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The phosphoketolase pathway in Lactobacillus pentosus.

Posthuma, C.C.

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Chapter 3

Cloning, sequencing and functional characterization of ackA, the gene encoding acetate kinase from Lactobacillus pentosus MD363

C. Posthuma, R. Bader, P.W. Postma, P.H. Pouwels
3.1 ABSTRACT

The gene encoding acetate kinase, ackA, from Lactobacillus pentosus MD363 was cloned and sequenced. It encodes a protein of 398 amino acids with acetate kinase activity, as shown by functional expression of the gene in an Escherichia coli ackA deletion mutant. The deduced amino acid sequence of ackA showed high similarity to that of acetate kinases from other organisms. The highest similarity was observed with the proteins encoded by ackA (64% identical residues) and ackB (71% identical residues) from Lactobacillus sakei. An ackA knock-out mutant of L. pentosus lacked acetate kinase activity and was unable to grow on sugars which are fermented via the phosphoketolase pathway. Northern blot analysis indicated that the ackA gene is transcribed into monocistronic mRNA.

Activity assays in the wild type strain of L. pentosus showed that acetate kinase activity is comparable after growth on fructose, gluconate, ribose, xylose or arabinose, but a four-fold lower activity was observed after cultivation on glucose. In a ccpA knock-out mutant of L. pentosus, the repression of the acetate kinase synthesis was relieved after growth on glucose, suggesting the involvement of catabolite repression (CR) mediated by CcpA. Repression of acetate kinase synthesis by glucose was also relieved in a mutant of L. pentosus that was affected in the mannose phosphotransferase system (PTS) activity.
3.2 Introduction

*Lactobacillus pentosus* is a facultative heterofermentative lactic acid bacterium, which possesses the enzymes of both the glycolytic and the phosphoketolase pathway (PKP; see also Introduction and Chapter 2). Hexoses like glucose and fructose are fermented *via* the glycolytic pathway, whereas pentoses are metabolized by their appropriate enzymes into xylulose 5-phosphate, which is converted by xylulose 5-phosphate phosphoketolase (XpkA), the central enzyme of the PKP, into glyceraldehyde 3-phosphate (GAP) and acetyl-phosphate. Further metabolism of GAP by glycolytic enzymes results in lactate as an end-product, while acetyl-phosphate is converted into acetate by acetate kinase. An ATP molecule is produced during this latter reaction by substrate level phosphorylation. During growth on gluconate, part of the acetyl-phosphate that is produced in the PKP is also converted into ethanol in order to reoxidize the NADH that is formed by 6-phosphogluconate dehydrogenase, one of the enzymes involved in the conversion of gluconate into xylulose 5-phosphate (see Figure 1.1, General Introduction).

Acetate kinase activity also plays a role during homolactic fermentation of lactic acid bacteria under aerobic conditions. In *Lactobacillus plantarum*, pyruvate resulting from glycolysis is converted into acetyl-phosphate by pyruvate oxidase [40, 117, 118] and is further metabolized by acetate kinase into acetate, yielding an extra molecule of ATP. NADH is simultaneously reoxidized by an oxygen inducible NADH-oxidase (for reviews: see [13, 59, 142]). Since *L. plantarum* is evolutionary closely related to *L. pentosus* [12], a similar role might be expected for acetate kinase in *L. pentosus*.

Acetate kinase does not play a role during growth on acetate in lactic acid bacteria, since these organisms are unable to utilize acetate as an energy or carbon source. Other organisms, like *Methanosarcina thermophila* and *Corynebacterium glutamicum* can utilize acetate by converting it into acetyl-CoA through acetate kinase and phosphotransacetylase, encoded by *ackA* and *ptaA*, respectively. The latter enzyme catalyses the conversion of acetyl-phosphate into acetyl-CoA. The expression of *ptaA* and *ackA*, which form an operon in these organisms, is induced in the presence of acetate in both organisms [97, 123].

In contrast, phosphotransacetylase and acetate kinase are primarily operative in the acetyl-CoA-to-acetate direction in *Bacillus subtilis*, and are involved in acetate excretion during growth on glucose [42]. The expression of the *ackA* and *ptaA* genes of *B. subtilis* is activated in the presence of excess glucose by a catabolite control protein A (CcpA)-mediated mechanism in which the same components are involved as in the general
mechanism of CR in Gram positive bacteria [42, 94, 122, 143]. Growth on acetate by \textit{B. subtilis} depends on the presence of acetyl-CoA synthetase, encoded by \textit{acsA}, which converts acetate directly into acetyl-CoA [43]. Also in \textit{Escherichia coli}, acetate is metabolized by acetyl-CoA synthetase, although it can be converted into acetyl-phosphate by acetate kinase and phosphotransacetylase as well [57, 58, 67].

The interest in acetate kinase from \textit{L. pentosus} originates from previous research on xylose metabolism in this organism, in which the specific part of xylose metabolism, i.e. xylose uptake and the conversion into xylulose 5-phosphate was studied. Xylose is transported by facilitated diffusion without being phosphorylated by the mannose PTS in \textit{L. pentosus}, that generally transports glucose or mannose with concomitant phosphorylation [10]. The genes that are involved in the conversion of the pentose into xylulose 5-phosphate, \textit{xylA} encoding xylose isomerase and \textit{xylB} encoding xylulose kinase, form an operon which is regulated by the repressor protein XylR [75]. Expression of \textit{xylAB} is induced in the presence of xylose and repressed by glucose mediated by CcpA [73, 74].

Since the regulation of the specific part of the xylose metabolism, i.e. the conversion of xylose into xylulose 5-phosphate, has been elucidated, we were also interested in the enzymes involved in the general part. The characterization as well as a study of the regulation of the synthesis of one of these enzymes, XpkA, is reported in Chapter 2 of this thesis. This Chapter deals with the cloning, sequencing and functional expression of \textit{ackA}, encoding acetate kinase, from \textit{L. pentosus} MD363.

