Butanoate pathway in production of acetate in *Thermoanaerobacterium sp.* AK17

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Raunvísindadeild
Háskóli Íslands
2016

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15 ECTS ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í sameindalíffræði

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Raunvísindadeild
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Skráningarupplýsingar:
Einar Baldvin Haraldsson, 2016, *Butanoate pathway in production of acetate in Thermoanaerobacterium sp. AK17* BS ritgerð, Raunvísindadeild, Háskóli Íslands, 28 bls.

Prentun: Háskólaprent
Reykjavík, júní 2016

Útdráttur

Markmið rannsóknarinnar var að kanna hvort etanól þolnara afbrigði af stökkbreyttum etanólframleiðandi stofni af *Thermoanaerobacterium sp.* AK17 sé fært um að nota ensím úr bútanóat ferlinum til að framleiða asetat í stað ensíma úr asetat ferlinum, en þeim ferli hafði verið eytt úr erfðamengi AK17 til þess að auka etanólframleiðslu AK17. Frumuhreinsuðum innanfrumupróteinum úr frumuræktum þriggja afbrigða af AK17 var safnað: villigerð (WT), tvöfalt stökkbrigði (DM) og aðlagað tvöfalt stökkbrigði (AD). Ensímpróf voru notuð til að staðfesta virkni bútanóats ferils ensímanna, fosfóstransbútyrylasa (PTB) og bútirat kínasa (BK), ásamt virkni fosfótransasatylasa (PTA) og asetat kínasa (AK) úr asetat ferlinum. Niðurstöður okkar renna stoðum undir þá kenningu að bútanóats ferils ensímin eru fær um að hvata myndun asetats í AK17, þó með lægri virkni heldur en ensímin úr asetat ferlinum.

Abstract

The main objective of this study was to test if an ethanol resistance adapted strain of the ethanologenic mutant of *Thermoanaerobacterium sp.* AK17 strain is capable of using the butanoate pathway enzymes in the production of acetate in place of the acetate pathway enzymes, whose genes have been knocked-out from AK17s genome in order to increase ethanol production. Cell-free cytosolic protein extract was harvested from batch cultures of three AK17 strains, i.e. wild type (WT), double mutant (DM) ethanologenic strain and adapted double mutant (AD). To test this, enzymatic assays were used to verify activity of the butanoate pathway enzymes, phosphotransbutyrylase (PTB) and butyrate kinase (BK), and phosphotransacetylase (PTA) along with acetate kinase (AK) in the acetate pathway. Our results support that butanoate pathway enzymes are capable of catalyzing the formation of acetate in AK17, but at a lower catalytic efficiency than acetate pathway enzymes.

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Abbreviations

AB media	Minimal growth media for anaerobic bacteria
AD	Adapted DM strain
AK	Acetate kinase
BK	Butyrate kinase
CFU	Colony forming units
DM	Double mutant (lac-/ace-)
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
erm	Erythromycin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
kan	Kanamycin
LC	Large culture
PCR	Polymerase chain reaction
PTA	Phosphotransacetylase
PTB	Phosphotransbutyrylase
RT	Room temperature
SC	Small culture
TCA	Trichloroacetic acid
WT	Wild type

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Þakkir

Ég vil þakka Bryndísi Björnsdóttur sérstaklega vel fyrir að hafa veitt mér leiðsögn, tækifæri og skemmtilegt verkefni, sem gaf mér reynslu og sjálfstæði í vinnubrögðum við rannsóknir. Guðmundi Ó. Hreggviðssyni var ávallt hjálplegur og tilbúinn að ræða um verkefnið, og fær hann bestu þakkir fyrir aðstoðina. Ég kunni vel að meta allt það starfsfólk hjá Matís sem ég kynntist og vann með, enda var það alltaf reiðubúið að svara spurningum og hjálpa til þegar á þurfti.

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1 Introduction

1.1 Biofuel

The demand for and production of fuel as an energy source increases every year, and consequently there is an increase in pollution. This calls for clean and renewable fuel sources. One type of renewable fuels are biofuels, which can come in many forms, i.e. liquid, gas and even solids. They can be produced by fermentation, chemical or thermal conversion from organic waste, natural or specifically produced biomass. Biomass is considered to be organic matter from contemporary sources, like organisms that are alive in the present day. There are various sources of biomass, from waste, such as lignocellulosic plant material and other organic materials, even animals. Biomass and biofuel may also be sourced from microalgae that produce high amounts of lipids, carbohydrates from macroalgae or simple sugar and starch from corn and sugarcane.

Biofuel production was commonly divided into two categories, first and second generation, but recently there has been defined a third (Behera et al., 2015). In first generation production, starch and sugars are fermented into ethanol or other biofuels, also oils from for example rapeseeds and palm oil which are turned into biodiesel. These sources of sugars, starch and oils also happen to be food crops for humans and animals alike. This method of production is the least favorable in economic terms, but the most convenient, considering ease of production, and it is not often viable without government subsidies, as a consequence can have very negative effects on food prices and available land that can support edible crops (Martin, 2010).

Second generation biofuel production is a more advanced process, utilizing organic waste, often lignocellulosic plant based material from forestry, agriculture or specially grown non-food crops. Lignocellulose is the main carbon source, but any source of carbohydrate can be used. This type of biofuel production often requires more intense and harsh approaches in the initial processing and conversion of the source material into biofuel as the biomass contains recalcitrant biomass (Brethauer and Studer, 2015).

Recently there has been defined a new category of biofuel production, which may be referred to as the third generation, based on micro- and macroalgae. This new source has many advantages, one of which is that the algae can be grown in salt water and does not require arable land, therefore there is no competition with current crops for land and water. Algae can be grown in great density and they are also very efficient photosynthesizers with an average photosynthetic efficiency of 6-8 % compared to an average of 1.8-2.2 % for terrestrials (Aresta et al., 2005). Biofuel from microalgae is most often in the form of oils/lipids to be converted into biodiesel, while the macroalgae contains no lignin but various structural and storage polysaccharides, which potentially can be fermented into ethanol or other biofuels (Alvarado-Morales et al.,2013). This conversion of macroalgae polysaccharides may be a simpler and milder process than the conversion of lignocellulose to biofuels. However, there are a few problems that need to be overcome, including finding an organism that can do this conversion of carbohydrate efficiently and consistently, as there

are not many organisms known that have the enzymatic toolkit necessary to metabolize all the available macroalgae polysaccharides. To solve this, we need to turn to bioengineering.

1.2 Bioengineering

Bioengineering has become a very valuable and important tool in making biofuel production more efficient and versatile, allowing for new and novel methods that increase the variety of biomass sources and conversion methods available for biofuel production. Bioengineering can be applied directly on the primary biofuel source as in microalgae were by increasing lipid levels that are then extracted. Another approach is modifying the conversion organisms, bacteria and yeast, by editing metabolic pathways, such as adding genes for enzymes which metabolize complex carbohydrates and thereby expand the metabolic range of conversion organisms. The addition of enzymes capable of preprocessing and metabolizing macroalgal carbohydrates, for example alginate lyases or laminarinase to be secreted by the fermenting organism to facilitate a single step bioconversion of macroalgal carbohydrates (Wargacki et al, 2012). Ethanol is not the only end product of anaerobic fermentation, lactate and acetate are common end products but not always desired when ethanol is the primary product and optimization of the organism's fermentative pathway is needed. Optimization of bioconversion by fermentation can be in the removal of genes required in pathways that lead to undesirable byproducts and what was done in AK17.

