The phosphoketolase pathway in Lactobacillus pentosus.

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Citation for published version (APA):
A xylulose 5-phosphate phosphoketolase-like protein was found in *Synechocystis* sp. PCC6803, however no phosphoketolase activity could be detected.
A BLAST search with the amino acid sequence of xylulose 5-phosphate phosphoketolase (XpkA) from Lactobacillus pentosus revealed a hypothetical protein with a calculated molecular mass of 92447 Da, encoded by slr0453, of Synechocystis sp. PCC6803 with significant similarity (48% identical and 64% conserved residues), suggesting that it may catalyse a phosphoketolase reaction. Western blot analysis indicated that a protein of 90 kDa is synthesized that cross-reacts with the antibody against XpkA during mixotrophic growth of Synechocystis on glucose and during heterotrophic growth on glucose in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of linear photosynthetic electron transfer. The 90 kDa protein was absent during photoautotrophic growth. However, XpkA activity could not be detected in extracts from heterotrophically grown cells.

The gene slr0453 was expressed in Escherichia coli, yet phosphoketolase activity could not be detected. These results do not support the hypothesis that slr0453 encodes a phosphoketolase.
5.2 INTRODUCTION

*Synechocystis* is a unicellular oxygenic phototrophic bacterium that belongs to the group of cyanobacteria, formerly known as blue-green algae. Photoautotrophy, i.e. carbon dioxide fixation driven by light, is the common way of growth. The reductive power for anabolism is provided from the light-driven water-splitting by photosystem 2, which leads to a photosynthetic electron transfer and a proton gradient across the thylakoid membrane and which results finally in the synthesis of ATP, NADPH and oxygen. During photoautotrophic growth, a carbon dioxide molecule is accepted by ribulose 1,5-bisphosphate to form a six-carbon compound, which is rapidly converted into two molecules of 3-phosphoglycerate (PGA). This reaction is catalysed by ribulose 1,5-bisphosphate carboxylase, also known as RuBisCo. Via gluconeogenesis, PGA is converted into fructose 6-phosphate. The regeneration of ribulose 1,5-bisphosphate from PGA involves transketolase and transaldolase activities, amongst others, in a reaction in which one PGA and two fructose 6-phosphate molecules finally lead to the formation of three molecules of ribulose 1,5-bisphosphate. This latter pathway is sometimes also called the reductive pentose phosphate pathway. Via the Calvin cycle, six molecules of carbon dioxide are fixed into one hexose, which is stored as glycogen. In the dark, glucose that is derived from glycogen is used as an energy source, and is degraded in a similar manner as during photomixotrophic or photoheterotrophic growth [125].

Exogenous glucose, which is transported by a specific glucose permease, GlcP [113, 170], can be used as a carbon source under mixotrophic growth conditions in *Synechocystis* when cells grow at a low light intensity. Several pathways for the metabolism of glucose have been claimed in various cyanobacteria, of which the oxidative pentose-phosphate pathway, involving glucose 6-phosphate dehydrogenase, transketolase and transaldolase activities, is the most common one. In *Synechocystis*, the enzymes of the glycolytic pathway are also present [62] but this pathway is probably only active when glucose is fermented in the absence of oxygen [84]. In the cyanobacterium *Oscillatoria limosa*, heterolactic fermentation of glucose was claimed, since glycogen degradation led to the production of equimolar amounts of lactate, ethanol and carbon dioxide [50].

During so-called photoheterotrophic growth in the presence of the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), that inhibits photosystem 2, glucose is both used as a carbon source and as a reductant. Under these conditions, light is solely driving photosystem 1, resulting in the generation of ATP by cyclic photophosphorylation.

