Regulation of lactate metabolism in the acetogenic bacterium *Acetobacterium woodii*

Marie Charlotte Schoelmerich[1,†], Alexander Katsyv[1], Woung Sung[1], Vanessa Mijic[1], Anja Wiechmann[1], Patrick Kottenhahn[1] Jonathan Baker[2], Nigel Peter Minton[2] and Volker Müller[1]*

[1]*Molecular Microbiology & Bioenergetics, Goethe University Frankfurt, 60438 Frankfurt, Germany*

[2] *Clostridia Research Group, BBSRC/EPSRC Synthetic Biology Research Centre (SBRC), University of Nottingham, Nottingham, UK*

[†]*Present address: Department of Molecular Microbiology & Bioenergetics, University of Hamburg, 22609 Hamburg, Germany*

**Corresponding author.*

Volker Müller,

Molecular Microbiology & Bioenergetics,

Institute of Molecular Biosciences,

Goethe University Frankfurt,

Max-von-Laue-Str. 9,

60438 Frankfurt, Germany

Phone: +49-69-79829507

Fax: +49-69-79829306

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.14412

E-mail: vmueller@bio.uni-frankfurt.de

Running title: Metabolic regulation in anaerobic microorganisms

Originality-Significance Statement

This work describes the molecular regulatory basis of substrate utilization in an ecologically important group of anaerobic bacteria, the acetogens.

Summary

Acetogenic bacteria compete in an energy-limited environment by coupling different metabolic routes to their central metabolism of CO_2 fixation. The underlying regulatory mechanisms are often still not understood. In this work, we analysed how lactate metabolism is regulated in the model acetogen *Acetobacterium woodii*. Construction of a Δ*lctCDEF* mutant and growth analyses demonstrated that the genes are essential for growth on lactate. Subsequent bridging PCR and quantitative PCR analyses revealed that the *lctBCDEF* genes form an operon that was expressed only during lactate metabolism. The *lctA* gene was cloned, expressed in *Escherichia coli* and purified. LctA bound to the intergenic DNA region between *lctA* and the *lct* operon in electromobility shift assays and binding was revoked in the presence of lactate. Further restriction site protection analyses consolidated the lactate-dependent binding of LctA and identified the binding site within the DNA. Cells grew mixotrophically on lactate and another energy source and showed no diauxic growth. From these data we conclude that the

catabolic lactate metabolism is encoded by the *lct* operon and its expression is negatively regulated by the DNA binding repressor LctA.

Introduction

Acetogenic bacteria are a specialized group of strictly anaerobic bacteria that grow by the oxidation of a number of different substrates such as sugars, acids, alcohols or molecular hydrogen (Drake et al., 2008). Oxidation of these substrates is coupled to the reduction of CO_2 as electron acceptor yielding acetate. While laboratory cultivation usually is conducted with a single substrate, in nature, these organisms encounter multiple substrates and mixotrophic growth has been reported (Braun and Gottschalk, 1981; Breznak and Blum, 1991; Liu and Suflita, 1995; Peters et al., 1998). CO_2 is the common electron acceptor and reduced to acetate in the Wood-Ljungdahl pathway (WLP). In this pathway, 2 mol CO_2 are reduced *via* two branches to 1 mol acetate using eight electrons (Wood and Ljungdahl, 1991; Drake et al., 2008). Since there is no net ATP gain from substrate-level phosphorylation in the WLP, ATP must be formed by a chemiosmotic mechanism under lithotrophic conditions (Schuchmann and Müller, 2014). The chemiosmotic gradient is established by a Fd^{2-}:NAD^+ oxidoreductase (Rnf complex), that couples the exergonic electron transfer to the translocation of Na^+ across the cytoplasmic membrane (Biegel and Müller, 2010; Hess et al., 2013). A Na^+-dependent F_1F_O ATP synthase then uses this gradient to phosphorylate ADP to ATP (Fritz et al., 2008).

The WLP in *A. woodii* requires 1 mol H_2 or reduced ferredoxin (Fd^{2-}), 2 mol NADH and 1 mol Fd^{2-}. The electrons gained from substrate oxidation are converted to the required reductants by two adaptor modules, the membrane bound Rnf complex (a ferredoxin:NADH transhydrogenase) and the electron bifurcating hydrogenase. Using these two adaptor

modules, acetogens such as *A. woodii* gain access to different substrates independent of the electron acceptor used for the initial oxidative reaction (Schuchmann and Müller, 2016). Lactate is a very good carbon and energy source for the acetogen *A. woodii* (Weghoff et al., 2015). Lactate is oxidized to pyruvate by the electron confurcating lactate dehydrogenase, that requires reduced ferredoxin as co-reductant, yielding 2 mol NADH. Pyruvate is then oxidized to acetyl-CoA, reduced ferredoxin and CO_2. A redox-balanced model of acetogenesis from lactate is presented in Fig. 1. First *in silico* analyses suggested that the LDH/Etf complex is encoded by three adjacent genes on the genome. Upstream is a gene in reverse orientation, encoding a putative transcriptional regulator. Downstream are two genes that encode a lactate permease and racemase. These six genes were termed *lctABCDEF* (Weghoff et al., 2015).