### 3.2 MATERIALS AND METHODS

\textbf{Bacterial strains, plasmids and growth conditions} - The strains and plasmids used in this study are listed in Table 3.1. \textit{Escherichia coli} was cultivated on Luria-Bertani (LB) agar or in LB broth. Ampicillin if required was added to a final concentration of 100 \(\mu\)g/ml. \textit{Lactobacillus pentosus} was cultivated without agitation at 37 \(^\circ\)C in MRS medium ([16]; Difco Laboratories, Detroit, Mich.), or M-medium [74] containing 1\% (wt/vol) of the indicated sugar. Erythromycin was added at a final concentration of 5 \(\mu\)g/ml, when necessary. For plating, media were solidified with 1.5\% (wt/vol) agar.

\textbf{Materials} - Enzymes for the acetate kinase activity assay were obtained from Boehringer Mannheim. Enzymes for DNA manipulations were obtained from Boehringer Mannheim or New England Biolabs and used according to the specifications of the manufacturer. \([\alpha\text{-}^{32}\text{P}]\text{dATP (3,000 Ci/mmol)}\) was obtained from Amersham.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source Reference</th>
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<tbody>
<tr>
<td><strong>Lactobacillus pentosus</strong></td>
<td></td>
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<tr>
<td>MD353</td>
<td>wild type strain</td>
<td>[75]</td>
</tr>
<tr>
<td>MD363</td>
<td>wild type strain</td>
<td>[73]</td>
</tr>
<tr>
<td>LPE4</td>
<td>MD363, ccpA::pEl2, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[73]</td>
</tr>
<tr>
<td>LPE5</td>
<td>2DG&lt;sup&gt;R&lt;/sup&gt; mutant of MD353</td>
<td>[10]</td>
</tr>
<tr>
<td>LPE116</td>
<td>MD363, ackA::pLPA15, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
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<tr>
<td>KS24</td>
<td>del(ackA) supE thi del(lac-proAB) hsdR4</td>
<td>[58]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pRV300</td>
<td>3.5 kb Lactobacillus integration vector, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[70]</td>
</tr>
<tr>
<td>pBCP367</td>
<td><em>E. coli</em> expression vector, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[148]</td>
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<tr>
<td>pLPA14</td>
<td>pUC18 with an 0.8 kb blunt internal PCR fragment of ackA inserted in Smal</td>
<td>This study</td>
</tr>
<tr>
<td>pLPA15</td>
<td>pRV300, 600 bp HindIII fragment from pLPA14 inserted in HindIII; ackA integration plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pPLA27</td>
<td>pUC18 with a 2.4 kb inverse PCR fragment of ackA, inserted between BamHI and EcoRI</td>
<td>This study</td>
</tr>
<tr>
<td>pLPA34</td>
<td>pUC18 with PCR amplified ackA (total gene) inserted between Ndel and BamHI</td>
<td>This study</td>
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<tr>
<td>pLPA35</td>
<td>like pLPA34, but independent PCR clone ligated between Ndel and BamHI</td>
<td>This study</td>
</tr>
<tr>
<td>pLPA37</td>
<td>pBCP367 with PCR amplified ackA ligated between Ndel and BamHI (expression in <em>E. coli</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>pLPA38</td>
<td>pUC18 with a 0.4 kb PCR amplified fragment containing upstream region of ackA inserted between EcoRI and BamHI</td>
<td>This study</td>
</tr>
<tr>
<td>pLPA39</td>
<td>like pLPA38, but independent PCR clone ligated between EcoRI and BamHI</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 3.1. Bacterial strains and plasmids.
DNA manipulations - Recombinant DNA procedures and transformation of E. coli were performed by standard methods [106]. Chromosomal DNA and RNA of L. pentosus were isolated as described [75, 93]. For the Northern blot assay, RNA was isolated from wild type cells during logarithmic growth on MRS liquid medium. For Southern blot analysis, 10 μg of chromosomal DNA was digested, separated on an 0.8 % (wt/vol) agarose gel and blotted on Hybond-N™ (Amersham) by using the VacuGene™ XL Vacuum Blotting System (Pharmacia). In both Southern and Northern blot experiments, the 780 bp internal PCR fragment of ackA that was amplified by using Ak140 and Ak400R (see Cloning and sequencing of ackA) was used as a probe after labelling with [α-32P]dATP by using the Prime-a-Gene Labelling System from Promega. The hybridization temperature was 65 °C. The blots were washed under stringent conditions: twice with 6xSSC, 0.5% (wt/vol) SDS at room temperature for 15 minutes, twice with 1xSSC, 0.5% (wt/vol) SDS at 37 °C for 15 minutes and once with 0.1xSSC, 0.5% (wt/vol) SDS at 65 °C. The signals were detected by autoradiography.

DNA-fragments were isolated from agarose gels using the QIAEX II Gel Extraction kit (QIAGEN GmbH). PCR reactions were performed using Expand™ high fidelity PCR system (Boehringer Mannheim), unless indicated otherwise. Automated sequencing was performed by BaseClear, Leiden, The Netherlands.