The photoautotrophic and gluconeogenetic pathways on the one hand and the glucose degradation pathways on the other, are strictly regulated
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in cyanobacteria, mainly at the level of enzyme activity [50, 84, 144]. For instance, light-dependent regulation of the activity of enzymes of the Calvin cycle takes place by thioredoxin: thioredoxin is reduced under the influence of light and in the reduced form it activates enzymes by reduction of disulfide bridges [14]. Glucose 6-phosphate dehydrogenase activity, which is needed for the degradation of glucose, is regulated by metabolites: high concentrations of ATP, NADPH and ribulose 1,5-bisphosphate and a high pH, all the result of photosynthetic activity, lead to a lower activity of glucose 6-phosphate dehydrogenase [108]. In some cases, pathways are also regulated at the level of gene expression. The anabolic carbon flow via the Calvin cycle and the gluconeogenetic pathway from the catabolic carbon flow via the glycolytic pathway are regulated by two iso-enzymes of glyceraldehyde 3-phosphate dehydrogenase, encoded by gap1 and gap2. The product of gap2 is constitutively synthesized and its activity is essential during photosynthesis, whereas the protein encoded by gap1 is only synthesized during glucose breakdown [62].

As described above, glycogen degradation in the cyanobacterium Oscillatoria limosa is probably exerted via heterolactic fermentation [50]. However, the presence of xylulose 5-phosphate phosphoketolase (XpkA) activity in this bacterium was not investigated and thus no conclusions about the presence of the phosphoketolase pathway in O. limosa can be drawn. A gene (slr0453) with high similarity to xpkA, which encodes XpkA in Lactobacillus pentosus, was found in the genome sequence of Synechocystis. This finding led to the hypothesis that the phosphoketolase pathway may be used in some cyanobacteria for glucose degradation and we assumed that XpkA activity could be demonstrated in this organism. For this purpose, Synechocystis sp. PCC6803 was cultivated under different physiological conditions and phosphoketolase activities were determined in cell-free extracts. To test if slr0453 encodes a phosphoketolase, the gene was expressed in Escherichia coli.

5.3 MATERIALS AND METHODS

Strains and growth conditions - Synechocystis sp. PCC 6803 was cultivated under gentle shaking in BG11 medium [101] at 32 °C. Three different growth conditions were used: photomixotrophically under moderate light (30 μmol photons·m⁻²·s⁻¹) in the presence of 10 mM glucose, photoheterotrophically in the presence of 10 mM glucose and 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and photoautotrophically under medium high light (100 μmol photons·m⁻²·s⁻¹) in the absence of glucose. Lactobacillus pentosus ATCC8041 was cultivated without agitation at 37 °C in M-medium [74] containing 1% (wt/vol) of ribose. Escherichia coli
M15[pREP4] was cultivated aerobically on Luria-Bertani (LB) agar plates or in LB broth at 37 °C. Ampicillin and kanamycin were added to a final concentration of 100 μg/ml and 25 μg/ml, respectively, when necessary.

Expression of slr0453 in *E. coli* - slr0453 from *Synechocystis* sp. PCC6803 was amplified by PCR using the Expand™ high fidelity PCR system (Boehringer Mannheim) with the primers Fksynbam (CGCGGATCCGTGGGTTCTACCCTGGTAG; the additional BamHI site is underlined) and Fksynkpn (ATAGGTACCGTCTAGAGCCAGCG, the additional Kpnl site is underlined). The primer Fksynbam was designed against the nucleotide sequence encoding amino acids 1 to 6 from the hypothetical protein and Fksynkpn against the nucleotide sequence about 70 nucleotides downstream of the stop codon of the gene. After restriction of the amplified fragment, it was ligated between the BamHI and Kpnl sites of pQE30 (QIAGEN) in frame with a 6xHis affinity Tag at the N-terminus of the protein. The resulting plasmid, pLPA23, was used to transform to *E. coli* M15[pREP4]. The synthesis of recombinant protein was induced by addition of various concentrations of isopropyl β-D-thiogalactopyranoside (IPTG) during aerobic cultivation in LB at various temperatures, as specified in the Results and Discussion.

![Figure 5.1](image-url) - Western blot of cell-free extracts from *Synechocystis* sp. PCC6803 using antibodies raised against XpkA from *L. pentosus* MD363. 1: photoheterotrophic growth in the presence of glucose; 2: photoheterotrophic growth in the presence of glucose and DCMU; 3: photo-autotrophic growth; M: marker.