Since nothing is known about regulation of catabolic genes in acetogens, we chose the lactate metabolism in *A. woodii* as a model system. The aim of this study was to elucidate the physiological role of the *lct* genes and their regulation.

Results

Construction and characterization of a ΔlctCDEF mutant

To address the physiological role of the *lct* genes of *A. woodii*, a markerless deletion mutant of the genes *lctCDEF* was generated. To this end the genes *Awo_c08720* (*lctC*) coding for the electron transferring subunit of the lactate dehydrogenase (EtfA), *Awo_c08730* (*lctD*) coding for the major subunit of the lactate dehydrogenase, *Awo_c08740* (*lctE*) coding for a potential L-

lactate permease (LctP) and *Awo_c08750* (*lctF*) coding for a potential lactate racemase were deleted from the chromosome of *A. woodii.* The mutagenesis was performed in a *pyrE* deletion mutant (Westphal et al., 2018) by double homologous recombination using a suicide plasmid (pMTL-JPB24, plasmid sequence S1) containing homology arms with the upstream and downstream region of the *lctCDEF* genes leaving 6 bp behind the start codon of *lctC* and 3 bp in front of the stop codon of *lctF*. After homologous recombination, the strain was tested for plasmid loss through thiamphenicol sensitivity and the 5565 bp deletion was confirmed by a flanking polymerase chain reaction (PCR) using primers AW_lct_FlankF and AW_lct_FlankR (Table S1) followed by Sanger sequencing.

The generated deletion mutant *A. woodii ΔlctCDEF* was tested for growth on different substrates. The mutant grew on 20 mM fructose, 50 mM ethanol, 60 mM methanol, 15 mM 1,2-propanediol, 50 mM glycine betaine, 100 mM pyruvate, 100 mM formate or 2.0×10^5 Pa H_2+CO_2 (80:20 [v/v]) with a growth rate and final yield indistinguishable from the wild type. However, the mutant did not grow on lactate, providing the genetic proof that the *lct* genes are essential for growth on lactate and apparantly encode the enzyme essential for growth on lactate in *A. woodii*.

The metabolic lactate module is confined to one highly regulated operon

To establish that the *lctBCDEF* genes form an operon, RNA was isolated from cells grown on DL-lactate and complementary DNA (cDNA) was synthesized. Bridging PCR analyses revealed that all five genes (Fig. 2A) indeed make up one transcriptional unit (Fig. 2B). Subsequently, we analysed whether the expression level of the *lct* genes changes, when

A. woodii is cultivated on different growth substrates. Thus, cDNA was prepared using RNA isolated from cultures grown on lactate, fructose, methanol, H_2+CO_2 or ethylene glycol. The relative transcript level of the *lct* operon obtained from quantitative PCR (qPCR) analyses was by far the highest in lactate-grown cells during the exponential growth phase. The relative expression was downregulated in cells grown on H_2+CO_2, fructose, methanol and on ethylene glycol (Fig. 2C). In line with this, when screening crude extracts prepared from cultures grown on H_2+CO_2, fructose, methanol or ethylene glycol for LDH-specific lactate:ferricyanide oxidoreductase activity as described previously (Weghoff et al., 2015), only protein extract prepared from lactate-pregrown cells exhibited this activity. Crude extracts prepared from cells grown until the exponential or stationary growth phase exhibited a lactate:ferricyanide oxidoreductase activity of 166 or 27 mU/mg. This activity drop in cells from the stationary compared to the exponential growth phase was also reflected by a log2 fold change of -0.8 for *lct* transcript. Thus, the results clearly show that the metabolic lactate module is highly regulated and only expressed when *A. woodii* is grown on lactate.

LctA controls the metabolic lactate module

The gene preceding the *lct* operon is inversely orientated and first *in silico* analyses indicated that it might encode a transcriptional regulator protein (Weghoff et al., 2015). To substantiate this, *lctA* was first cloned in *E. coli* DH5α together with a His_6-tag (at the N- or at the C-terminus), subsequently overexpressed in *E. coli* BL21(DE3) and finally purified *via* Ni^{2+}-NTA affinity chromatography (Fig. S1). Furthermore, the entire intergenic DNA region between *lctA* and *lctB* or fragements of this region were amplified *via* PCR (Fig. 3A). Next, we assessed