1.3 AK17

AK17 is a Gram positive thermophilic anaerobic bacterium that was isolated from hot springs in 2004, in a geothermal area in NE Iceland, Víti in the Krafla area (Örlygsson and Baldursson, 2007). Phylogenetic analyzes of its 16S rRNA and its ability to utilize thiosulfate placed AK17 in the genus of *Thermoanaerobacterium* (Örlygsson and Baldursson, 2007; Sveinsdóttir et al., 2009). AK17 produces high amounts of ethanol, 1.5 mol-EtOH per mol glucose, as well as acetate and lactate as fermentation end products. The strain is proficient in degrading a wide range of mono- and disaccharides, cellulose, pectin and amino acids (Örlygsson and Baldursson, 2007; Almarsdóttir, 2011). Because of these comprehensive metabolic capabilities of AK17, being an efficient ethanol producer and having moderate natural ethanol tolerance, 4 % v/v (Koskinen et al., 2008). It was of great interest to Matís to investigate the utilization of AK17 in the production of bioethanol from macroalgae, a highly underutilized source of biomass in Iceland. Another advantage of AK17 is its natural competence (Shaw et al., 2010). In order to further increase ethanol production and decrease byproduct formation of lactate, a knock-out strain of AK17 was made (Sigurðardóttir, 2009). Acetate knock-outs were also performed on the AK17 genome (Matís, unpublished). The genes knocked out in the acetate pathway were Phosphotransacetylase (PTA), EC 2.3.1.8, and acetate kinase (AK), EC 2.7.2.1. The ethanol yield doubled in the double mutant (DM) (Lac-/Ace-) strain compared to the WT strain, and produced no lactate and only trace amounts of acetate. However, a downside to these knock-outs were markedly slower growth rates as the loss of acetate formation lead to a reduction in formation of adenosine triphosphate (ATP) (Matís, unpublished results). The predicted fermentative pathway of glucose in the DM strain is shown in Figure 1.1, along with the knocked-out enzymes, two enzymes in the acetate pathway and a single enzyme involved in lactate formation.

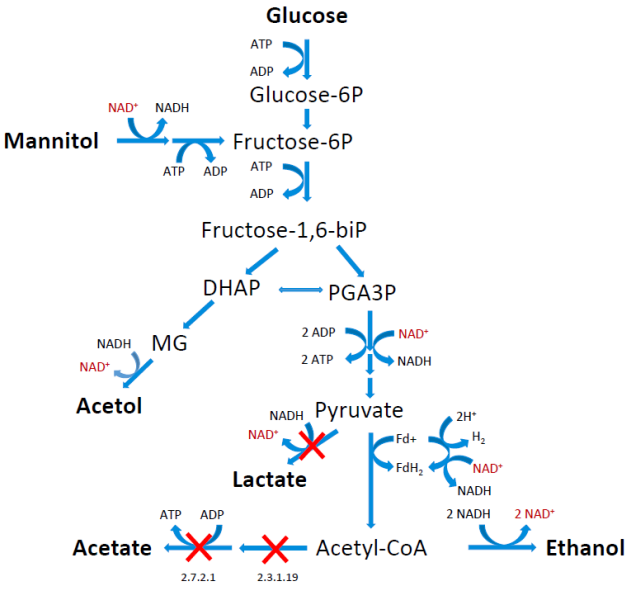

Figure 1.1 Fermentation of glucose in AK17 DM and AD strain. Knocked-out enzymes are shown with the respective EC numbers for the acetate pathway enzymes (Matís, unpublished).

1.4 Renewed acetate production

The driving force of life is survival and reproduction. There is fierce competition for available resources and therefore it is crucial to be able to use these sources of energy and material as efficiently as possible (Maitra and Dill, 2015). It is highly disadvantageous for an organism when certain energy producing pathways are lost, or as in the case of the bioengineered AK17 organism, removed. A result of 20 % lower cell yield has been reported as a consequence to the knock-out of AK in *Thermoanaerobacterium saccharolyticum* during xylose fermentation, due to a loss of equivalent of 23 % ATP per mole xylose fermented (Shaw et al., 2008).

In order to increase ethanol tolerance of AK17 DM, the strain was grown in a continuous cultivation (850 hours) with increasing ethanol concentrations. The tolerance increased stepwise, but an undesired change also occurred, an increased production of acetate (SINTEF, unpublished results). This was unexpected as, the DM was supposed to be incapable of producing acetate due to the double knock-out of genes needed in the acetate pathway. Due to the loss of AK the DM strain had also lost an ATP and consequently had a slower growth rate and lower yield than the wild type (WT). The renewed acetate formation in the adapted strain (AD) apparently lead also to regain of the ATP formation as this new strain had a marked increase in growth rate in comparison to the DM and was therefore enriched during the continuous culture.

1.5 Acetate and butyrate pathways

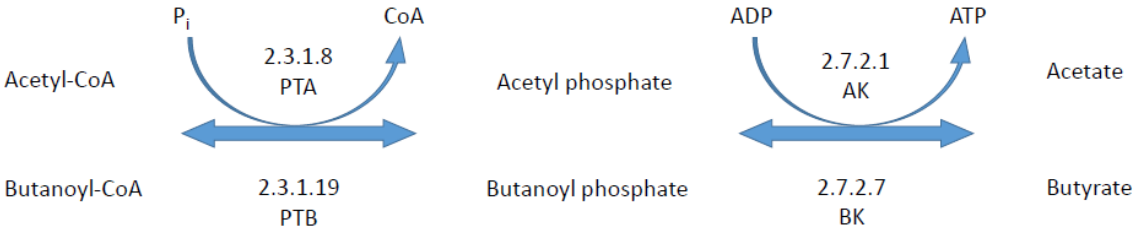

Figure 1.2 Acetate and butyrate pathways, their substrates and products. EC numbers and abbreviations of respective enzymes involved are shown.

Annotation of the AK17 genome showed the presence of two predicted genes that might be able to make up for the loss of PTA and AK in the double mutant (DM), these are phosphotransbutyrylase (PTB), EC 2.3.1.19, and butyrate kinase (BK), EC 2.7.2.7. PTA and PTB belong to the same family of acyltransferases It has been previously reported that butanoate pathway enzymes are able to compensate for acetate production (Kuit et al. 2012). Enzyme assays can be used to determine the presence of an enzyme capable of metabolizing a specific substrate. A forward reaction is the reaction as formation of products from reactants as it proceeds from left to right in a reversible reaction, and the reverse is termed the backward reaction. Here we will look at the forward and backward reaction of AK and BK. These enzymes are part of the butanoate pathway in the metabolism of butyrate that is also a short chain carboxylate as acetate and could possibly compensate for the loss of PTA and AK from the acetate pathway. Another gene, acylphosphatase, EC 3.6.1.7, was found in the AK17 genome that is able to hydrolyze acetyl phosphate to acetate.

1.6 Objective

The main objective of this study was to test if the AK17 AD strain is capable of using butanoate pathway enzymes in place of the acetate pathway in production of acetate. To test this an enzymatic assay in the acetyl- and butanoyl phosphate forming direction by release of CoA will be applied to detect the presence of PTA and PTB enzymes. In addition, assays in formation of acetate and butyrate from acetyl- and butanoyl phosphate in both the backward and forward direction in detection of the enzymes BK and AK.

2 Methods and materials

2.1 Culture

2.1.1 Media

AB medium for anaerobic bacteria and adjusted to AK17 (Sveinsdóttir et al., 2009, Sigurðardóttir, 2009). All growth of AK17 strains was done in an anaerobic environment. Serum flasks with butyl stoppers and Hungate type tubes were used. AB media was added to flasks and tubes, containers flushed with N_2 and autoclaved. Media and containers were stored in a dark place at RT until needed. Before inoculation, glucose, C_1 (vitamins, minerals and trace elements) and C_2 (oxygen absorber) were added to the media and incubated at 55 °C until all oxygen was reduced. When using agar plates same AB media was prepared with the addition of 1.5 % agarose. Plates were dried at 55 °C and allowed to cool to RT before spreading the culture as this was done aerobically and the lower temperature reduces AK17 activity and increases survival when plating in aerobic environment. Plates were placed in BD GazPak EZ Pouch System with an oxygen reducing sachet, left at RT until the oxygen was reduced, for 2-3 h, using a color indicator. Thereafter plates were incubated at 55 °C for up to 4 days.