Preparation of cell-free extracts - 70 ml of cell culture of *Synechocystis* in the log phase was harvested by centrifugation and cells were resuspended in 5 ml 50 mM MOPS NaOH buffer (pH 7), 0.5 mM EDTA, 0.5 mM DTT. About one half volume of glass beads (φ=0.1 mm) was added and cells were broken by shaking at 5000 rpm in a Mini BeadBeater (Biospec Products) during 4 times 30 s. In between the samples were cooled on ice. Cell debris was removed by centrifugation for 5 min at maximal speed in an Eppendorf centrifuge. *E. coli* cell-free extracts were prepared by one passage through a French pressure cell (11,000 lb/in²), as described in Chapter 2. Materials
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and Methods. Cell debris was removed by centrifugation (10,000 x g, 4 °C, 20 min). The protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH), using BSA as a standard.

*Phosphoketolase activity assays* – Xylulose 5-phosphate phosphoketolase (XpkA) activity was determined as described in Chapter 2, Materials and Methods. Fructose 6-phosphate phosphoketolase activity was measured by determining the amount of acetyl-phosphate produced by phosphoketolase as described in [35], with the modification that 12.5 µl instead of 300 µl 28% (w/v) hydroxylamine was added in the second step. The volume was adjusted by addition of 287.5 µl demi water. Acetyl-phosphate in a concentration range from 0.05 to 5 mM was used as a standard. 100 mM fructose 6-phosphate was used as the substrate (20 mM end concentration).

*Western blot analysis* – 10 µg total protein of the *Synechocystis* or *E. coli* cell-free extracts were loaded onto a 10 % SDS-polyacrylamide gel and separated by gel electrophoresis. After transfer of the proteins to nitrocellulose [140], the blots were incubated with 5,000 times diluted polyclonal anti-XpkA antiserum (Chapter 2, Materials and Methods, preparation of antibodies) and a horseradish peroxidase-conjugated secondary antibody. For detection, Pierce Super Signal was used according to the method specified by the manufacturer.

5.4 **RESULTS AND DISCUSSION**

*Presence of a xpkA-like gene in Synechocystis* – A BLAST search (http://www.ebi.ac.uk/blast2/) with the amino acid sequence of xylulose 5-phosphate phosphoketolase (XpkA) from *L. pentosus* revealed several genes encoding proteins with significant similarity (see also Table 2.5 from Chapter 2). Amongst them was slr0453 from *Synechocystis* sp. PCC 6803 (EMBL Gene bank accession number P74690), encoding a hypothetical protein of 821 amino acids and a calculated molecular mass of 92447 Da. The deduced amino acid sequences of XpkA and of the protein encoded by slr0453 shared 48% identical and 64% conserved residues spread over the total proteins. slr0453 was located 125 nucleotides downstream of slr0452, which encoded a putative dihydroxy-acid dehydratase involved in the biosynthesis of valine, leucine, isoleucine, pantothenate and Coenzyme A (*ilvD*, EMBL Gene bank accession number P74689). Upstream of *ilvD* a putative ribosome binding site and -35 and -10 sequences were found. In contrast, such sequences were not clearly identified in the intergenic region between *ilvD* and slr0453. Both genes had the same reading frame orientation and might form an operon.
Growth conditions | Specific activity nmol·min⁻¹·mg⁻¹ protein
--- | ---
Synechocystis sp. photomixotrophic, glucose | 15 ± 7
Synechocystis sp. photoheterotrophic, glucose, DCMU | 20 ± 5
Synechocystis sp. photoautotrophic | 19 ± 10
*L. pentosus* ribose | 634 ± 4

**Table 5.1.** XpkA activities in cell-free extracts of *Synechocystis* sp. PCC6803 grown under three different conditions. The cell-free extract of *L. pentosus* ATCC8041, cultivated on ribose, was used as a positive control for the assay. The activities are the mean of four independent measurements.

XpkA from *L. pentosus* showed also similarity to another putative protein encoded by s!10529 (EMBL Gene bank accession number Q55517) in the genome of *Synechocystis*. This gene was smaller than *xpkA* and encoded a protein of 731 amino acids with a calculated mass of 78316 Da. The similarity between XpkA and this protein was much less: 28% identical and 43% conserved residues.