whether LctA binds to the DNA fragments *via* an electromobility shift assay (shift assay). The principle of this assay is that if protein binds to the DNA, the DNA-protein complex will migrate less in a native gel than the DNA without protein bound. And indeed, preincubation of either version of purified LctA (LctA-His_6 in Fig. 3B or His_6-LctA in Fig. 3C; 9.8 µM) with DNA (50 nM), resulted in a shift in the assays containing the entire intergenic DNA fragment. In assays containing shorter versions of the template DNA, a shift was encountered only with LctA-His_6 and fragments 2 and 4. These results show that the N-terminally located tag interferes with DNA binding. Subsquent analyses were carried out using the C-terminally tagged purified LctA (LctA-His_6). Furthermore, the results showed that LctA binds to the intergenic region of 100-178 bp (Fig. 3B). Since control assays where LctA was omitted or substituted with BSA never resulted in a shift, the binding of LctA to the intergenic DNA was specific.

Next, we analysed whether potential effector molecules (D-lactate, L-lactate, pyruvate, alanine) influence the binding of LctA to the DNA. And indeed, assays containing protein-DNA probe in the bound state (0.45 µM LctA-His_6 and 7.5 nM DNA fragment 1), showed a reversal of the binding when preincubated with lactate in either stereoisomeric form, but not with alanine or pyruvate (Fig. 3D). These data indicate, that LctA acts as a transcriptional repressor of the *lct* operon, which decreases its affinity to the DNA in response to elevated levels of the potential substrate lactate.

To elucidate the binding motif within the DNA fragment, restriction site protection analyses (RSPA) were performed. If LctA bound to the DNA covers a restriction site, a subsequent digest using the specific restriction endonuclase will result in an intact DNA fragment, as opposed to a digested fragment in a control without the repressor. Based on the band shift results, a closer examination of the intergenic region between 100-178 bp revealed a palindromic sequence (TGGT(CTG)ACCA) that could be the LctA binding site. This

sequence is cut by the commercially available endonuclease Hpy188I. Hence, we analysed the fragment pattern of RSPA assays containing the DNA fragment (covering the intergenic region and parts of *lctB* to aid visualization due to larger fragments) incubated with or without LctA-His_6, Hpy188I and lactate. The PCR product alone contained one fragment at 645 bp (Fig. 4 a) and preincubation with Hpy188I led to the anticipated fragmentation of this product into two fragments at 425 and 220 bp (Fig. 4 b). The assay containing DNA and LctA-His_6 resulted in one fragment only, although the assay was subjected to a Hpy188I digest (Fig. 4 c). Thus, LctA protects the binding site within the DNA. Interestingly, this protection was relieved in the presence of lactate. Samples preincubated in the presence of L-lactate (50, 100 or 200 mM) still facilitated a partial protection (Fig. 4 d-f), but D-lactate abolished the protection entirely, already at the lowest concentration tested (50 mM; Fig. 4 g-i). Therefore, the RSPA assays substantiate that LctA is released from its binding site TGGT(CTG)ACCA upstream of the *lct* operon in response to (D-)lactate.

Mixotrophic growth of A. woodii on lactate

The before mentioned data demonstrate that the expression of the *lct* genes in *A. woodii* is negatively regulated. To analyze a potential second, positive regulatory mechanism we examined growth of *A. woodii* on lactate plus a second carbon and energy source, but for none of the conditions tested, diauxic growth was observed. The optical density reached by a culture grown on lactate and fructose (Fig. 5C) was the sum of the densities observed with the single substrates (Fig. 5A, B). Acetate production was also not diauxic and the level of acetate produced was nearly the sum of the level obtained with either substrate. Lactate and fructose

were consumed simultaneously (Fig. 5C). Diauxic growth was also not observed for lactate and methanol (data not shown).

Discussion

This work revealed that metabolic flexibility in acetogenic bacteria is inducible in response to environmental availability of the metabolite. The mutant studies, qPCR and enzyme activity analyses clearly demonstrated that the metabolic lactate module is required, expressed and produced only for growth on lactate, but repressed when lactate is unavailable. The *lct* operon was tightly downregulated by the LctA repressor, which was revoked once lactate became available. The observation that the binding of LctA to the DNA was more responsive to the D-stereoisomer of lactate matches the reported specificity of the LDH/Etf complex to D-lactate (Weghoff et al., 2015).