Figure 3.1. Physical map and organization of L. pentosus ackA locus. The sequenced part is indicated. The primers used for cloning are indicated, as well as the constructed plasmids. The arrow indicates the putative transcriptional start, the stem-loop structure indicates a putative transcriptional terminator, cre means catabolite responsive element. 1: Ak140; 2: Ak400R; 3: Ackinv1; 4: Ackinv6; 5: Akgen1; 6: Akgen2; 7: Akgen3; 8: Akgen4. The exact position of the Sful restriction sites is unknown, as indicated by the question marks.
Cloning and sequencing of ackA - The primers used for cloning of ackA and the constructed plasmids are shown in Figure 3.1. With the degenerate primers Ak140 (TTRGCICICTTTRCAYAAYCC, complementary to the nucleotides encoding residues 117 to 123) and Ak400R (ATCATYAAYTCTRIYIGTIGG, complementary to the nucleotides encoding residues 387 to 380), a 780 bp internal fragment of ackA from *L. pentosus* MD363 was amplified by touch-down PCR [21] using *Taq* polymerase. The annealing temperatures ranged from 55 °C to 40 °C. The primers were designed on the basis of regions with high similarity, that were identified by the alignment of the amino acid sequences from 13 acetate kinases (*Bacillus subtilis*, EMBL gene bank accession number P37877; *Clostridium acetobutylicum*, P7110; *Corynebacterium glutamicum*, P77845; *Escherichia coli*, P15046; *Haemophilus influenzae*, P44406; *Mycoplasma capricolum*, Q49113; *Mycoplasma genitalium*, P47599; *Mycoplasma pneumoniae*, P75245; *Mycobacterium tuberculosis*, P96255; *Synechocystis* sp., P73162; *Methanosarcina thermophila*, P38502; *Thermoanaerobacterium thermosaccharolyticum*, Q59331; Chlorante-Aster yellows phytoplasma, AAB51345) and two propionate kinases from the database (*Escherichia coli* PI 1868; *Salmonella typhimurium*, O06961). The alignment was done with ClustalW at http://www.ebi.ac.uk/clustalw/. The amplified fragment was treated with T4 DNA polymerase to remove the 3' A overhang generated by *Taq* polymerase and the resulting blunt fragment was ligated into the *Smal* site of pUC18. The resulting plasmid (pLPA14) was used for automated sequencing. The flanking regions of ackA were cloned by inverse PCR. For this purpose, chromosomal DNA of *L. pentosus* MD363 was digested by *Sful* and ligated at a concentration of 1 ng/µl. Ligated DNA was precipitated and dissolved at a concentration of 10 ng/µl. 30 ng was used in a PCR experiment using Ackinv1 (CGCGGATCCGGTAACAGATCAGGAAAC, corresponding to residues 132 to 138) containing an additional *BamHI* site (underlined) and Ackinv6 (CGCGGAATTCCATCACATGCACGGCAAGG, corresponding to the nucleotides encoding residues 364 to 358), containing an additional *EcoRI* site (underlined). The amplified fragment (about 2.4 kb) was cloned between the *BamHI* and *EcoRI* sites of pUC18 to obtain pLPA27 and sequenced.

The upstream region of ackA was amplified in two independent PCR reactions with Akgen1 (CGCGGAATTCCCGTAACAGATCAGGAAAC, located about 350 nucleotides upstream of the translational start of ackA) containing an additional *EcoRI* site (underlined) and Akgen2 (CGCGGATCCCTCAACCATTGCAGGAAAC, complementary to the nucleotides encoding residues 33 to 26), containing an additional *BamHI* site (underlined). Both fragments were ligated between the *EcoRI* and *BamHI* sites of pUC18, which resulted in plasmids pLPA38 and pLPA39.
Figure 3.2. Strategy for the construction of the ackA integration vector pLPA15 for \textit{L. pentosus}. pLPA14 was digested with HindIII and the 600 bp fragment was ligated in HindIII digested pRV300. The resulting plasmid was named pLPA15. \textit{Em}, erythromycin resistance; \textit{Ap}, ampicillin resistance. Open and shaded squares indicate multiple cloning sites originating from pRV300 and pLPA14, respectively.

With Akgen3 (GGGAATTCCATATGGCAAAAATTTTAGCAATTAACGCCG; the added Ndel site is underlined and starts at the translational start of \textit{ackA}) and Akgen4 (CGCGGATCCACTGAAGGTAAATC1TGACGAAC, located about 60 nucleotides downstream of \textit{ackA} and containing an additional BamH1 site which is underlined), \textit{ackA} was amplified in two independent PCR reactions. Ligation of these fragments between the Ndel and BamH1 sites of pUC18 resulted in pLPA34 and pLPA35. The sequences of pLPA34 and pLPA35 were used to verify the initially obtained sequence of \textit{ackA}. If
the sequence at a specific place differed in the two plasmids, nucleotide(s) were chosen that occurred in two of the three obtained sequences. In the same way, pLPA38 and pLPA39 were used to verify the initially obtained sequence of the upstream region of ackA.

Construction of ackA knock-out mutant - pLPA15 (ackA integration vector) was constructed as follows (Figure 3.2): Restriction of pLPA14 with HindIII, cutting inside of the inserted PCR fragment and the multiple cloning site, resulted in a 600 bp internal fragment of ackA. The fragment was cloned in the HindIII site of pRV300. The orientation of the fragment was checked by restriction analysis and was opposite to that of EmR. The construction of pLPA15 is shown in Figure 3.2. pLPA15 was used to transform L. pentosus MD363 by electroporation and EmR transformants were isolated. The correct integration of pLPA15 into the chromosome of one of the transformants was checked by Southern blot analysis (as described in the Results). The ackA knock-out mutant of L. pentosus MD363 was named LPE116.

Preparation of cell-free extracts - L. pentosus cells in the exponential phase of growth were harvested by centrifugation (3700× g, 4°C, 10 min) and washed twice in 20 mM Hepes HCl (pH 7). Residual liquid was removed by vacuum drying and the pellet was stored at -20°C until further use. For the preparation of cell-free extracts, the pellets were thawed and resuspended in 1/20 of the culture volume in 20 mM Hepes HCl (pH 7), 0.5 mM EDTA, 0.5 mM DTT. Cells were broken by three passages through a French pressure cell (11,000 lb/in²). Cell debris was removed by centrifugation (10,000 x g, 4°C, 20 min). For E. coli, cells were not vacuum dried and only one passage through a French pressure cell was needed. E. coli extracts were centrifuged at 100,000×g at 4°C for 1 h and the supernatant was used for the determination of acetate kinase activity. Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH), using BSA as a standard.