**Expression of slr0453 in Synechocystis** - In the cyanobacterium *O. limosa*, glycogen is degraded via heterolactic fermentation [50]. The fact that a gene encoding a protein with high similarity to XpkA was found in the genome sequence of *Synechocystis* prompted us to test whether such activity could be detected in this cyanobacterium, although there are no claims that glucose is degraded via heterolactic fermentation in this organism. For this purpose, *Synechocystis* sp. PCC6803 was cultivated under photoautotrophic, photomixotrophic and photoheterotrophic conditions (see Materials and Methods). Cell-free extracts of the cultures were prepared and the proteins of these extracts were separated by SDS-PAGE. After transfer of the proteins to a nitrocellulose membrane, a Western blot analysis was performed using the antibodies raised against XpkA from *L. pentosus* MD363 (Figure 5.1). A protein of about 90 kDa reacted with the antibody in the cell-free extracts from *Synechocystis* cells that were cultivated in the presence of glucose. The size of this protein correlated with the theoretical size of the protein encoded by slr0453. The amount of protein that reacted with the antibody was considerably reduced during photoheterotrophic growth in the presence of DCMU compared to the amount of protein during photomixotrophic growth, whereas the protein was absent during photoautotrophic growth.
XpkA activity was measured in these cell-free extracts, the results of which are shown in Table 5.1. As a positive control, XpkA activity was also measured in a cell-free extract of *L. pentosus* ATCC8041, cultivated on ribose. The specific activities detected were between 15 and 20 nmol-min⁻¹·mg⁻¹ protein in the three *Synechocystis* extracts which was comparable to the levels of activity detected in *L. pentosus* under non-inducing conditions (see Chapter 2). In *L. pentosus* ATCC8041, grown on ribose, much higher activities were found. Using MnCl₂ in stead of MgCl₂ as a cofactor in the activity assay, or changing the pH of the assay to pH 7.0 or 7.8 instead of pH 6.0 resulted in the same amounts of XpkA activity (data not shown).

Since the XpkA activity that was detected in the extracts did not correlate with the quantity of the protein that reacted with the antibodies against XpkA, it seems that *Synechocystis* does not express a protein with XpkA activity.

Expression of slr0453 in *E. coli* - To analyse whether slr0453 encodes a phosphoketolase, the gene was expressed in *E. coli*. For that purpose, the gene was amplified in a PCR reaction and ligated in the *E. coli* 6xHis affinity Tag expression vector pQE30. The resulting plasmid, pLPA23, was used to transform *E. coli* M15[pREP4]. pREP4 contains the lacI gene, encoding the lac repressor. The multiple copies of pREP4 in the cell ensure high levels of the lac repressor and tight regulation of expression.

In a first experiment, *E. coli* M15[pREP4] containing pLPA23 was cultivated at 37 °C in LB to an OD₆₀₀ of about 0.6. Synthesis of the protein was induced by addition of 1 mM IPTG. The cultures were harvested 3 hours after induction and cell-free extracts were prepared. An SDS-PAGE
An SDS-PAGE gel showed that a protein of about 90 kDa was synthesized in *E. coli* cells containing pLPA23. This protein, which reacted with antibodies against XpkA, was absent in *E. coli* containing pQE30, the vector without slr0453. After breakage of the bacteria in the French Pressure cell, all of the 90 kDa protein was located in the pellet fraction which suggests that it was present as inclusion bodies (data not shown).

To limit the formation of inclusion bodies, *E. coli* cells were cultivated at 25 °C to an OD<sub>600</sub> of 0.4 after which production of the protein was induced with 7.5 mM IPTG. The cultures were harvested one hour after induction. Cell-free extracts and the pellet fraction (containing cell debris) of *E. coli* after passage through a French Pressure cell were analysed by SDS-PAGE, followed by a Western blot experiment (Figure 5.2). Again, a 90 kDa protein which reacted with the antibodies against XpkA was found in *E. coli* containing pLPA23. The protein was present in both the supernatant and the pellet fraction, indicating that a part but not all of the protein formed inclusion bodies. In the negative control, *E. coli* containing pQE30, a faint band of approximately the same size was seen both in the supernatant and in the pellet fraction. The protein band from the pellet fraction migrated a little faster than that from the supernatant fraction. The same faint protein band that reacted with the antibodies against XpkA was also observed in the pellet and supernatant fraction of *E. coli* containing pLPA23. Since a gene encoding a protein with significant similarity to XpkA from *L. pentosus* is absent from *E. coli*, it was concluded that these faint protein bands were the result of an aspecific reaction of the antibodies with protein(s) in the cell-free extracts of *E. coli*. XpkA activity was measured in the cell-free extracts of *E. coli* as shown in Table 5.2. The activity found in *E. coli* containing pLPA23 was comparable to the activity detected in *E. coli* containing the empty vector. These results, together with the absence of XpkA activity in *Synechocystis* extracts, do not support the hypothesis that slr0453 encodes a XpkA.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Specific activity (nmol·min&lt;sup&gt;-1&lt;/sup&gt;·mg&lt;sup&gt;-1&lt;/sup&gt; protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M15[pREP4] * pLPA23</td>
<td>with slr0453</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>M15[pREP4] * pQE30</td>
<td>empty vector</td>
<td>39 ± 17</td>
</tr>
</tbody>
</table>