2.1.2 Clone selection

Frozen aliquots, 200 µL, of the three AK17 strains of interest (WT, DM and AD) were thawed and inoculated into 10 mL of AB media in Hungate tubes under N_2, 2 % inoculation, incubated at 55°C, 24h. Of each strain 100 µL were plated onto 2 AB agar plates, incubated at 55°C, 48h. The strains were derivatives of the following isolates; W12 for the wildtype, DM13 for the double mutant and AD46 for the adapted strain. Inoculation of two colonies from each strain were made into 10 mL Hungate tubes by dissolving the selected colony in 100 µL of dH_2O and injected into the Hungate tube, incubated at 55 °C, 24h.

2.1.3 DNA isolation

DNA was isolated from 5 mL of each culture, 6 cultures, 2 of each strain for PCR analyses for the presence of Kanamycin (*kan*) and Erythromycin (*erm*) resistance. This was done using the MasterPure^{TM} Gram Positive DNA Purification Kit (epicenter). DNA concentration was measured using NanoDrop^{TM}.

2.1.4 PCR

Validation of strains was done using PCR with primers, Table 2.1, targeting the *erm* and *kan* resistance genes that are present in both the DM and AD but not in the WT as the resistance genes were inserted into the strains during mutant construction for strain selection purposes. PCR was done on purified DNA to validate strains before inoculation of large cultures. Furthermore, colony PCR was also done from samples taken at the end of incubation of cultures used for enzymatic assays. Mutant construction plasmids were used as positive controls containing either *erm* or *kan* primer sites, plasmids PEO3 and pBB16, respectively,

dH_2O for negative control. Reaction mixture volume used was 20 µL. The master mix of reactants and buffers is shown in Table 2.2 and thermal cycles for the PCRs in Table 2.3.

Table 2.1 Primers used for amplification of resistance genes

Primer	Sequence
kan-mfe-f	AATTGTAGAGGAGGATGTT
kan-eco-bam-r	AATTCTCAAAATGGTATGC
erm-mfe-f	GGCGATGTAATGAATAAGAACATAAAGTAC
erm-bam-r	CTTGGATCCTTACTTCCTTCCATTGAATAACAAATAG

Table 2.2 Master mix for purified DNA PCR and Colony PCR

Master Mix	µL
F Primer (20 mM)	0.5
R Primer (20 mM)	0.5
dNTPs	0.5
10x Standard Taq Buffer	2.0
Taq Polymerase	0.15
dH_2O	15.35
Template	0.5 DNA / 1.0 for Colony PCR

Table 2.3 Thermal cycles for resistance genes and Colony PCR

erm		***kan***		**Colony**		
Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**	
94 °C	2 min	94 °C	2 min	94 °C	10 min	
94 °C	20 sec	94 °C	20 sec	94 °C	20 sec	35x
52 °C	15 sec	55 °C	20 sec	55 °C	20 sec	
72 °C	50 sec	72 °C	1 min	72 °C	50 sec	
72 °C	7 min	72 °C	7 min	72 °C	7 min	
4 °C	∞	4 °C	∞	4 °C	∞	

PCR products were analyzed using electrophoreses on 1 % agarose gel with SYBR Safe, 90 V, 45 min, 1 kb marker. Into each sample 7 µL of loading dye was added before loading onto gel, 25 µL was loaded into each well.

2.1.5 Cultures

Two cultures were set up in order to have a biological replicate. Also to ensure a good supply of enzymes for the assays, one culture containing 300 mL (LC) to be used during enzymatic assay validation and another 50 mL (SC) liquid volume as a biological replicate, were inoculated into serum flasks. The LC and SC flasks were flushed with N_2 for 5 min and 2 min, respectively, then autoclaved before inoculation with 1 % v/v of each strain. Cultures were incubated at 55 °C. A 50 mL control culture containing the complete growth media but without inoculation of AK17 was also prepared. Growth of cultures was stopped by storing them at 4 °C, visual estimation of cloudiness was used in determining when to stop each culture. Incubation of WT and AD cultures was stopped after 24h and DM cultures after 48h, the control culture was also given 48h. Cultures were stored at 4 °C until all cultures were ready for harvest.

2.1.6 Cell-free cytosolic protein extraction

The Optical Density at 600 nm (OD600) of each culture was measured at time of harvest using the control culture as blank. Cell biomass was estimated by weighing the cell pellet after centrifugation. Cells were harvested from cultures by centrifugation, 2,500x g, 25 min, 4 °C. Cells were then resuspended in 50 mM, pH 6.2 KPO_4 buffer. Addition of buffer was estimated according to cell pellet weight to achieve a cell biomass suspension of 0.1 g/mL. Cells were then lysed using sonication on ice. The cell suspension from LC were sonicated 2x 3 min and 2x 2 min for the SC. Lysed cells from LC were spun down at 2500x g, 20 min, 4 °C and SC 8,000x g, 4 min, 4 °C. The supernatant was then filtered through syringe filters, 0.45 µm. Extracts from AD and WT cultures were diluted with suspension buffer to a concentration of total protein to that of ~1.85 mg/mL for LC and ~1.5 mg/mL for SC, both DM cultures were not diluted further before freezing. Cell-free extracts were aliquoted 0.5

mL in Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80 °C. The process of cell-free extraction was carried out aerobically.

2.1.7 Bradford

Bradford protein assay, Quick Start™ Bradford Protein Assay (BioRad), was used to determine total protein concentration of the cell-free extract, with bovine serum albumin as standard.

2.1.8 Dionex HPLC

To quantitatively analyze the products and glucose utilization in each culture, Dionex HPLC was used. Samples were collected at inoculation and at cell-free extraction from every culture, ~1.5 mL. Samples were then spun down at 4000 rpm, 4 °C, and filtered through a 0.45 µm syringe filter, frozen at -20 °C until analysis. Dionex ICS-3000 with Rezex ROA-Organic Acid H+ (8%) column, 0.005 N sulphuric acid running phase, 0.6 mL/min flow rate and RI detector at 512 nm was applied. A quantitative standard of; lactic acid, acetic acid, glucose, 1,2-propanediol, glycerol and ethanol was prepared and concentration of standards is shown in Table 2.4. Quantitative analysis of the culture growth medium at 0h and then at harvest, the ratio of consumed glucose to produced ethanol, lactic acid and acetic acid was compared.

Table 2.4 Concentrations of analyte standards for quantitative analysis of culture media

Analyte	Concentration
Lactic acid	0.25 – 8.0 mg
Acetic acid	0.125 – 2.0 %
Glucose	0.25 – 8.0 mg
1,2-Propanediol	0.125 – 2.0 %
Glycerol	0.125 – 2.0 %
Ethanol	0.125 – 2.0 %

2.2 PTA-PTB Assay

Activity of PTA and PTB was assessed by the release of CoA from butyryl-CoA and acetyl-CoA in consequent formation of acetyl phosphate and butanoyl phosphate. Free CoA reacts with DTNB and can be read at 412 nm. Method was adopted from Andersch (1983) and Cary (1988). The specific activity was defined as µmole of released CoA per minute relative to mg of total protein (U/mg). End-point absorptions were measured. The standard curve was created using L-Cysteine, 0.0 – 6.0 mM (0.0 – 1.8 µmole), incubated at 55 °C, 60 min. A stock solution of 100 mM L-Cysteine was made in a serum flask, dH_2O was degassed by sonication and then flushed with N_2 to reduce the risk of formation of Cysteine-Cysteine

dimers, stored at 4 °C. Stock solutions for the assay are shown in Table 2.5. Stock solution of DTNB 4 mM was made in 100 mM KPO_4, pH 7.20, 0.1 mM EDTA, stored at 4 °C. The assay buffer, 300 mM KPO_4, pH 7.29 (pH 7.20 at 55 °C), 10 mM stock of acetyl-CoA in dH_2O needed to be made fresh daily, butyryl-CoA could be aliquoted and stored at -20 °C, for at least 4 weeks. The assay was done in a 96 well flat bottom plate, total assay volume was 300 µL, incubated at 55 °C, 20 min, read immediately at 412 nm. Dilutions of cell-free extract done as needed. Preparation of a 1x master reaction mix for the assay is shown in Table 2.6.