Expression of ackA in E. coli - ackA was amplified using Ack-gen3 and Ack-gen4 (see Cloning and sequencing of ackA) and cloned between the Ndel and BamHI sites of pBCP367. This plasmid was named pLPA37. E. coli KS24 cells (containing pLPA37) were grown aerobically in 200 ml LB-broth containing ampicillin at 37°C. Expression of ackA was induced at OD600 1.8 with 60 μM isopropyl β-D-thiogalactopyranoside (IPTG). The culture was harvested two hours after induction and a soluble protein fraction prepared (see Preparation of cell-free extracts). As a negative control, E. coli KS24 containing pBCP367 was used.
Acetate kinase activity assay - Acetate kinase activity was measured as described [133]. The ADP formation was coupled to NADH consumption by addition of phosphoenolpyruvate (PEP), pyruvate kinase and lactate dehydrogenase. The reaction mixture contained 0.75 ml 100 mM Imidazole HCl, pH 7.2; 0.02 ml water; 0.06 ml 32 mM PEP; 0.02 ml 1M MgCl₂; 0.03 ml 2.5 M KCl; 10 µl 100 mM ATP, pH 7; 2 µl pyruvate kinase (10 mg/ml in glycerol); 2 µl lactate dehydrogenase (10 mg/ml in glycerol) and 7 µl 22.5 mM NADH. Cell-free extract (in total 4 to 15 µg total protein for *L. pentosus* and *E. coli* KS24 containing pBCP367; 50 to 100 ng protein for *E. coli* containing pLPA37) was added to the reaction mixture in a 1 ml cuvet and the NADH consumption was followed in time at 340 nm at 30°C in a spectrophotometer. An initial decrease in NADH concentration was observed in the absence of acetate. Acetate kinase activity started upon addition of 100 µl 2M sodium acetate. Acetate kinase activity was calculated as the difference between both rates and is expressed in µmol·min⁻¹·mg⁻¹. The decrease in absorption at 340 nm was linearly dependent on the protein concentration.

3.3 Results

Cloning and sequencing of ackA - In order to clone and sequence the gene encoding acetate kinase from *L. pentosus*, degenerate primers were designed on the basis of regions with high similarity between several acetate kinases. Chromosomal DNA from *L. pentosus* MD363 was used in a touchdown PCR experiment with the primers Akl40 and Ak400R to obtain an internal fragment of the acetate kinase gene (Figure 3.1). A fragment of about 800 nucleotides was amplified, purified and sequenced. The amino acid sequence deduced from this fragment showed similarity to other acetate kinases, which suggested that indeed a part of a gene encoding acetate kinase had been amplified. For the isolation of the remaining part of the acetate kinase gene, new primers were designed (Ackinv1 and Ackinv6) and used in an inverse PCR experiment after digestion of chromosomal DNA by SfuI. A 2.5 kb fragment was amplified and sequenced, by which the sequence of the gene and its flanking regions could be completed. The gene was named ackA. A physical map of the ackA locus, as well as the cloning strategy, is shown in Figure 3.1.

To verify the obtained sequence, two additional, independent PCR-clones of the gene and of the upstream region were sequenced as well. Ambiguous nucleotides occurred in 12 and in 6 positions of the gene and the upstream region, respectively. The definite sequence was based on nucleotides that occurred in two out of the three sequences (Figure 3.3). ackA consists of 1194 nucleotides and encodes a protein of 398 amino acids with a
ACETATE KINASE

TGTGCCCATGCGTCAACGCTGAGGCGGTTCTAAAAACTAGTGATGAGGGTGAGCAATGC
CAGTAGCGTTCCAACGACGAGTAGGAGCGTTGCAAAGCCGTTGCTCAAAGTCCAAGTAAT
TCTTTTCATCTGATTTGCGCTCTACCTTTCTGCTATATATTTAATGCTAATTAGTGTTTA
TGAAAGAAATAATATGCTGACCAAATCGCGTATTTTTACAACTGTTATGAAATATCATGAGT