The elucidated regulatory mechanism behind lactate metabolization in *A. woodii* is reminiscent of the regulation of lactate utilization in the well-studied model organisms *E. coli*, *Corynebacterium glutamicum* and *Pseudomonas aeruginosa* (Jiang et al., 2014). These three organisms employ an LldR repressor protein that similarly silences gene expression of lactate utilization genes in the absence of lactate. The three LldR proteins and LctA from *A. woodii* belong to the FadR subfamily of the GntR transcription factor family. As typical for these regulators, LctA probably forms a dimer and *in silico* analyses indicate the presence of two binding motifs. Located at the N-terminus is a 65 amino acid long helix-turn-helix motif that facilitates DNA binding. Located at the C-terminus is the ligand-binding site comprising 124 amino acids. Structural modelling (Waterhouse et al., 2018) of LctA using the solved crystal

structure of LldR from *C. glutamicum* (Gao et al., 2008) unfortunately did not lead to a suitable model for LctA. This is probably because LctA of *A. woodii* only shares sequence identities between 25-29% with the three different LldR proteins. However, the binding site we identified for LctA (TGGT(CTG)ACCA) is identical to the conserved amino acids in the binding motif of LldR in *C. glutamicum* and *P. aeruginosa* (Gao et al., 2008; Gao et al., 2012).

Growth on lactate is less favourable than growth on fructose, because 2.15 or 0.27-0.37 mol ATP are theoretically produced per mol acetate when *A. woodii* grows on fructose or lactate, respectively (Schuchmann and Müller, 2016). This could lead to a sequential utilization of fructose and then lactate, leading to diauxic growth. Since we did not encounter biphasic growth, there seems to be no second, positive regulatory mechanism, as described for the *lac* operon in *E. coli*, for example (Loomis and Magasanik, 1967). The simultaneous utilization of two (or more) substrates reflects the necessity of acetogens to immediately exploit the traces of energy sources available in an energy-limited environment. The mixotrophic growth could be the reason why acetogens can compete with methanogenic archaea and sulfate reducing microorganisms despite their position at the bottom of the trophic chain. Mixotrophic behaviour of acetogens is also of substantial advantage for industrial applications, as it enables the simultaneous influx of electrons from different industrial feedstocks.

The elucidation of the regulatory mechanism controlling the catabolic lactate module marks a very valuable asset for expanding the genetic toolbox for synthetic biology approaches, as it depicts an inducible promoter-repressor system (= regulatory Lct system). This is of particular interest, since acetogens have emerged as industrial production platforms to produce organic building blocks in an eco-friendly way (Bertsch and Müller, 2015; Liew et

al., 2016). A lot of advances have been made throughout the past years to improve the employed strains *via* genetic engineering. However, the applied expression systems often rely on the use of constitutive promotors such as the thiolase or acetate kinase promoter (Schiel-Bengelsdorf and Dürre, 2012). The regulatory Lct system could be used in *A. woodii* itself, or the entire gene segment encoded by *lctA* and the subsequent intergenic region could be introduced into other related Clostridia, to facilitate a tightly regulated lactate-inducible expression system.

Experimental Procedures

Growth of A. woodii

Acetobacterium woodii (DSM 1030) was cultivated at 30°C under anaerobic conditions in complex medium that was prepared using the anaerobic techniques described previously (Hungate, 1969; Bryant, 1972). The components of the complex medium are listed in (Heise et al., 1992). A similar medium was used for genetic manipulations, however yeast extract was omitted and higher amounts of KH_2PO_4 (0.2 g/l), NH_4Cl (1.35 g/l) and selenite-tungsten solution (1.5 ml/l) were used and 10 µg/ml of D/L-panthothenic acid was added. If necessary, 50 µg/mL uracil was added. For RNA isolation, cells were cultivated in 120-ml-serum bottles (Glasgerätebau Ochs, Bovenden/Lenglern, Germany) containing 50 or 20 ml complex medium with 80 mM DL-lactate, 20 mM fructose, 60 mM methanol, 50 mM ethylene glycol or 2.0×10^5 Pa H_2+CO_2 (80:20 [v/v]) until the mid-exponential growth phase. When mixotrophic growth was analysed, *A. woodii* was cultivated in 50 ml complex medium containing substrate-limiting

concentrations of 40 mM DL-lactate or 2.5 mM fructose. 1-ml samples were withdrawn to measure the OD_{600} and subsequently, samples were centrifuged (12 000 × g, 1 min, room temperature (RT)) and the supernatant was stored at -20°C for metabolite analyses *via* high pressure liquid chromatography (HPLC) or gas chromatography (GC).

Construction of the lctCDEF *deletion mutant*

The suicide plasmid (pMTL-JPB24, Plasmid sequence S1) for the in-frame deletion of the *lctCDEF* genes without deletion of *lctB* in *A. woodii* was constructed in *E. coli* HB101 (Promega, Madison, USA). Plasmid pMTL-JPB24 is an allelic exchange KO vector which lacks a Gram-positive replicon, but carries the *Clostridium perfringens catP* marker (specifying resistance to thiamphenicol) and a heterologous *pyrE* (cac_0027) from *Clostridium acetobutylicum* ATCC 824 that is used as a counter selectable marker (Westphal et al., 2018). For construction of the deletion cassette, homology arms (HA) were designed to flank the *A. woodii lctC* and *lctF* genes of 997 bp (Left HA) (NC_016894.1, 1004845-1005841) and 982 bp (Right HA) (NC_016894.1, 1011407-1012388), respectively, thus retaining the three starting codons of *lctC* and two end codons of *lctE* without affecting the 5`-untranslated region (UTR) and 3`-UTR. The two PCR products were assembled as a single DNA fragment using splice-by-overlap-extension PCR (SOE-PCR) and the oligonucleotides listed in Table S1, and cloned, following cleavage with the restriction enzymes NotI and NheI, into the equivalent sites for creation of pMTL-JPB24. pMTL-JPB24 was then transformed into the *A. woodii pyrE* (NC_016894.1, *Awo_c16210*) deletion mutant as previously described (Leang et al., 2013; Westphal et al., 2018) and was plated onto minimal agar media supplemented with 1 µg/mL