Table 2.5 List of stock solutions for the PTA and PTB assay and storage conditions

Stock solutions [mM]		**Storage**
300 mM	KPO_4, pH 7.49	Store at RT
10 mM	Acetyl-CoA / Butyryl-CoA	Fresh daily, rapid degradation*
4 mM	DTNB	Store at 4 °C

*Butyryl-CoA can be stored at least 4 weeks at -20 °C.

Table 2.6 Master mix for PTA-PTB assay reaction

Reaction mixture	**Volume [µL]**
KPO_4 Buffer	150
Acetyl-CoA / Butyryl-CoA	6
DTNB	6
dH2O	128
Total:	**290**

Procedure:

1. 10 µL of enzyme was added to each well, dH_2O as control, 10 µL dH_2O for blank.
2. Reaction was started by the addition of 290 µL of reaction mixture. Lid was placed on the plate and incubated at 55 °C while shaking for 20 min.
3. Plate was taken directly to be read at 412 nm, lid was removed prior to reading.

2.3 AK-BK Assay

Activity of AK and BK was tested in both the forward and backward reaction. The forward reaction is ATP formation from reaction of acetyl- and butyryl phosphate with adenosine diphosphate (ADP), the reaction is shown in Figure 1.2. The backward reaction is the formation of acetyl- and butanoyl phosphate and ADP from the reaction of ATP with acetate and butyrate, also shown in Figure 1.2. Formation of acetyl- or butanoyl hydroxamate is from the reaction between acetyl- or butanoyl phosphate with neutral hydroxylamine. The acetyl-, butanoyl hydroxamate forms a colored complex with ferric ions and the complex can be detected at 540 nm. The forward reaction is an adaptation of a method by Fowler (2011), and the backward reaction a method described by Rose (1955). Both assays are discontinuous, were the assay is stopped by addition of trichloroacetic acid (TCA). Measurements needed to be taken within 20 min after the addition of the development solution (DS), TCA and $FeCl_3$, the ferric-hydroxamate complex may start to degrade if measurement was delayed beyond 20 min. The neutral hydroxylamine hydrochloride solution was pH adjusted with potassium hydroxide pellets. Buffers were adjusted to the right pH by addition of a monovalent base, 1 M NaOH or acid, 1 M HCl. A list of all stock solutions and storage conditions is shown in Table 2.7.

Two standard curves were made, with acetyl phosphate and butanoyl phosphate, 0.00 – 0.90 µmole (0.0 – 3.0 mM), incubated at 55 °C, 30 min.

Table 2.7 Stock solutions for AK and BK assays and storage conditions

Stock solutions [mM]		**Storage**
3.2 M	Potassium acetate / Sodium butyrate	Store at RT
1.0 M	Tris-HCl buffer, pH 8.39 (7.4 at 55 °C)	Store at RT
1.0 M	$MgCl_2$	Store at RT
0.1 M	ATP / ADP	Aliquot and freeze, -20 °C
2.0 M	Hydroxylamine hydrochloride, pH 7.0	Store at 4 °C
1.25 %	$FeCl_3$, 1 M HCl	Store at RT
10 %	Trichloroacetic acid (TCA)	Store at RT
0.1 M	Acetyl phosphate / Butanoyl phosphate	Aliquot and freeze, -20 °C

DS was made fresh daily, a mixture of stock 1.25 % $FeCl_3$ and 10 % TCA in the ratio of 4:1, respectively. For each reaction 1 mL of DS was needed.

2.3.1 Backward reaction

As acetyl- and butanoyl phosphate is formed it reacts with hydroxylamine and is removed from the reaction as it forms driving the reaction backward. Concentrations and volumes for

1x reaction mix is shown in Table 2.8. The specific activity for the backward reaction was defined as µmole of formed acetyl- and butanoyl phosphate per minute relative to mg of total protein (U/mg).

Table 2.8 Concentration and volumes of solutions for 1x reaction mix in the backward direction for AK and BK assay

Reaction mix	**mM**	**µL**
Tris	50	10
$MgCl_2$	10	2
Acetate / Butyrate	800	50
ATP	10	20
Hydroxylamine	700	70
dH_2O		38
Total:		**190**

Procedure:

1. Reaction was started when 190 µL of reaction mixture was added into a 1.5 mL Eppendorf containing 10 µL of cell-free extract, 10 µL dH_2O for blank.
2. Incubated at 55 °C, 20 min.
3. Reaction was stopped by addition of 1000 µL DS and let develop for 5 min at RT.
4. Samples spun down at 16.1k RCF, 1 min, to remove any precipitated protein.
5. 300 µL transferred to a 96 well flat bottom plate and read at 540 nm. Bubbles were prone to form in the wells and were removed when possible.

2.3.2 Forward reaction

For the forward reaction the amount of remaining acetyl- and butanoyl phosphate following the reaction is subtracted from a sample blank. Thereby acetyl- and butanoyl phosphate consumed by the enzyme can be calculated. Hydroxylamine is added after the initial reaction incubation, therefore it acts also as a reaction stop in addition to the DS, after the addition of hydroxylamine the reaction needs to be incubated at 60 °C, 5 min, to ensure quick conversion of remaining acetyl- and butanoyl phosphate to its hydroxamate form. Concentrations and volumes for 1x reaction mix are shown in Table 2.9. The specific activity of the forward reaction was defined as µmole of consumed acetyl- and butanoyl phosphate per minute relative to mg of total protein (U/mg).

Table 2.9 Concentration and volumes of solutions in a 1x reaction mix in the forward direction for AK and BK assay

Reaction mix	mM	µL
Tris	100	12
$MgCl_2$	10	1.2
Acetyl phosphate **Butanoyl phosphate**	2	2.4
ADP	5	6
dH_2O		98.4
Total:		**120**

Procedure:

1. Reaction was started by addition of 120 µL of reaction mix into 10 µL of cell-free extract in a 1.5 mL Eppendorf, 10 µL dH_2O for blank.
2. Incubated at 55 °C, 20 min.
3. Reaction was stopped by addition of 70 µL of neutral hydroxylamine solution, incubated at 60 °C, 5 min.
4. 1000 µL of DS was added and let develop at RT, 5 min.
5. Samples were spun down at 16.1k RCF, 1 min, to remove any precipitated protein.
6. 300 µL transferred to a 96 well flat bottom plate and read at 540 nm. Bubbles were prone to form in the wells and were removed when possible.
7. Absorption of sample was subtracted from the control in order to determine consumed substrate.

2.4 Data presentation

Measurements were performed in biological duplicates and repetition triplicates. Data presented is the average of measurements from two independent cultures (LC, SC) of each strain and error is given as ± Standard Error Mean (± SEM), n = 2, unless otherwise stated.

2.5 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare differences in cytosolic protein expression between each strain. The samples were prepared as follows; 5 µL sample, 3 µL SDS Loading Buffer, 5 µL dH_2O, boiled at 100 °C, 5 min. A precast Mini-PROTEAN® TGX 15 well, 15 µL/well 12% acrylamide gel (BioRad) with Precision Plus Protein™ Dual Xtra Standards (BioRad). Gel electrophoresis was done with 30 mA, 45 min. Gel was stained with Coomassie Blue overnight before being decolored and scanned.

2.6 GAPDH

In addition to using Bradford to estimate the protein content of the cell-free cytosolic extract, a reference enzyme was tested to estimate the relative expression of target enzymes in comparison with a possible reference enzyme. The glycolytic enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected for this purpose using GAPDH Activity Assay Kit (BioVision) to determine the activity according to manufacturer's instruction. The reaction was incubated at 55 °C, 5 min and a single endpoint measured. A ratio comparison of specific activity of butyrate and acetate pathway enzymes against that of GAPDH (U mg^{-1}/U mg^{-1}) was made.

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3 Results

3.1 Cultures

3.1.1 Strain verification

Strains AD and DM were verified as they showed positive results for both resistance markers, *kan* and *erm*, results shown in Figure 3.1. One culture of each strain was chosen for inoculation of SC and LC. One of the tested WT strains seemed positive for *erm* and therefore the double negative sample was used for further inoculation.