-35

ATAAATACCTTGAAAGGCGCATGGAATTTTATGCTAAACTGACTAGGACACTCTTGAATG

-10

cre

GATGGAGGAATAATGTCTGCCAAGAATTTTGGCAATATTACGCCGGGACATTGCGACTTTT

******

M A K I L A I N A G S S T L

AAATGGAAACTTTTCCATGTTTCCAGAGAGAACGTTATCCATCGAAGAATGTTGACCAG
K W L K F S V P E T V I A S G M V D R

TTGGGTTACCCGACTCTGCTTTTTACAAATTTAAAAACGATGGGGATTAAGAGACCGAA
L G L P N S V F T I K K A D G S K E T E

ACCAAGGATAGGAGTTCGCGCAACAGAAGGATGCAGCCGCAATGTTTTTAAACCGGGTGAAGAAT
T K D T A A K E E A A A M V L T R L K N

GATAACACTGTCGACAACACTCAGTATATCAAAAGGTTGGACACCGGGTCGTCGCTGT
DNIVDQTLTDIKGVHRVAG

GGCGGAGAACACTTCCAGACTGACTGCTGTGTGTATTAGCGGCGCAAAACATTTGAAAGAATAATCGAGAT
GEDFKDSVIPQTLLKKID

TTATCTGATTACCCGCGTTCTATATAACCCAGCGAAGCTTTCACAGTATATGTTGCTCGGT
LSEYAPLHNPQAYYYIEVR

GATTTGTTCACAAAAAGCGCCTCAGTACGCGGCTTTTGTATACGCTGTTGTATGCGGATATG
DLLPKAVQVAVFDTSLYADM

CCAAAAGTTAATTTATTTATACAGCATTACCCATATGCAHTAAGAAAAATTCGTTGCAGAC
PKVNLYSIIPYDYEYKFGR

AAGTATGTTATGCACGGGACACTGTATCGTTACGTGCCAAGCCGACCGGTCTCAATTACTG
KYGAHGTSHVRVANRTAQL

GGCAAGGCGCTCGATGGAATTAAAGTATTAGTCACCTGAGGCTTGGCAGTGTTCAATC
GKPDLKVLTVLHLGSAGSI

ACGCCCTTTGATCATGTTGACAGCTATTTGATCTATCGCAATATGGAAGGTTTACGCGGTTACCGG
1020
TAFDHGQAIDTSMGFTPLAG

ATTACGATGAGTACGGCGTTCTTGTTGATACTGATGCGTGCTTTTCTTATTATGCGG
1080
ITMSSTRPIDDASIIIPFLMR

CACCTCGTATTTTCAAGACGTGTTGAAAGACTTTCAGCTATATTTTAGACCACAAAGTCCGGCTT
1140
HLGISDDEVFDIDLNHKSGL

TTGGGTAATTTCCAGCTTTCACCAAGACATGCCTGAGCTTGGATAAGGCAGACACCGGCTTG
1200
LGISGLSPMDRDLKTQDTR

GAACAGTCAAGCTTCGCAAGTATCCCTGTAAGGCCGGTGTGGGATGACTTTTGGTGAG
1260
EQSRLAIEIFVNRVVKYVGS

TACATTGCCGAATAAGCGATTATGGATGACACTGCTTTTACCGCCGCGATTGGTGAAGAAT
1320
YIAEMNGIDALVFTAGMGEN

TCAATGATGATTCGCAAGTCATCGCCCATCTGAGTTCTTACATCGGTATACAGTCGAC
1380
SWMIERIAKSLSYFGETVD
calculated molecular mass of 43897 Da. A BLAST search (http://www.ebi.ac.uk/blast2/) with the deduced amino acid sequence revealed that acetate kinase from *L. pentosus* showed similarity with many acetate kinases from other organisms. The similarity was highest for the proteins encoded by *ackA* (64% identical and 75% conserved residues) and by *ackB* (71% identical and 90% conserved residues) from *L. sakei* (M. Zagorec and S. Chaillou, personal communication) and with acetate kinase encoded by *ackA* from *B. subtilis* (52% identical and 73% conserved residues). An alignment of acetate kinase from *L. pentosus* with the acetate kinases mentioned in the Materials and Methods that were used to design
the degenerate primers Ak140 and Ak400R is shown in Figure 3.4. Acetate kinase encoded by ackA from *L. sakei* was also included in the alignment. Putative -35 and -10 promoter sequences were found 113 and 78 nucleotides upstream and a putative Shine-Dalgarno element 15 basepairs upstream of the translational start of ackA, respectively. Downstream of the -10 sequence, 69 nucleotides upstream of the start codon of ackA, a catabolite responsive element (cre) was found containing two mismatches compared to the consensus sequence: the A at position 10 is a T and the G at position 12 is an A or T in the consensus sequence [51].

An ORF with its reading frame opposite to that of ackA was found downstream from ackA, encoding a putative protein of at least 180 amino acids. This protein aligned with residues 26 to 164 of ElaA from *E. coli*, showing 36% identical and 54% similar residues (EMBL Gene bank accession number P52077). ElaA is a small hypothetical protein with unknown function of 153 amino acids that belongs to the unidentified protein family UPF0039. It is located in the *ela* locus, that comprises the genes menF, encoding an isochorismate synthase involved in biosynthesis of menaquinone, elaB and elaC (both encoding hypothetical proteins with unknown function) and elaD, encoding a putative sulfatase or phosphatase. Since only a part of the ORF of *L. pentosus* aligned with ElaA and no translational start methionine was found, the exact size and function of the protein is unclear. Hypothetical proteins from several other organisms belong to the UPF0039 protein family, all with approximately the same size as ElaA from *E. coli*, like YS51 from *Mycobacterium tuberculosis* (O05808), YYBD from *B. subtilis* (P37500), YAT3 from *Staphylococcus aureus* (P52080), Y351 from *Synechocystis* sp. and YA02 from *Schizosaccharomyces pombe*. To determine the size of the ackA transcript, mRNA was isolated from *L. pentosus* MD363 and MD353, another *L. pentosus* wild type strain, after cultivation on glucose. Hybridization with the 780 bp internal PCR fragment of ackA as a probe revealed a single mRNA band of approximately 1.2 kb in size, as shown in Figure 3.5. This suggested that ackA does not form an operon with other genes but is transcribed as a monocistronic mRNA in *L. pentosus*.

**Expression of the ackA gene in E. coli** - In order to ascertain that ackA encoded a functional acetate kinase, the gene was expressed in *E. coli* KS24, an ackA deletion mutant. For this purpose, the gene was amplified with PCR, as described in Materials and Methods, and cloned behind the trc promoter in pBCP367, which is inducible by IPTG. The resulting plasmid, pLPA37, was used to transform *E. coli* KS24. As a negative control, cells transformed with pBCP367 were used.
CHAPTER 3

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Ak140
CHAPTER 3

Figure 3.4. Comparison of the primary sequences of acetate kinase from L. pentosus (Lpe); Lactobacillus sakei (Lsa; EMBL gene bank accession number: Q9X4M1); Clostridium acetobutylicum (Cac); Thermoanaerobacterium thermosaccharolyticum (Tth); Methanosarcina thermophila (Mth); Bacillus subtilis (Bsu); Chlorante-Aster yellows phytoplasma (Cas); Mycoplasma genitalium (Mge); Mycoplasma pneumoniae (Mpn); Mycoplasma capricolum (Mca); Synechocystis sp. (Syn); Corynebacterium glutamicum (Cgl); Mycoplasma tuberculosis (Mt); Escherichia coli (Eco); Haemophilus influenzae (Hin). Identical amino acids are shown in white letters in a solid background, similar residues are shown in white letters on a grey background. The numbers on the left of the alignment correspond to the amino acid positions for each protein. The positions of the degenerate primers Akl 40 and Ak400R are indicated with arrows. The alignment was done with ClustalW (http://www.ebi.ac.uk/clustalw/); shading with Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

The Coomassie stained SDS-PAGE gel from Figure 3.6 shows that a 45 kDa protein was synthesized in cells containing pLPA37. Upon induction with IPTG, the amount of the 45 kDa protein largely increased. In the negative control, this protein band was absent. The acetate kinase activity...
in the supernatant after ultracentrifugation of a cell-free extract of *E. coli* KS24 containing pLPA37 was about 800 times elevated upon induction with IPTG compared to the activity in the supernatant of the negative control (Table 3.2). These results showed that *ackA* from *L. pentosus* indeed encoded a functional acetate kinase.