uracil and 35 µg/mL thiamphenicol. Thiamphenicol resistant colonies, representing single crossover integrants, were restreaked onto agar minimal media containing 1 µg/mL uracil and 1 mg/mL 5-fluoroorotic acid (5-FOA) agar plates to select for 5-FOA^R cells in which the plasmid had excised and been lost from the population. The resulting colonies were screened with flanking oligonucleotides listed in Table S1 followed by Sanger sequencing of the entire cloned region to distinguish wild type revertants from the desired mutants containing the 5565 bp *lctCDEF* deletion.

RNA isolation and cDNA synthesis, bridging PCR and qPCR analyses

To analyze whether the *lctBCDEF* genes form one transcriptional unit and to quantify gene expression, *A. woodii* was grown in complex medium on lactate (or fructose, methanol or H_2+CO_2) and harvested in the mid-exponential growth phase by centrifugation (10 000 × g, 10 min, 4°C), frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was isolated using the InviTrap Spin Cell RNA Mini kit (Stratec Molecular GmbH, Berlin, Germany) with some modifications. After lysozyme treatment, the lysis solution was added to the cells where they were fully lysed in a cell disrupter (Retsch GmbH, Haan, Germany) at 30 Hz for 5 min. The cell debris was separated by centrifugation (14 000 × g, 10 min, 4°C) and the supernatant was used as described in the kit protocol. 10 µg nucleic acids were used for DNaseI treatment (Promega, Madison, USA) in the presence of Rnasin (Promega, Madison, USA). The nucleic acids were precipitated using 2.5 volumes 100% ethanol and 1/10 volume 3 M sodium acetate (pH 6.5). Subsequently, the sample was incubated at - 20°C for 1 h and centrifuged (11 000 × g, 30 min, 4°C). The precipitate was washed in 500 µl ice-cold 70%

ethanol and centrifuged (11 000 × g, 30 min, 4°C). The supernatant was discarded and the pellet was air-dried and then dissolved in 20 µl RNase-free MQ water. The RNA was checked using an agarose gel (1% [w/v] in 1×TBE buffer containing 89 mM Tris-HCl, 89 mM boric acid and 1 mM EDTA). Afterwards, 1 µg RNA was used for reverse transcription using M-MLV Reverse Transcriptase according to the manufacturer's protocol (Promega, Madison, USA). For bridging PCR analyses, 100 ng synthesized cDNA, chromosomal DNA or DNase-digested RNA served as template for a PCR containing primer pairs *b_lctB_for* and *b_lctC_rev*, *b_lctC_for* and *b_lctD_rev*, *b_lctD_for* and *b_lctE_rev* or *b_lctE_for* and *b_lctF_rev* (Table S1). All quantitative PCRs were performed in triplicate, using SYBR Green qPCR Kits (2x without ROX) (Thermo Fisher Scientific, Waltham, USA). Expression of the the *lct* operon was analysed using primers raised against *lctD, q_lctD_for* and *q_lctD_rev* and the gyraseA as housekeeping gene, *q_gyrA_for* and *q_gyrA_rev* (Table S1). The relative expression was calculated according to (Livak and Schmittgen, 2001) and a subsequent log2 fold change to visualize downregulation. Primers used are listed in the Table S1. The relative mRNA levels were calculated from two biological independent experiments.

Cloning, expression and purification of LctA from A. woodii *in* E. coli

The *lctA* gene was amplified from chromosomal DNA of *A. woodii* by PCR with primers *HislctA*_for and *HislctA_rev* or *lctAHis*_for and *lctAHis_rev* (Table S1). The PCR product was cloned into the expression vector pET-28a(+). pET-28a(+) was used to transform *E. coli* BL21(DE3), and the resulting strain was grown at 37°C in LB medium to an optical density at 600 nm of 0.9. The expression of *lctA* was induced by the addition of 1 mM isopropyl-D-