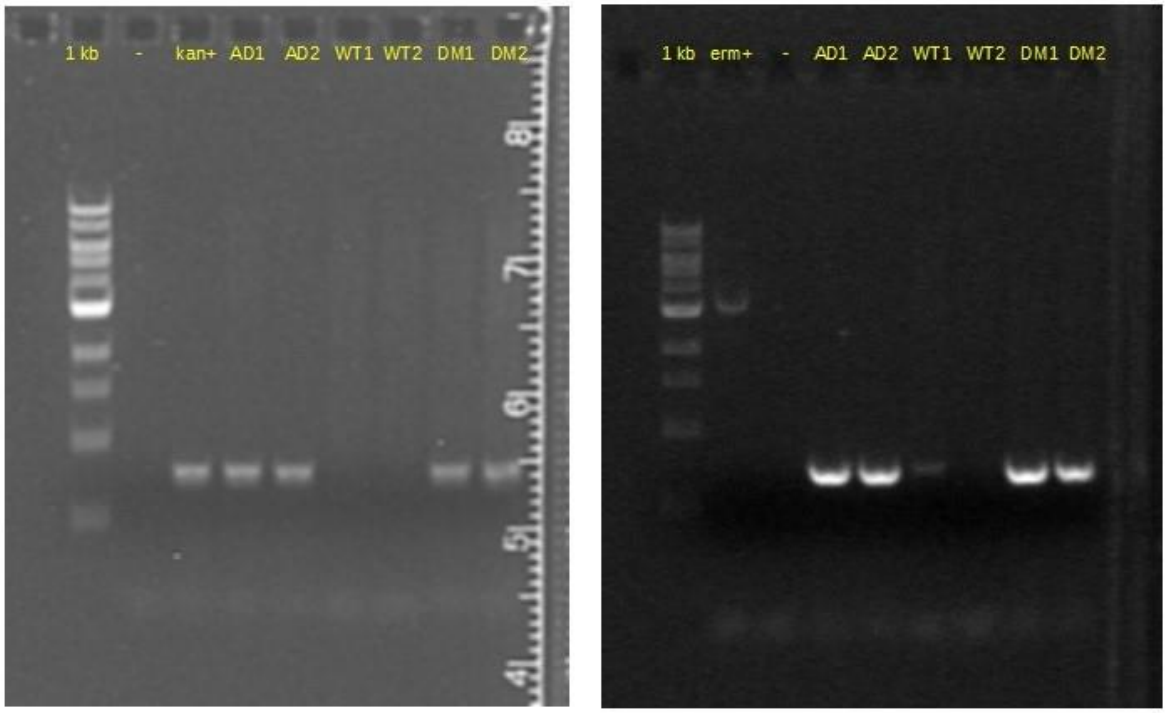

Figure 3.1 PCR products for detection of erm and kan resistance in selected clones. Left, kan and right, erm resistance. Positive control plasmids, erm+ and kan+, - no template, six cultures, two for each strain, WT, DM and AD.

Colony PCR was performed on LC and SC cultures to validate that each culture had the resistance as expected from initial inoculation, Figure 3.2. Cultures for AD and DM were both positive for *kan* and *erm*, while WT was negative.

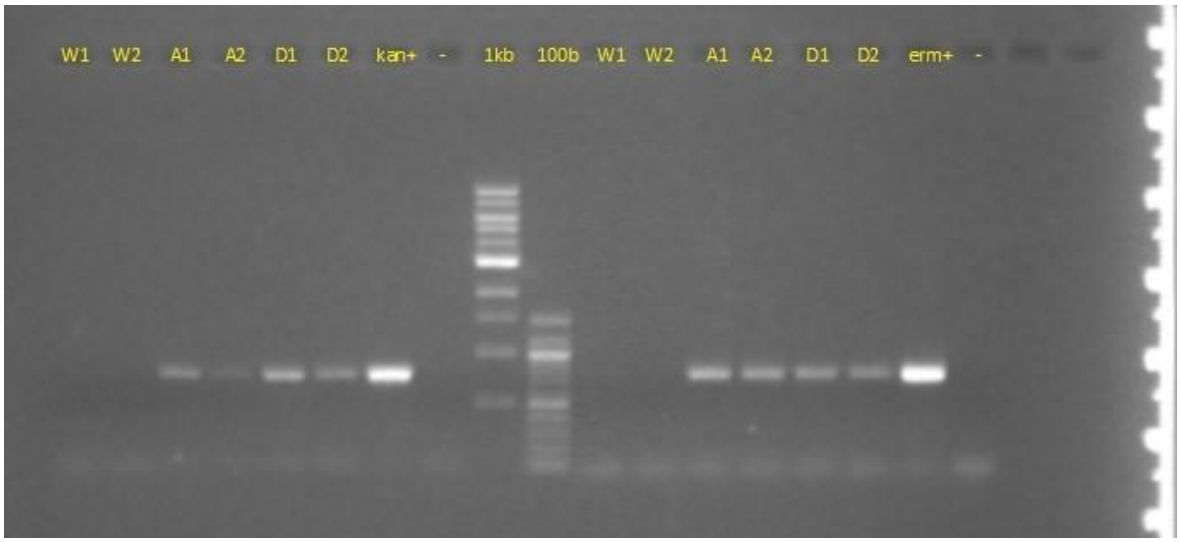

Figure 3.2 Colony PCR on LC and SC cultures for erm and kan resistance genes. W1, A1 and D1 mark the LC and W2, D2 and A2 the SC, 1kb and 100 b markers. kan+ and erm+ for positive control plasmids and - negative control with no template.

3.1.2 Growth of cultures

Measurements of OD600 and cell biomass weight at the time of harvest is shown in Table 3.1. Data shown in Table 3.1 are from single measurements.

Table 3.1 OD600 (absorption at 600 nm) of cultures LC and SC along with biomass of cell pellet at time of harvest, control culture used as blank for OD600.

Culture	OD600	Cell biomass [g]
WT-SC	0.656	0.15
WT-LC	0.623	0.78
DM-SC	0.492	0.10
DM-LC	0.508	0.50
AD-SC	0.689	0.12
AD-LC	0.688	0.72

3.1.3 Cell-free extract

Bradford assay was repeated on the initial samples of aliquoted frozen cell-free cytosolic extracts, data shown in Table 3.2. Four independent measurements of LC samples were used to determine total protein content for LC cultures of each strain. Only one measurement was made of each SC strain sample.

Table 3.2 Total protein concentration of cell-free extract form cultures LC and SC, data presented as mean ± SEM (n=4) for LC. SC samples were measured only once.

Culture	WT [mg/mL]	DM [mg/mL]	AD [mg/mL]
SC	1.54	0.80	1.53
LC	1.81 ± 0.14	0.85 ± 0.05	1.82 ± 0.12

3.2 Fermentation end products

With Dionex HPLC we could determine and therefore differentiate AD from DM based on their fermentation end products; ethanol, lactic acid and acetic acid, and validate that all strains behaved similarly as previous results have shown. Both AD cultures, SC and LC, had consumed all available glucose in the growth medium. Results of glucose conversion ratio into fermentation end products is shown in Table 3.3. No 1,2-Propanediol was detected in any of the cultures. There was no detectable difference measured in concentrations of analytes in the control culture.

Table 3.3 End product formation (in mM) of ethanol, lactic acid and acetic acid from glucose (20 mM) for the three AK17 strains.

Strain	Ethanol	Lactate	Acetate
WT	16.7 ± 0.4	2.9 ± 0.9	12.2 ± 0.1
DM	21.8 ± 1.3	0.1 ± 0.1	1.8 ± 0.2
AD	22.0 ± 1.2	0.2 ± 0.1	9.1 ± 0.2

3.3 Cytosolic protein profiles

SDS-PAGE was performed on samples from LC, both as stock concentrate and with all samples equalized to a total protein concentration relative to that of the DM sample by addition of 50 mM, pH 6.2 KPO_4 buffer, results are shown in Figure 3.3. Some minor variance was detected in cytosolic protein expression.