**Acetate kinase activities** - To study the conditions under which acetate kinase activity was present in *L. pentosus* MD363, wild type cells were cultivated on several energy sources and acetate kinase activity was measured in cell-free extracts. The results are shown in the first column of Table 3.3. Acetate kinase activity was detected in extracts that were cultivated on all six sugars tested. The acetate kinase activities were roughly the same in the extracts of cells grown on fructose, gluconate, xylose, arabinose or ribose, varying between 1.2 and 1.9 μmol·min⁻¹·mg⁻¹ protein. A 3 to 4 fold lower acetate kinase activity was found in extracts of cells that were grown on glucose.

![Figure 3.5. Northern blot analysis of mRNA isolated from *L. pentosus* MD363 and MD353.](Image)

In order to study whether the lower acetate kinase activity observed after growth on glucose was due to CR mediated by CcpA, the same experiment was performed in a *ccpA* knock-out mutant, LPE4. Indeed, acetate kinase activity was elevated during growth on glucose in the mutant and was comparable to the activities detected in the wild type bacteria after growth on the other energy sources. Acetate kinase activity was also determined in extracts of a 2-deoxyglucose resistant (2DGᵣ) mutant of *L. pentosus*, LPE5, cultivated on the same energy sources. This strain is mutated in the mannose PTS and is therefore unable to transport glucose via the PTS.
system [10]. Also in LPE5, the acetate kinase activity during growth on glucose was elevated to the levels observed during growth on the other sugars (Table 3.3).

In order to study the acetate kinase activity during aerobic growth, the wild type strain of *L. pentosus* was also cultivated under aerobic conditions in the presence of glucose. An acetate kinase activity of 0.3 μmol·min⁻¹·mg⁻¹ protein was detected in the cell-free extracts of these cells, which was similar to the activity under anaerobic conditions.

The acetA knock-out mutant - To test whether acetA was the only gene that encoded a functional acetate kinase in *L. pentosus* and to study the effect of deleting this activity on growth, an acetA knock-out mutant was constructed by transforming *L. pentosus* MD363 with pLPA15. pLPA15 was obtained by ligation of an internal part of acetA in pRV300, a suicide vector for lactobacilli [70], as shown in Figure 3.2. An Em<sup>R</sup> transformant was isolated and the integration of the vector in the acetA locus was verified by Southern hybridization after digestion of chromosomal DNA with *SalI* or *EcoRV*. The 780 bp PCR fragment of acetA was used as a probe. A schematic representation of the integration of pLPA15 in the chromosome and of the resulting fragments after digestion of wild type and mutant chromosomal DNA is drawn in Figure 3.7. The results of the Southern hybridization are shown in Figure 3.8. The sizes of the hybridizing DNA fragments showed the expected values. From these results it can be concluded that pLPA15 was correctly integrated in the acetA locus in *L. pentosus* LPE116. The
Acetate kinase activity
(µmol·min⁻¹·mg⁻¹ protein)

<table>
<thead>
<tr>
<th></th>
<th>KS24*pBCP367 (-ackA)</th>
<th>KS24*pLPA37 (+ ackA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 ± &lt; 0.1</td>
<td>322 ± 18</td>
</tr>
</tbody>
</table>

Table 3.2. Acetate kinase activities in soluble protein fractions of *E. coli* KS24 (ΔackA) containing pBCP367 (empty vector) or pLPA37 (ackA expression vector). Values are the average of two separate experiments.

<table>
<thead>
<tr>
<th>sugar</th>
<th>MD363 (wild type)</th>
<th>LPE4 (ΔccpA)</th>
<th>LPE5 (2DG⁰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>0.5 ± &lt; 0.1</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>D-fructose</td>
<td>1.2 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>D-gluconate</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>D-xylose</td>
<td>1.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>n.g.</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>D-ribose</td>
<td>1.9 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>3.4 ± 0.6</td>
</tr>
</tbody>
</table>

Table 3.3. Acetate kinase activities measured in cell-free extracts of wild type *L. pentosus* (MD363), a ccpA knock-out mutant (LPE4) and a 2DG⁰ mutant (LPE5). Cells were cultivated in M-medium supplemented with 1% of the indicated sugar. Activities are expressed in µmol·min⁻¹·mg⁻¹ protein. All values are the average of two separate experiments. Before acetate was added, a decrease of NADH was already observed in the enzyme assay. This decrease was dependent on the presence of ATP and the presence of pyruvate kinase and lactate dehydrogenase (not shown). This decrease might be due to ATPase activity, or to the presence of glycerol kinase in the cell-free extracts that converts the glycerol in which lactate dehydrogenase and pyruvate kinase that are both added to the reaction buffer were stored. The rate of the background reaction was linearly dependent on the total protein concentration. Acetate kinase activities were calculated as the difference in reaction rate before and after acetate was added to the reaction mixture. n.g. no growth.

Intensities of all hybridization fragments were equal and it is therefore likely that only a single copy of pLPA15 was integrated in the ackA locus: if multiple copies of pLPA15 were integrated, the intensities of the hybridization bands of 4.2 kb of both the Sall and the EcoRV digestion would be higher than those of the other bands.