thiogalactopyranoside (IPTG). The culture was shaken for 3 h at 37°C. Cells of *E. coli* were harvested and washed once in buffer A (50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 10 mM Imidazole). After centrifugation, the cells were resuspended in buffer A with 0.5 mM PMSF and 0.1 mg/ml DNAseI and passed twice through a French pressure cell (110 MPa). Cell debris was removed by centrifugation at 24 000 × g for 20 min. Afterwards, membranes were removed by centrifugation at 200 000 × g for 30 min. The supernatant containing the cytoplasmic fraction was applied on Ni-nitrilotriacetic acid agarose (Ni-NTA column) (Macherey-Nagel, Düren, Germany) equilibrated with buffer A. The Ni-NTA column was washed twice by adding 10 bed volumes of buffer W (50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 20 mM imidazole). Afterwards, the protein was eluted five times in 2 ml fractions with buffer EI (50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 150 mM imidazole) and 5 times with buffer EII (50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 800 mM imidazole). The fractions were stored at 4°C. After the purification the concentration of the purified protein was measured according to Bradford (Bradford, 1976). To verify the purification, samples were separated in a 12% denaturing polyacrylamide gel (Laemmli, 1970) and stained with Coomassie Brilliant Blue G250.

Electromobility shift assays

DNA fragments (1-5) covering the *lct* promoter region were obtained by amplifying regions between *lctA* and the *lct* operon *via* PCR, using chromosomal DNA of *A. woodii* as template and the following primer pairs: *lctA1_for* and *lctA1_rev* (fragment 1), *lctA2_for* and *lctA2*_rev (fragment 2), *lctA3_for* and *lctA1*_rev (fragment 3), *lctA4_for* and *lctA2_rev* (fragment 4) or

lctA5_for and *lctA1_rev* (fragment 5) (Table S1). Shift assays were prepared in an overall reaction volume of 12 µl reaction mixture (10 mM NaH_2PO_4 (pH 7.5) and 1 M NaCl) and incubated for 25 min at RT. LctA-His_6 or His_6-LctA was incubated with purified DNA fragments 1-5 at concentrations of 9.8 µM protein and 50 nM DNA. For effector screening studies, 0.45 µM LctA-His_6 was incubated with 7.5 nM DNA fragment 1 in the presence of D-/L-lactate, pyruvate or alanine at a final concentration of 200 mM. In control assays, protein was omitted (-). After adding loading buffer (10 mM NaH_2PO_4 (pH 7.5), 10% [v/v] glycerol, bromphenol blue and xylene cyanol) the samples were loaded onto a 5% native polyacrylamide gel (Toyoda et al., 2009) or 10% native polyacrylamide gel for effector studies (Carey, 1988). Electrophoresis was performed at 150 V in 1×TBE at RT. The gel was stained with ethidium bromide and DNA was visualized on a ultraviolet (UV) transilluminator (Intas Science Imaging, Göttingen, Germany).

Restriction site protection analyses

For RSPA assays, a 645 bp long DNA fragment covering the intergenic region and part of the first gene of the cluster (*lctB*, to aid visualization of fragmentation) was amplified *via* PCR using the primer pair *lctA*1_for and *lctA*3_*rev* (Table S1) and chromosomal DNA of *A. woodii* as template. Purified LctA-His_6 (0.2 µg) was incubated with purified PCR product (20 ng) in RSPA buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 50 mM $MgCl_2$ and 0.5 mg/ml of bovine serum albumin) at a final volume of 20 µl. For effector screening studies, D-/L-lactate was added at a final concentration of 50, 100 or 200 mM. The mixture was first incubated for 25 min at RT, then supplemented with Hpy188I (0.5 U; New England Biolabs, Ipswich, USA) and further

incubated for 30 min at 37°C. The resulting DNA-protein complexes were loaded onto a 1.5% agarose gel containing ethidium bromide (0.01% [v/v]). Electrophoresis was performed at 120 V in 1×TAE (40 mM Tris-HCl (pH 8), 20 mM acetic acid and 1 mM EDTA) at RT. DNA was visualized on a UV transilluminator (Intas Science Imaging, Göttingen. Germany).

Analytical Methods

The metabolites acetate and lactate were quantified *via* HPLC on an Acclaim organic acid column (5 µm, 4.0 × 250 mm; Thermo Fisher Scientific, Waltham, USA) coupled to a photodiode array detector (PDA-100) (Dionex, Idstein, Germany). 300 µl samples were acidified with 3 µl 20% H_2SO_4, centrifuged (12 000 × g, 15 min, RT) and the supernatant was filtered (Millex-LH PTFE membrane filter 0.45 µm, 4 mm; Merck KGaA, Darmstadt, Germany). 10 µl samples were loaded onto the column and separated using 0.1 M Na_2SO_4 (pH 2.65) as solvent at a flow rate of 0.6 ml/min and a constant oven temperature of 30°C. Lactate and acetate concentrations were detected at 210 nm. Fructose was quantified from centrifuged (12 000 × g, 15 min, RT) and filtered samples separated on a HyperREZ XP Carbohydrate Ca^{2+} column (8 µm, 7.7 × 300 mm; Thermo Fisher Scientific, Waltham, USA) using MQ-H_2O as solvent at a flow rate of 0.6 ml/min and a constant oven temperature at 80°C. Fructose was detected with a refractory index (RI) detector at 55°C. Acetate concentrations were additionally verified *via* GC-flame ionization detector (FID) as described previously (Weghoff and Müller, 2016). This protocol also detects alcohols, but they were not detected in our samples.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. The contribution of Anna Knieper in the early stage of experiments is gratefully acknowledged.