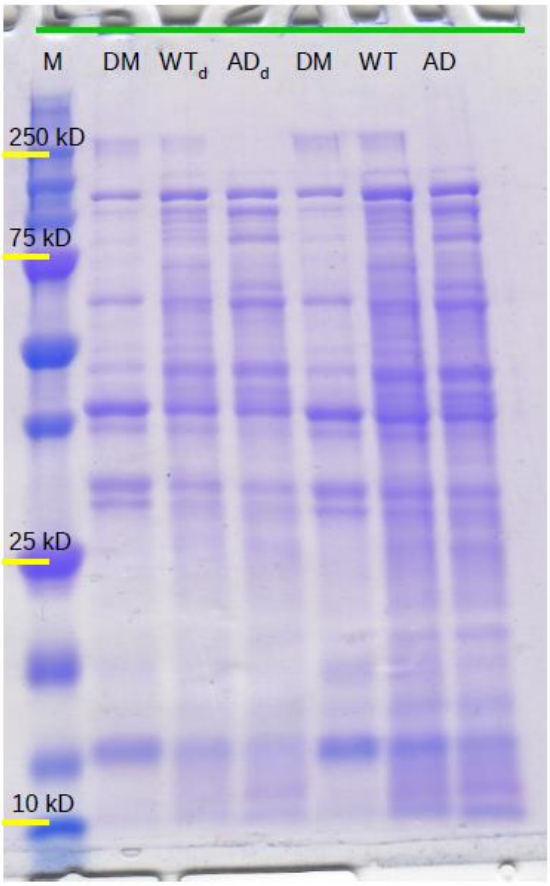

Figure 3.3 SDS-PAGE of LC cultures, M is the standard, DM, AD and WT for each strain. Samples WT_d and AD_d have been diluted relative to that of total protein concentration in DM sample. Green line marks the wells and 4 protein markers have been labeled with size (kD).

3.4 PTA-PTB Activity

Specific activity (U/mg) of PTA and PTB in direction of acetyl- and butanoyl phosphate formation by release of CoA form acetyl- and butyryl-CoA, and free CoAs subsequent reaction with DTNB that can then be detected, results shown in Figure 3.4. WT shows good activity for acetyl-CoA, negligible in DM as expected, and increased activity in AD along with an increase in butanoyl-CoA activity

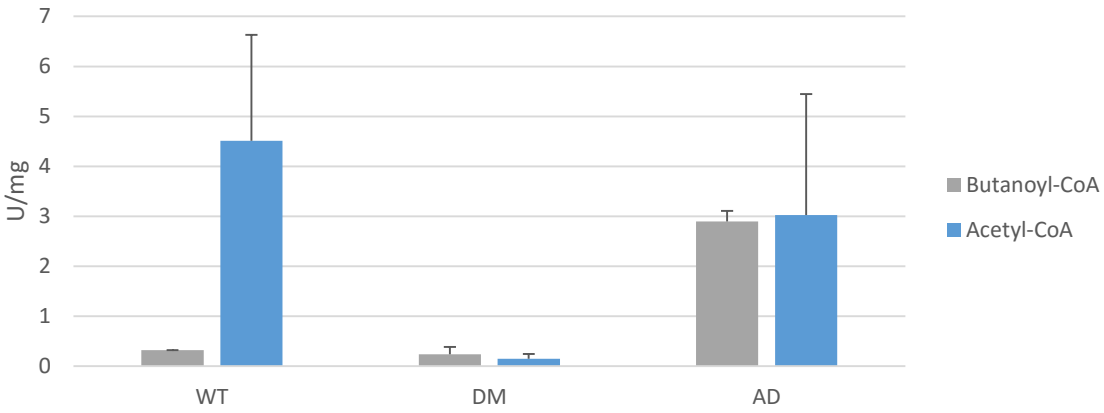

Figure 3.4 Specific activity (U/mg) of PTA and PTB in formation of acetyl- and butyryl phosphate. WT shows good activity for acetyl-CoA, negligible in DM as expected, and increased activity in AD along with an increase in butanoyl-CoA activity. The data is reported as mean ± SEM (n=2).

3.5 AK-BK Activity

3.5.1 Formation of acetate and butyrate

Specific activity (U/mg) of AK and BK in the forward direction, results for the AK activity in the direction of acetate formation in WT was omitted in Figure 3.5 as the activity was too high relative to other activities, its activity was 11.5 ± 0.4 U/mg. No AK activity was detected in DM. Data is shown in Figure 3.5.

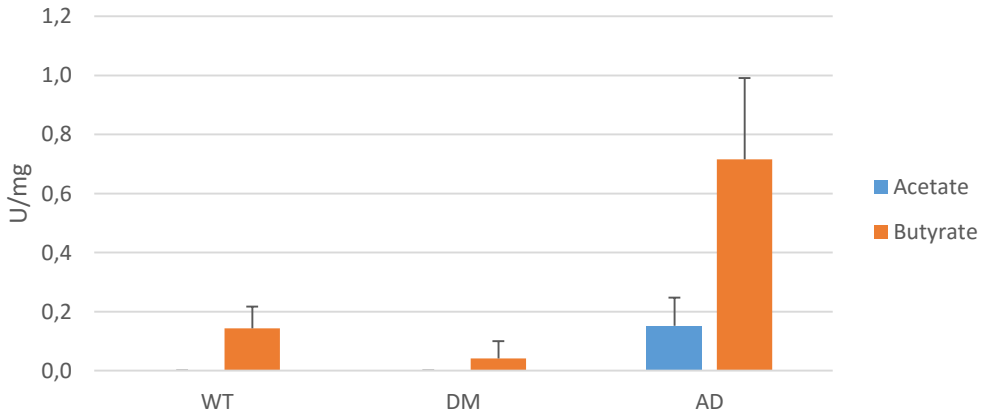

Figure 3.5 Specific activity (U/mg) in formation of acetate and butyrate in AK17 strains WT, DM and AD. Activity of acetate formation in WT was 11.5 ± 0.4 U/mg and could not be shown in relation to other activities. An increase in acetate formation was detected in AD along with a stronger increase in butyrate formation. DM showed little to no activity in acetate and butyrate formation, respectively. The data is reported as mean ± SEM (n=2).

3.5.2 Formation of acetyl- and butanoyl phosphate

Specific activity of AK and BK in the acetyl- and butanoyl phosphate forming direction is shown in Figure 3.6. The specific activity of AK in the acetyl phosphate formation was omitted from the figure for the WT strains as its activity was too high relative to DM and AD, 3.2 ± 0.3 U/mg.

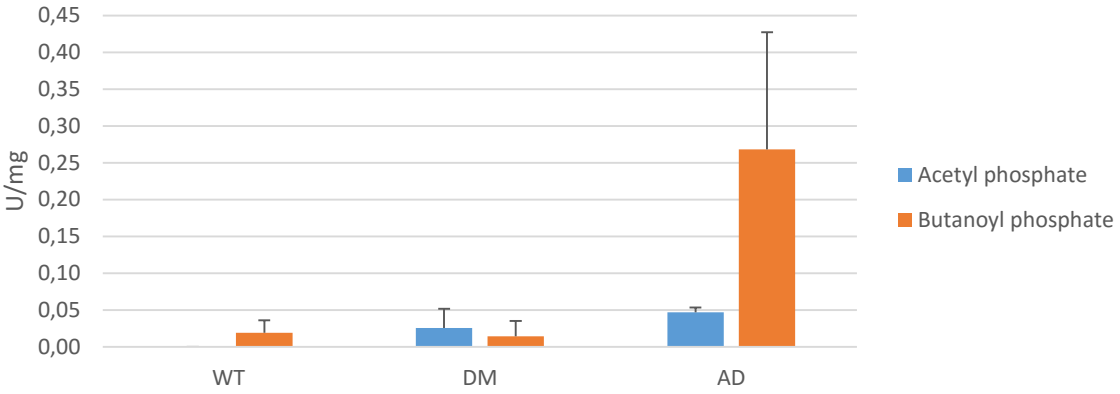

Figure 3.6 Specific activity (U/mg) in formation of acetyl- and butanoyl phosphate in AK17 strains WT, DM and AD. Acetyl phosphate formation activity for the WT strains were omitted as the values were too high in relation to DM and AD. AD showed increased activity in acetyl phosphate formation alongside a greater increase in butanoyl phosphate formation. Negligible activity in DM in formation of acetyl- and butanoyl phosphate. The data is reported as mean ± SEM (n=2).