The ackA knock-out mutant was cultivated on glucose or fructose and acetate kinase activities were measured in cell-free extracts. No acetate kinase activity was detected in the ackA knock-out mutant under both...
Figure 3.7. Schematic representation of the integration of pLPA15 in ackA on the chromosome of L. pentosus MD363. A. ackA locus of wild type L. pentosus and plasmid pLPA15, containing an internal fragment of ackA. B. ackA locus in the ackA knock-out mutant. Integration has resulted in two truncated copies of ackA. The theoretical sizes of fragments after digestion of the chromosome with restriction enzymes are shown. S: Sall; E: EcoRV; H: HindIII. Arrows indicate the putative transcriptional start site, stem loop structures indicate the putative transcriptional terminator.
conditions, as shown in Table 3.4. This indicated that *ackA* was the only acetate kinase encoding gene that was expressed in *L. pentosus* after growth on glucose or fructose. To test the influence of inactivation of acetate kinase on the growth on various sugars, LPE116 was plated on M-agar plates supplemented with different energy sources. Table 3.5 shows that, whereas the wild type strain grew on all sugars tested, there was no growth of the *ackA* knock-out mutant LPE116 on the sugars that are fermented via the phosphoketolase pathway: pentoses and gluconate. Apparently, a functional acetate kinase was necessary for the fermentation of sugars by this pathway.

### 3.5 DISCUSSION

A gene encoding acetate kinase from *L. pentosus* MD363, *ackA*, was cloned and sequenced. The gene coded for a protein of 398 amino acids, that showed high similarity with many acetate kinase proteins from other organisms. Expression of the gene from *L. pentosus* in an *E. coli* acetate kinase deletion strain confirmed that *ackA* encoded a functional acetate kinase. A putative promoter sequence was identified upstream of *ackA* and a stem loop terminator sequence was found immediately downstream of
Table 3.4. Acetate kinase activities in cell-free extracts of wild type (MD363) and ackA knock-out mutant (LPE116) of L. pentosus. Cells were cultivated in M-medium supplemented with 1% (wt/vol) of the indicated sugar. The activities are the average of two separate experiments and expressed in μmol·min⁻¹·mg⁻¹ protein. In the absence of acetate, a background NADH consumption was observed in cell-free extracts of the mutant of 0.5 and 0.6 μmol·min⁻¹·mg⁻¹ protein after growth on glucose or fructose, respectively. Addition of acetate to the reaction mixture did not lead to an increase of NADH consumption, indicating that no residual acetate kinase activity was present in these extracts.

<table>
<thead>
<tr>
<th></th>
<th>MD363 (wild type)</th>
<th>LPE116 (ΔackA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>0.5 ± 0.1</td>
<td>-0.1 ± 0.1</td>
</tr>
<tr>
<td>D-fructose</td>
<td>1.2 ± 0.1</td>
<td>-0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.5. Growth of L. pentosus MD363 (wild type) and LPE116 (ackA knock-out mutant) on M-agar plates supplemented with 0.5 % of the indicated sugar. Ribose was added to a final concentration of 0.25 %. Mutant plates contained erythromycin. +, growth; -, no growth.

<table>
<thead>
<tr>
<th></th>
<th>MD363 (wild type)</th>
<th>LPE 116 (ΔackA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-gluconate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-xylose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-ribose</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

ackA. These results suggested that ackA was transcribed from its own promoter and was terminated directly after the gene. Indeed, an RNA transcript of 1.2 kb corresponding to the size of ackA was found in a Northern blot experiment, indicating that ackA was transcribed into monocistronic mRNA (Figure 3.5). The genetic organization of the ackA locus is similar to that of the ackA operon of B. subtilis, that comprises only the acetate kinase gene [42]. In many other organisms, however, ackA forms an operon together with ptaA, which encodes a phosphotransacetylase. For instance, in C. acetobutylicum, M. thermophila and C. glutamicum, ackA is cotranscribed with the upstream located ptaA [5, 123, 97], and in E. coli,
ptaA is located downstream of ackA [57]. In contrast to these organisms, no ORF encoding a putative phosphotransacetylase was identified in the downstream region or in the approximately 300 nucleotides that were sequenced from the upstream region of ackA in L. pentosus. Phosphotransacetylase is involved in the production of ethanol from acetylphosphate during heterofermentation of gluconate via the PKP in L. pentosus by catalysing the conversion of acetyl-phosphate to acetyl-CoA (see Figure 1.1, General Introduction). Although the presence of phosphotransacetylase activity during gluconate fermentation has not been investigated in L. pentosus, a ptaA gene is expected to be present. This gene is probably located at another position on the chromosome, like in B. subtilis [122].

Acetate kinase activity was absent in an ackA knock-out mutant, suggesting that under the conditions studied only one gene encoding acetate kinase was expressed in wild type L. pentosus. On the basis of the results from L. sakei, another facultative heterofermentative Lactobacillus in which two ack genes were reported (M. Zagorec and S. Chaillou, personal communication), the presence of a second ack gene in L. pentosus might be expected. The deduced amino acid sequences of both ack genes from L. sakei show similarity to other acetate kinases, but neither of the genes has been functionally characterized. One of the genes in L. sakei, ackA, is located upstream of the operon involved in ribose utilization [127], while the other, ackB, is located at an unknown position on the chromosome. The absence of acetate kinase activity in the ack knock-out mutant of L. pentosus indicated that if a second ack gene were present in L. pentosus, this gene was not expressed under the conditions studied or that this gene did not encode a functional acetate kinase.