References

Bertsch, J., and Müller, V. (2015) Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria. *Biotechnol Biofuels* **8**: 1-12.

Biegel, E., and Müller, V. (2010) Bacterial Na^+-translocating ferredoxin:NAD^+ oxidoreductase. *Proc Natl Acad Sci USA* **107**: 18138-18142.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of proteine-dye-binding. *Anal Biochem* **72**: 248-254.

Braun, K., and Gottschalk, G. (1981) Effect of molecular hydrogen and carbon dioxide on chemo-organotrophic growth of *Acetobacterium woodii* and *Clostridium aceticum*. *Arch Microbiol* **128**: 294-298.

Breznak, J.A., and Blum, J.S. (1991) Mixotrophy in the termite gut acetogen, *Sporomusa termitida*. *Arch Microbiol* **156**: 105-110.

Bryant, M.P. (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr* **25**: 1324-1328.

Carey, J. (1988) Gel retardation at low pH resolves *trp* repressor-DNA complexes for quantitative study. *Proc Natl Acad Sci U S A* **85**: 975-979.

Drake, H.L., Gößner, A.S., and Daniel, S.L. (2008) Old acetogens, new light. *Ann N Y Acad Sci* **1125**: 100-128.

Fritz, M., Klyszejko, A.L., Morgner, N., Vonck, J., Brutschy, B., Müller, D.J. et al. (2008) An intermediate step in the evolution of ATPases: a hybrid F_1F_O rotor in a bacterial Na^+ F_1F_O ATP synthase. *FEBS J* **275**: 1999-2007.

Gao, C., Hu, C., Zheng, Z., Ma, C., Jiang, T., Dou, P. et al. (2012) Lactate utilization is regulated by the FadR-type regulator LldR in *Pseudomonas aeruginosa*. *J Bacteriol* **194**: 2687-2692.

Gao, Y.G., Suzuki, H., Itou, H., Zhou, Y., Tanaka, Y., Wachi, M. et al. (2008) Structural and functional characterization of the LldR from *Corynebacterium glutamicum*: a transcriptional repressor involved in L-lactate and sugar utilization. *Nucleic Acids Res* **36**: 7110-7123.

Heise, R., Müller, V., and Gottschalk, G. (1992) Presence of a sodium-translocating ATPase in membrane vesicles of the homoacetogenic bacterium *Acetobacterium woodii*. *Eur J Biochem* **206**: 553-557.

Hess, V., Schuchmann, K., and Müller, V. (2013) The ferredoxin:NAD^+ oxidoreductase (Rnf) from the acetogen *Acetobacterium woodii* requires Na^+ and is reversibly coupled to the membrane potential. *J Biol Chem* **288**: 31496-31502.

Hungate, R.E. (1969) A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology*. Norris, J.R., and Ribbons, D.W. (eds). New York and London.: Academic Press, pp. 117-132.

Jiang, T., Gao, C., Ma, C., and Xu, P. (2014) Microbial lactate utilization: enzymes, pathogenesis, and regulation. *Trends Microbiol* **22**: 589-599.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Leang, C., Ueki, T., Nevin, K.P., and Lovley, D.R. (2013) A genetic system for *Clostridium ljungdahlii*: a chassis for autotrophic production of biocommodities and a model homoacetogen. *Appl Environ Microbiol* **79**: 1102-1109.

Liew, F., Martin, M.E., Tappel, R.C., Heijstra, B.D., Mihalcea, C., and Köpke, M. (2016) Gas fermentation-a flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. *Front Microbiol* **7**: 694.

Liu, S., and Suflita, J.M. (1995) H_2 as an energy source for mixotrophic acetogenesis from the reduction of CO_2 and syringate by *Acetobacterium woodii* and *Eubacterium limosum*. *Curr Microbiol* **31**: 245-250.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**: 402-408.

Loomis, W.F., Jr., and Magasanik, B. (1967) Glucose-lactose diauxie in *Escherichia coli*. *J Bacteriol* **93**: 1397-1401.

Peters, V., Janssen, P.H., and Conrad, R. (1998) Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. *FEMS Microbiol Ecol* **26**: 317-324.