3.6 GAPDH enzyme activity

Results for the GAPDH assay as a potential reference enzyme, are shown in Figure 3.7. Samples from LC cultures, AD and WT were diluted to a protein concentration relative to DM and specific activity was linear in relation to dilutions (data not shown).

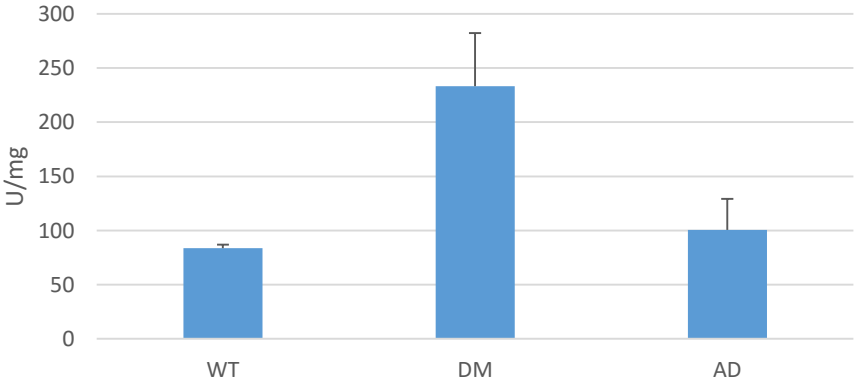

Figure 3.7 Specific activity (U/mg) for GAPDH in AK17 strains WT, DM and AD. Specific activity for the DM strain was significantly greater than for the WT and AD strains, which showed similar activity. The data is reported as mean ± SEM (n=2).

4 Discussion

The main objectives of this study was to determine if the butanoate pathway enzymes PTB and BK could compensate for the loss of the acetate pathway enzymes PTA and AK in the production of acetate in the AK17 AD strain. We also observed differences in growth rate of the three strains of AK17, i.e. WT, DM and AD, as well as difference in fermentation end products.

4.1 Growth and fermentation end products

Fermentation end products for each strain of AK17 are mostly in accordance with previous results, (Matís and SINTEF, unpublished) with the ethanol as the single end product in DM and increased acetate production in the AD strain. However, and for unknown reasons, ethanol production was not double in the DM compared to the WT strain as previously shown (Matís, unpublished). Cultures of AK 17 strains AD and WT grew at relatively equal rates according to OD600 (Table 3.1). The AD cultures had slightly higher OD600 values than WT, indicating possible higher cell density, however, cell biomass weight was greater in WT cultures. Growth rate of AK17 strain DM was considerably lower than that of AD and WT, even with the additional 24h incubation. Consumption of total glucose in the growth media between the strains was interesting in respect to OD600 and biomass weight, 71%, 89% and 100%, for WT, DM and AD, respectively. No correlation was evident between consumed glucose, cell biomass and OD600. A cell count of colony forming units (CFU) from the cultures was not performed as AK17 had previously been reported having low survival during plating and giving unreliable plate count results (Matís, unpublished). The rapid growth of the AD strain was not expected, and as it consumed all available glucose in the growth medium some results may not be strictly comparable to WT and DM cultures which had glucose remaining in growth medium. Therefore we conclude that the slight increase in acetate formation and the additional ATP it provides, enables the AD strain to achieve a growth rate similar to its original WT strain, while still retaining greater yield of mol-EtOH per mol-Glucose, as can be seen in Table 3.3.

We wanted to test for 1,2-propanediol as it is a valuable precursor in production of polymers and formation of 1,2-propanediol has been reported in the genus of *Thermoanaerobacterium* (Altras et al., 2001). AK17 can produce acetol which is a precursor to 1,2-propanediol (University of Akureyri, unpublished results).

The use of GAPDH as a reference enzyme was not done as the enzyme appears to be quite up-regulated in DM, while at a relatively similar concentration in WT and AD. It is possible that due to the DM strains inability to produce acetate as an end product, up-regulation of glycolytic enzymes occurs in order to increase ATP production.

4.2 Acetate formation restored with PTB and BK

Enzymatic assays with specific substrate for butanoate and acetate pathway enzymes, as shown in Figure 3.4, shows notable catalytic activity on the substrate butyryl-CoA in the AD strain in relation to strains DM and WT, thereby confirming the presence of PTB. This

presence of PTB and the observed increase in catalysis of acetyl-CoA in the AD strain compared to the DM strain, strongly indicates that PTB is able to catalyze acetyl-CoA. Similarly, the catalytic activity on butyryl-CoA in the WT strain, supports the conclusion that both enzymes are able to catalyze to some extent other carboxylates than their primary substrate. The next step in the pathway is the formation of acetate by AK and butyrate by BK. Results from the forward reaction, Figure 3.5, and the backward reaction, Figure 3.6, supports evidence of BK activity in the AD strain, negligible activity in the DM strain and slight activity in the WT strain. The AD strain also shows catalytic activity on acetyl phosphate and acetate, and supports strongly the original hypothesis that PTB and BK are able to facilitate in acetate production and subsequent gain of ATP, even though at a lower rate of turnover. Despite the suboptimal enzyme activity in acetate formation, the AD strain gains a rate of growth near to its WT strain predecessor.

4.3 Review of procedure

PCR on purified DNA and colony PCR gave conclusive results, with one exception, the positive control plasmid for *erm* resistance in Figure 3.1 failed, probably due to too high plasmid concentration, the band can be seen at the 4 kb mark. The assays performed well in detection of substrate specific enzymes. By addition of the forward reaction in the AK/BK assays we wanted to see if acylphosphatase could facilitate increased production of acetate or butyrate by hydrolysis of acetyl- and butanoyl phosphate. The substrate acetyl-CoA was highly unstable and degraded rapidly on ice, in a few hours nearly half of the substrate had been hydrolyzed. Butyryl-CoA was more stable and a stock solution could be kept at -20 °C for at least 4 weeks, whereas acetyl-CoA had become completely hydrolyzed. In all the enzymatic assays, except GAPDH assay, we wanted to minimize the error due to time as incubation was done at 55 °C and had to be done in an incubator. For this reason a relatively long time for enzyme assays was chosen, 20 min. When the AK-BK assays were done, care needed to be taken in looking for air bubbles as they formed spontaneously in the wells.

4.4 Next steps

To further develop AK17 as a proficient ethanol producer the next step would be to knock-out the butanoate pathway genes. However, a knock-out of only the PTB gene should be sufficient as no other PTB gene is predicted in the genome of AK17 and would be preferential as there is a predicted gene between PTB and BK, a leucine dehydrogenase, EC 1.4.1.9. These genes appear to be a part of the same operon according to a promoter region found using analysis by BPROM (Softberry), see Figure 0.1 (Appenix). Use of homologous recombination has proven a useful tool in transforming *Thermoanaerobacterium* (Shaw et al., 2010; Sigurðardóttir, 2009) and marker recycling method has been developed for the strain, enabling unlimited numbers of genetic modifications (Matís, unpublished). Whole genome sequencing will be performed in order to try and identify the mutation(s) required to activate the butanoate pathway in the AK17 AD strain for acetate production.

5 Conclusions

Activation of the butanoate pathway enzymes PTB and BK in the AK17 AD strain seems to have occurred during the extended cultivation. This study shows that these enzymes are able to facilitate production of acetate, but at a much lower rate of turnover than PTA and AK. Regardless of the low catalytic efficiency, the rate of growth for the AD strain is greatly increased in comparison to the DM strain, rivaling that of the original WT strain.