Inactivation of acetate kinase activity in the ackA knock-out mutant of L. pentosus resulted in the absence of growth on ribose, xylose, arabinose and gluconate (Table 3.5), which are all metabolized via the PKP. This indicated that acetate kinase activity was essential for the fermentation of energy sources via this pathway. Because of the absence of acetate kinase activity, fermentation of pentoses in the ackA knock-out mutant might lead to the accumulation of acetyl-phosphate. Conversion of acetyl-phosphate into ethanol, which would decrease the internal acetyl-phosphate concentration, results in the net production of NAD$^+$ and thus in an imbalance in the production and consumption of reduction equivalents. If an excess of acetyl-phosphate is produced, phosphate is trapped, resulting in the death of the cells. Gluconate metabolism requires both the 'acetate-' and the 'ethanol-branch' of the PKP for the conversion of acetyl-phosphate (see Figure 1.1, General Introduction), in order to maintain a balance in reduction equivalents. In the ackA knock-out mutant, an increasing
amount of NAD$^+$ is produced resulting in a lethal phenotype after growth on gluconate. The metabolism of glucose or fructose, which occurs via the Embden-Meyerhoff-Parnas (EMP) pathway, results in the production of lactate as the main end-product under anaerobic conditions. Under these circumstances, acetate kinase activity is not expected to play an important role. This explains why inactivation of acetate kinase was had no significant effect on growth of *L. pentosus* on glucose or fructose.

Although acetate kinase activity is not necessary during anaerobic growth on glucose or fructose, a specific activity was detected in the wild type strain of *L. pentosus* of 0.5 and 1.2 pmol-min$^{-1}$-mg$^{-1}$ protein under these conditions, respectively (Table 3.3). Cultivation on ribose, xylose, arabinose and gluconate resulted in specific activities that were comparable with the activity found in cells grown on fructose. For the growth on these energy sources, acetate kinase activity was needed as shown by the absence of growth of the *ackA* knock-out mutant, but no higher acetate kinase activities were found than the activity after growth on fructose. The reason why acetate kinase activity was constitutively synthesized in *L. pentosus*, thus also after growth on glucose and fructose, is unclear.

In the facultative heterofermentative *L. plantarum*, a species that is related to *L. pentosus*, it was shown that during aerobic growth on glucose, a pyruvate oxidase is induced that converts pyruvate which is produced in the EMP pathway into acetyl-phosphate, which is converted into acetate by acetate kinase [40, 87, 118, 141]. During aerobic growth of *L. plantarum* strains, increased acetate kinase activities and an increased acetate production were observed compared to anaerobic growth [88, 141]. Some preliminary experiments to study the role of acetate kinase activity during aerobic growth on glucose in *L. pentosus* showed that, in contrast to *L. plantarum*, the acetate kinase activity in the cell-free extracts of *L. pentosus* during aerobic growth was comparable to the activity during anaerobic growth. Also, no difference in growth rate on glucose was observed during aerobic or anaerobic growth of the *L. pentosus* wild type strain. Also, in initial experiments with the *ackA* knock-out mutant of *L. pentosus* LPE116, we showed that the mutant was not impaired in growth on glucose under aerobic conditions. These experiments suggest that acetate kinase probably does not play a major role during aerobic growth on glucose of *L. pentosus*.

A cre sequence was identified in the promoter region of *ackA* (Figure 3.3), which led to the suggestion that expression of *ackA* might be under the control of CR mediated by CcpA. Indeed, the acetate kinase activity detected in the *ccpA* knock-out mutant LPE4 after cultivation on glucose, was four times elevated compared to the activity in the wild type strain under the same conditions. Growth of LPE4 on the other sugars led to acetate kinase activities that were comparable to the activities detected in the wild type
strain, suggesting that CR mediated by CcpA of ackA did not play a role after growth on these energy sources.

A similar relief of CR during growth on glucose was seen in the 2DG<sup>R</sup> mutant of <i>L. pentosus</i> LPE5, which is defected in the mannose PTS activity (Table 3.3). Like in the ccpA knock-out mutant, there was no difference in the acetate kinase activities detected in LPE5 and the wild type strain during growth on ribose, gluconate or arabinose. Glucose transport by the PTS is absent in the 2DG<sup>R</sup> mutant and probably takes place by a specific glucose permease [10]. The lack of glucose transport by the PTS in LPE5 might lead to increased concentrations of HPr phosphorylated at His15. It was found that both HPr(HisP) and HPr doubly phosphorylated at His15 and Ser46 (the phosphorylation site involved in CR in Gram-positive bacteria) bind less efficiently to CcpA than HPr(SerP) [20]. As a result, less CR is exerted in the mannose PTS mutant strain under these conditions.

CR by fructose on acetate kinase synthesis was found as expected, since fructose is transported by a fructose-specific PTS in <i>L. pentosus</i> [6]. Fructose transport would thus lead to HPr(SerP) as the predominant form of HPr in a similar way as during glucose PTS transport. The acetate kinase activity after growth on fructose in the ccpA knock-out mutant was two fold elevated compared to the activity in the wild type strain, which would mean that a two fold CR on acetate kinase activity was exerted by fructose.

The result that the acetate kinase activity was repressed by CcpA in <i>L. pentosus</i> in the presence of glucose, differed from the results in <i>B. subtilis</i>, where the expression of ackA is activated about four times in the presence of glucose [42]. This activation mechanism is mediated by CcpA, that in complex with HPr(SerP) or Crh(SerP) binds to a cre sequence in the promoter region of ackA [143]. ptaA, which encodes phosphotransacetylase and is located in another operon as ackA in <i>B. subtilis</i>, is regulated in the same way [122]. Both ackA and ptaA are primarily active in acetate excretion in <i>B. subtilis</i> and respond to the availability of glucose in the medium. Anaerobic growth of lactic acid bacteria in the presence of high concentrations of glucose leads to the production of lactate, and not of acetate, as the main end-product [59]. The observation that acetate kinase activity in the presence of glucose in <i>L. pentosus</i> was not activated corroborates these findings.

### 3.6 Acknowledgements

We would like to thank M. Zagorec and S. Chaillou for providing the ackB sequence information prior to publication.