Schiel-Bengelsdorf, B., and Dürre, P. (2012) Pathway engineering and synthetic biology using acetogens. *FEBS Lett* **586**: 2191-2198.

Schuchmann, K., and Müller, V. (2014) Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nat Rev Microbiol* **12**: 809-821.

Schuchmann, K., and Müller, V. (2016) Energetics and application of heterotrophy in acetogenic bacteria. *Appl Environ Microbiol* **82**: 4056-4069.

Toyoda, K., Teramoto, H., Inui, M., and Yukawa, H. (2009) The *ldhA* gene, encoding fermentative L-lactate dehydrogenase of *Corynebacterium glutamicum*, is under the control of positive feedback regulation mediated by LldR. *J Bacteriol* **191**: 4251-4258.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R. et al. (2018) SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* **46**: W296-W303.

Weghoff, M.C., and Müller, V. (2016) CO metabolism in the thermophilic acetogen *Thermoanaerobacter kivui*. *Appl Environ Microbiol* **82**: 2312-2319.

Weghoff, M.C., Bertsch, J., and Müller, V. (2015) A novel mode of lactate metabolism in strictly anaerobic bacteria. *Environmental Microbiology* **17**: 670-677.

Westphal, L., Wiechmann, A., Baker, J., Minton, N.P., and Müller, V. (2018) The Rnf complex is an energy coupled transhydrogenase essential to reversibly link cellular NADH and ferredoxin pools in the acetogen *Acetobacterium woodii*. *J Bacteriol* in press.

Wood, H.G., and Ljungdahl, L.G. (1991) Autotrophic character of the acetogenic bacteria. In *Variations in autotrophic life*. Shively, J.M., and Barton, L.L. (eds). San Diego: Academic press, pp. 201-250.

Figure Legends

Fig. 1. Lactate metabolism in *A. woodii* (Weghoff et al., 2015). The lactate dehydrogenase/electron transferring flavoprotein (LDH/Etf) complex uses electron confurcation to drive NADH formation from lactate and reduced ferredoxin (Fd^{2-}) oxidation simultaneously.

Fd^{2-} is provided by the Rnf complex at the expense of the chemiosmotic gradient, which is established by the F_1F_O ATP synthase (ATPase) at the cost of ATP.

Fig. 2. Genetic organization and expression analyses of the *lctBCDEF* genes in *A. woodii*. A. Genetic organization of the *lctABCDEF* genes in *A. woodii*. The colours indicate the proposed functionality of the gene products. Red, putative transcriptional regulator; yellow, subunits of the LDH/Etf complex; blue, racemase; green, permease. **B.** Bridging PCR analyses was carried out for each intergenic region using complementary DNA (a), chromosomal DNA (b) or DNase-digested RNA (c) as template. The anticipated fragments are 665 bp for *lctB/C*, 584 bp for *lctC/D*, 684 bp for *lctD/E* or 701 bp for *lctE/F.* **C.** Relative expression of the *lct* operon under different metabolic conditions. cDNA was prepared from two biological replicates and qPCR was carried out in triplicate.

Fig. 3. Binding of LctA to intergenic DNA in response to lactate. A. Five different fragments of the intergenic region were used as templates for the shift assays in B and C. Fragment 1 covers the entire intergenic region (361 bp). **B.** Shift assays contained 9.8 µM purified LctA-His_6 and 50 nM DNA template (+) and control assays received only DNA template (-). Samples were separated on a 5% native polyacrylamide gel and the DNA was visualised with ethidium bromide. **C.** The same applies as for B with the exception that purified His_6-LctA was used. **D.** Shift assays contained 0.45 µM LctA-His_6, 7.5 nM DNA (fragment 1) and 200 mM potential effector molecules as indicated. The positive control (+) contained no effector and the negative control (–) contained only DNA template. The samples were separated on a 10% native polyacrylamide gel and the DNA was visualised with ethidium bromide.

Fig. 4. Restriction site protection analyses to determine the DNA binding site of LctA. DNA (20 ng) covering the intergenic region and part of *lctB* was incubated alone (a), with 0.5 U Hpy188I (b) or with Hpy188I and 0.2 µg purified LctA-His_6 (c). The other assays contained the same components as (c) and 200, 100 or 50 mM L- or D-lactate, respectively (d-i). Samples were separated on a 1.5% agarose gel and the DNA was visualised with ethidium bromide. The PCR fragment is 645 bp and a fragmentation with Hpy188I results in two fragments of 425 and 220 bp.

Fig. 5. Mixotrophic growth of *A. woodii* on lactate and fructose. *A. woodii* was cultivated in complex medium on 40 mM DL-lactate (**A**), 2.5 mM fructose (**B**) or a combination of both energy and carbon sources (**C**). Cellular growth was monitored by measuring the optical density at 600 nm (●) and the metabolites lactate (○), fructose (◇) and acetate (◆) were determined *via* HPLC analyses.