Table 5.2. XpkA activities in cell-free extracts of *E. coli* M15[pREP4] containing pLPA23 or pQE30. Numbers are the mean of two independent measurements. The XpkA activities were measured both in the presence and on the absence of inorganic phosphate (one of the substrates of XpkA). The activities detected under both conditions were approximately the same.
Table 5.3. Fructose 6-phosphate phosphoketolase activities measured in cell-free extracts. Synechocystis sp. PCC6803 was cultivated under different growth conditions and E. coli was transformed with an expression vector with or without slr0453. The activities were measured in the presence or in the absence of the substrate fructose 6-phosphate. The activity detected in the absence of the substrate was subtracted from the activity in the presence of fructose 6-phosphate. Activities are expressed in nmol·min⁻¹·mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis sp.</td>
<td>photomixotrophic, glucose</td>
</tr>
<tr>
<td>Synechocystis sp.</td>
<td>photoheterotrophic, glucose, DCMU</td>
</tr>
<tr>
<td>Synechocystis sp.</td>
<td>photoautotrophic</td>
</tr>
<tr>
<td>E. coli M15[pREP4] with pLPA23</td>
<td>induction of slr0453 with IPTG</td>
</tr>
<tr>
<td>E. coli M15[pREP4] with pQE30</td>
<td>empty vector</td>
</tr>
</tbody>
</table>

Fructose 6-phosphate phosphoketolase activity - A phosphoketolase catalysing a similar reaction as XpkA in Lactobacillus is present in bifidobacteria. This enzyme, fructose 6-phosphate phosphoketolase catalyses the conversion of fructose 6-phosphate and inorganic phosphate into erythrose 4-phosphate and acetyl-phosphate. Some of the fructose 6-phosphate phosphoketolase proteins that were (partially) purified from bifidobacteria were able to use both fructose 6-phosphate and xylulose 5-phosphate as a substrate [119]. No sequence information from fructose 6-phosphate phosphoketolase proteins is available at this moment, but it might be that fructose 6-phosphate phosphoketolase and XpkA share significant similarity at the amino acid level.

Because of the absence of XpkA activity in Synechocystis and since genes that might be involved in pentose transport or metabolism do not seem to be present in this organism (http://www.kazusa.or.jp/cyano/), we wanted to test whether slr0453 encoded a fructose 6-phosphate phosphoketolase rather than a XpkA. For this purpose, fructose 6-phosphate phosphoketolase activity was tested in the cell-free extracts of Synechocystis and of E. coli, in which slr0453 was expressed. The results are shown in Table 5.3. In all cell-free extracts, a very low activity was detected. Again, the activities that were found in the extracts did not correlate with the amount of protein that reacted with the antibody against XpkA (see Figure 5.1 and 5.2). It was therefore concluded that slr0453
might not encode a protein containing fructose 6-phosphate phosphoketolase activity.

It might be possible that technical imperfections of the assay conditions under which the xylulose 5-phosphate and/or fructose 6-phosphate phosphoketolase activities were determined prevented us to detect phosphoketolase activity in cell-free extracts of *Synechocystis*. For measuring XpkA activity, we have tried to optimize the assay conditions, for instance by changing the pH of the assay buffer, unfortunately without success. Further changes might be needed in order to improve the assay conditions before being able to detect possible phosphoketolase activities.