *et al.*, 2000).

Experiments in which C4 compound production in the *Ent. faecalis* wild-type strain was quantified indicated a sevenfold increase in the concentration of these compounds in cultures grown in the presence of pyruvate with respect to those supplemented with glucose. This finding may be related to the fact that upon addition of exogenous pyruvate to the medium, it accumulates in the cell, reaching the K_m of ALS, which leads to C4 compound production. It is important to note that the transcriptional increment for the *alsSD* operon in response to pyruvate observed in this work could also be increasing the amount of the corresponding enzymes.

In view of these results, the role of the *alsSD* operon when *Ent. faecalis* is grown in the presence of pyruvate at different pH values was studied. At pH 7.0, strains JH2-2 and JHGR2 showed similar optical density and μ values for

each pyruvate concentration tested (Supplementary Table S1). At this pH, the concentration of the protonated form of pyruvate (pyruvic acid), calculated with the Henderson–Hasselbalch equation, is in the micromolar range (varying from 1 to 4 μ M when the external pyruvate concentration increases from 50 to 150 mM). When the external pH was reduced to 5.5 and 150 mM pyruvate was added to the medium, the concentration of its protonated form increased to 0.1 mM. In this case, the wild-type strain was able to grow, whereas the *alsSD*-defective strain was partially inhibited (Fig. 4b). The complete arrest of strain JHGR2 occurred when the initial external pH value was set at 4.5 (Fig. 4d), a condition in which the external pyruvic acid concentration reaches 1.2 mM (when 150 mM pyruvate is added). These results showed that this 300-fold rise in the external concentration of pyruvic acid when the pH decreased from 7.0 to 4.5 provoked an inhibitory effect on *alsSD* mutant growth. We should bear in mind that the toxicity of this and other weak organic acids is related to their ability to permeate through the membrane and release protons in the more alkaline cytoplasm (Cotter & Hill, 2003). Interestingly, this effect is efficiently counteracted by the wild-type strain through the condensation of pyruvate to C4 compounds.

The variation of medium pH during pyruvate metabolism in batch cultures of JH2-2 and JHGR2 strains also showed significant differences. When the medium pH was set at 6.5, the pH remained fairly constant for the wild-type strain, reaching a value of 6.3 at 7 h of growth (Fig. 5a). Conversely, the *alsSD*-deficient strain showed an acidification of the external pH which amounted to 0.9 units after 7 h (Fig. 5a). Interestingly, when the initial pH was adjusted to 5.0, the wild-type strain showed a lag phase of 4 h in its growth curve during which an alkalinization of the external medium was detected (Fig. 5b). In this way, the JH2-2 strain resumed growth only after the external pH value increased by 0.4. This may be related to the activation of other enzymic activities besides ALS that can use pyruvate as a substrate. Conversely, the *alsSD* mutant strain was unable to raise the medium pH, leading to growth impairment. It has been reported that lactic acid bacteria possess numerous mechanisms to counteract acidic stress (Cotter & Hill, 2003). However, the C4 pathway seems to be the main mechanism under the conditions employed in our study, since the wild-type strain was the only one capable of raising the external pH when this parameter was set initially to 5.0 (Fig. 5b). These results were corroborated by the fact that when the pH was kept constant at 6.5, the *alsSD*-deficient strain behaved like the wild-type strain. This observation may indicate that when external pH remains near neutrality, pyruvate-utilizing enzymes are active, pyruvate toxicity is low and the role of the C4 pathway is not as relevant as when the pH is more acidic.

On the other hand, when the pH was maintained at 5.0 during the whole assay, none of the strains was capable of growing. This may be related to the fact that the

alkalinization provoked by the C4 pathway leads to pH values at which other pyruvate-utilizing enzymes become more active. Interestingly, when the pH was raised from 5.0 to 5.6, the *alsSD* strain resumed growth (Supplementary Fig. S2b). This observation suggests that this artificial pH shift replaces the external pH shift provoked by the C4 pathway in batch cultures of *Ent. faecalis*.

This phenomenon of growth medium alkalinization related to C4 compound production has also been reported for other Gram-positive and -negative micro-organisms. In *V. cholerae* and *B. subtilis*, it has been shown that glucose metabolism is redirected to the production of C4 compounds and consequently the pH of the medium increases. This capacity confers an adaptive advantage that, in the case of *V. cholerae*, leads to a prolonged growth period, which has an effect on pathogenicity (Yoon & Mekalanos, 2006), while for *B. subtilis* it results in a greater capacity to spread in the environment (Kinsinger *et al.*, 2005).

In agreement with the analysis of medium pH, our determination of internal pH showed that the wild-type strain has a greater capacity to alkalinize the cytoplasm in response to the addition of pyruvate at external pH 4.5 (Fig. 5c). These observations are consistent with the fact that the enzymic reactions catalysed by AlsS and AlsD (Fig. 1) contribute to pH homeostasis because they result in an alkalinization of the medium caused by scalar proton consumption (Lolkema *et al.*, 1995).

In summary, the findings obtained in this study improve our understanding of the role of the C4 pathway in *Ent. faecalis*, and this organism's strategies for growing on different nutrient sources and under different growth conditions. Our results show that pyruvate metabolism at low pH in this micro-organism requires the presence of an active C4 pathway, which can reduce the toxic levels of intracellular pyruvate while simultaneously contributing to the alkalinization of both the internal and the external medium.

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