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References

Altaras, N. E., Etzel, M. R., & Cameron, D. C. (2001). Conversion of sugars to 1,2-propanediol by Thermoanaerobacterium thermosaccharolyticum HG-8. *Biotechnology Progress*, *17*(1), 52–56. http://doi.org/10.1021/bp000130b

Andersch, W., Bahl, H., & Gottschalk, G. (1983). Level of enzymes involved in acetate, butyrate, acetone and butanol formation by Clostridium acetobutylicum. *European Journal of Applied Microbiology and Biotechnology*, *18*(6), 327–332. http://doi.org/10.1007/BF00504740

Aresta, M., Dibenedetto, A., & Barberio, G. (2005). Utilization of macro-algae for enhanced CO2 fixation and biofuels production: Development of a computing software for an LCA study. *Fuel Processing Technology*, *86*(14–15), 1679–1693. http://doi.org/10.1016/j.fuproc.2005.01.016

Arnheidur Ran Almarsdottir, M. A. S. (2010). Ethanol and Hydrogen Production from Lignocellulosic Biomass by Thermophilic Bacteria.

Behera, S., Singh, R., Arora, R., Sharma, N. K., Shukla, M., & Kumar, S. (2015). Scope of Algae as Third Generation Biofuels. *Frontiers in Bioengineering and Biotechnology*, *2*. http://doi.org/10.3389/fbioe.2014.00090

Cary, J. W., Petersen, D. J., Papoutsakis, E. T., & Bennett, G. N. (1988). Cloning and expression of Clostridium acetobutylicum phosphotransbutyrylase and butyrate kinase genes in Escherichia coli. *Journal of Bacteriology*, *170*(10), 4613–4618.

Fowler, M. L., Ingram-Smith, C. J., & Smith, K. S. (2011). Direct Detection of the Acetate-forming Activity of the Enzyme Acetate Kinase. *Journal of Visualized Experiments: JoVE*, (58). http://doi.org/10.3791/3474

Koskinen, P. E. P., Beck, S. R., Örlygsson, J., & Puhakka, J. A. (2008). Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. *Biotechnology and Bioengineering*, *101*(4), 679–690. http://doi.org/10.1002/bit.21942

Kuit, W., Minton, N. P., López-Contreras, A. M., & Eggink, G. (2012). Disruption of the acetate kinase (ack) gene of Clostridium acetobutylicum results in delayed acetate production. *Applied Microbiology and Biotechnology*, *94*(3), 729–741. http://doi.org/10.1007/s00253-011-3848-4

Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P., & Bernard, O. (2009). Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environmental Science & Technology*, *43*(17), 6475–6481. http://doi.org/10.1021/es900705j

Maitra, A., & Dill, K. A. (2015). Bacterial growth laws reflect the evolutionary importance of energy efficiency. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(2), 406–411. http://doi.org/10.1073/pnas.1421138111

Maney Sveinsdottir, M. A. S. (2011). Ethanol and Hydrogen Production with Thermophilic Bactera from Sugars and Complex Biomass. http://doi.org/10.5772/17404

Martin, M. A. (2010). First generation biofuels compete. *New Biotechnology*, *27*(5), 596–608. http://doi.org/10.1016/j.nbt.2010.06.010

Rose IA (1955) Acetate kinase of bacteria (acetokinase). Methods Enzymol 1:591–595

Shaw, A. J., Hogsett, D. A., & Lynd, L. R. (2010). Natural Competence in Thermoanaerobacter and Thermoanaerobacterium Species. *Applied and Environmental Microbiology*, *76*(14), 4713–4719. http://doi.org/

Shaw, A. J., Podkaminer, K. K., Desai, S. G., Bardsley, J. S., Rogers, S. R., Thorne, P. G., … Lynd, L. R. (2008). Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proceedings of the National Academy of Sciences*, *105*(37), 13769–13774. http://doi.org/10.1073/pnas.0801266105 10.1128/AEM.

Sigurðardóttir, S. H. (2009). Bioethanol: production of ethanol with anaerobic thermophilc mutant strains. Retrieved from http://skemman.is/handle/1946/3106

Sveinsdóttir, M., Baldursson, S. R. B., & Örlygsson, J. (2009). Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. Retrieved from http://skemman.is/item/view/1946/19950

Appendix

Sequence of the predicted PTB gene and promoter sequence, also upstream and downstream flanking sequences.

(Ribose ABC transport system, periplasmic ribose-binding protein)...GTGGTAGGATTCGATGGTACTGCTGATGGGTTGAATGCGATAAAAAA
CGGACAAATGACGGCCACAATAGCACAACAGCCAGAATTGATTGGTACACTTG
GCGTTGATACAGCAGATAAATATTTGAAAGGTGAAAAAGTAGAAAGAAAAAT
ACCTGCAGAAATAAAATTAGTAGAAAAAAAATAAAATAAAATTTGATCAATGT
TTTAAGATTAATAAAAAAAGAAAGGTTCTATGTTAGAGCTACAAACTAGCATA
TCTTGGGCGAAAAAGATATGCTAGTTTTTTACTGTTAAAGTATATTATAAAATA
AATATGATCATTATGCAATAGAACATTAAACATCTGAATTTTAATTTTGCTCAT
GAAGTGATAGAATTTTAACATATATGTAAATTAAAACTAATAATAAGCAAGTC
TATTAATCCCATTTTTGCATTATAAAGGTTAAAAAAATTATAAAACTAAGAAG
GAATTTTAAAATAAATGGAGAATATATTAAATATAAATTGTAAGCATTAAAAA
ATTTTAAGTAATAAACTTTTATAAAATAATAATTTTTCTCACTATTTCAAAATA
CAAGACAAGAATATGGAAGGTGCTTTCGGTGAATAGTTTTAAAGAAATGTATG
AGTTAGTTAAAGATTTGAATACTAAGATCGTGGCTGTTGCACAAGCGGCTGAT
GTCGATGTGCTTTTAGCTGTGAAAGATGCTTACGAAAAAGGAATAATAAAAGC
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ATCCAAGAAAGGATAAAAGTGAAGCATTATTTAGAAGCTTAGGCAGGTTTATA
GAGGGGCTTAATGGAAGGTATATAACCGCCGAAGATGTTGGGACAAATATGA
AAGACATGGATTATATAAGCATGGAAACAAACTATGTTGCTGGACTTGCTGAG
AAAAGTGGTGATCCATCTCCTTTTACAGCATATGGGGTGTTTAGAGGT...(leucine dehydrogenase EC 1.4.1.9)...(butyrate kinase EC 2.7.2.7)...(2-oxoglutarate oxidoreductase EC 1.2.7.3)

Between genes (421 nt), promoter prediction:

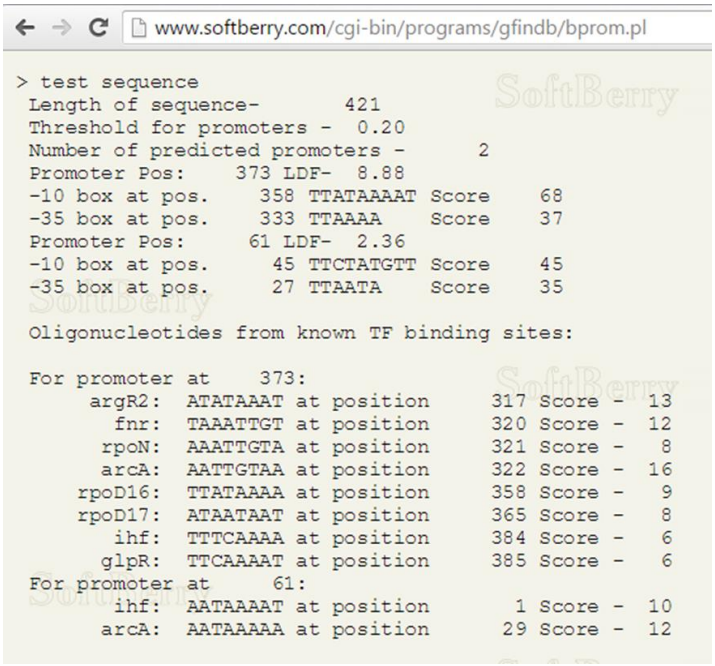

Figure 0.1 Promoter sqeuence prediction upstream of the PTB gene.