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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Towards an effective biosensor for monitoring AD leachate: a knockout *E. coli* mutant that cannot catabolise lactate

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Abstract Development of a biosensor for the convenient measurement of acetate and propionate concentrations in a two-phase anaerobic digestor (AD) requires a bacterium that will be unresponsive to the other organic acids present in the leachate, of which lactate is the most abundant. Successive gene knockouts of *E.coli* W3110 D-lactate dehydrogenase (*dld*), L-lactate dehydrogenase (*lldD*), glycolate oxidase (*glcD*) and a suspected L-lactate dehdrogenase (*ykgF*) were performed. The resulting quadruple mutant (IMD Wldgy) was incapable of growth on D- and L-lactate, whereas the wild type grew readily on these substrates. Furthermore, the O_2 consumption rates of acetate-grown IMD Wldgy cell suspensions supplied with either acetate (0.1 mM) or a synthetic leachate including acetate (0.1 mM) and DL-lactate (1 mM) were identical (2.79 and 2.70 mg l^{-1} min^{-1}, respectively). This was in marked contrast to similar experiments with the wild type which gave initial O_2 consumption rates of 2.00, 2.36 and 2.97 mg l^{-1} min^{-1} when cell suspensions were supplied with acetate (0.1 mM), acetate (0.1 mM) plus D-lactate (1 mM) or acetate (0.1 mM) plus L-lactate (1 mM), respectively. The knockout strain provides a platform for the design of a biosensor that can accessibly monitor acetate and propionate concentrations in AD leachate via O_2-uptake measurements.

Keywords Anaerobic digestion · Biosensor · Volatile fatty acids · Synthetic leachate

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Introduction

The primary objective of any anaerobic digestion (AD) deployment is to maximise methane yields, organic loading rates (OLR) and the destruction of volatile solids (VS), while minimising reactor volume sizes and hydraulic retention times (HRT) (Nizami et al. 2009; Nizami and Murphy 2010). Two-phase AD systems that comprise of a dry batch (DB) reactor coupled to an upflow anaerobic sludge blanket (UASB) (Nizami and Murphy 2011) or methane filter (MF) (Lehtomäki and Björnsson 2006) have been reported in the literature as best fulfilling this objective. However, Lehtomäki and Björnsson (2006) and Nizami et al. (2009) reported that the lack of monitoring and control mechanisms for two-phase AD systems substantially affected performance and suggested that further research with regard the development of chemical oxygen demand (COD), biological oxygen demand (BOD) and volatile fatty acid (VFA) sensing devices is required.

Wang et al. (2009) demonstrated that in a propionic acid concentration of 900 mg l^{-1}, there was a significant inhibition of methanogenic bacteria, whereas no effect was observed with even higher concentrations of acetic acid. Andersson and Björnsson (2002) demonstrated that the deviation of the propionate degradation rate in relation to that of acetate in a two-phase AD occurred before a change in COD reduction efficiency or drop in pH was observed, which are the usual parameters monitored to determine the efficient functioning of the AD, illustrating the need for an effective method of monitoring changes in VFA concentrations.

The ability to accurately measure an AD system's individual VFA (acetate, propionate, butyrate and valerate) concentrations is crucial for maintaining AD health (Ahring et al. 1995; Andersson and Björnsson 2002; Pind et al. 2003) and can also provide end users with information regarding substrate depletion patterns and substrate input compositions.

Total VFA concentrations within AD leachate samples are already identifiable by pre-existing technologies that include titration (Lange Cuvette Test), enzymatic assays (Rajashekhara et al. 2006; Zeravik et al. 2010) and inference from dissolved H_2 concentrations (Björnsson et al. 2001). However, methods that allow for AD leachate samples' individual VFA concentrations to be identified are sparse. Pind et al. (2003) proposed an automated method for identifying AD leachate samples' individual VFA concentrations by means of a gas chromatography coupled sample extraction and pre-treatment array. Although accurate, the expense of the proposed system and the extensive sample pre-processing requirements make this an unsuitable automated monitoring and control system for anything other than large-scale AD deployments.

A cost-effective alternative to the chemical analysis is a biosensor that can rapidly determine the concentrations of individual VFAs and enable timely alterations of OLRs in two-phase ADs. Whole-cell BOD sensors have been developed that map the oxygen consumption of immobilised microorganisms to a dataset containing known oxygen-usage-to-BOD correlations (Hikuma et al. 1981; Liu and Mattiasson 2002). As whole cell BOD sensors respire all available carbon, they cannot be applied to the measurement of individual VFAs. Nevertheless, a similar system with an appropriately engineered strain to discriminate the various VFAs could be designed. This would necessitate the deletion of genes coding for enzymes involved in the catabolism of the VFAs except those that are to be measured. This paper describes a proof-of-principle study in which systematic mutations of relevant genes in *Escherichia coli* were conducted to eliminate the bacterium's ability to catabolise lactate, which is the main VFA component in the leachate from two-phase grass silage-fed ADs. In this research, a mutant incapable of lactate metabolism but which selectively catabolised acetate was developed.

Materials and methods

Strains, plasmids and molecular biology methods

E. coli W3110 and P1vir were obtained from the *E.coli* Genetic Stock Centre (CGSC), Yale University, CT, USA. The bacterium was routinely grown on TSA and maintained on TSA slopes and glycerol −80 °C stocks. The sequences of the forward and reverse PCR primers for each of the *E.coli* knockout genes were specified by Baba et al. (2006) and were purchased from Eurofins Genomics (Ebersberg, Germany). All PCR reagents and pUC19 were purchased from New England Biolabs (Brennan and Co., Stillorgan, Dublin, Ireland). GeneRuler 1 kb Plus DNA Ladder was acquired from Thermo Scientific (Blanchardstown, Dublin, Ireland). The pCP20 plasmid was extracted from *E.coli* BT340 using a QIAGEN Plasmid Midi Kit.

Transduction of *E.coli* W3110 with a P1vir phage lysate containing the specific kanamycin-cassette-insertion gene knockout was performed using the Comprehensive Molecular BioEngineering (CMBE) (2010) P1 phage transduction protocol whereby the soft agar overlay method was employed to prepare P1vir phage lysate for each knockout strain (Table 1). Successful homologous recombination of the *E.coli*'s wild-type gene with the transduced kanamycin-cassette-insertion gene knockout resulted in a strain that was capable of growing on TSA supplemented with 50 μg ml^{-1} kanamycin. Further confirmation was provided by PCR amplification of the knockout gene, using the conserved k1 (5′-CAGTCATAGCCGAATAGCCT-3′) or k2 (5′-CGGTGCCCTGAATGAACTGC-3′) primers (Datsenko and Wanner 2000) coupled with the deleted gene's forward or reverse primers. These reactions yielded three amplicons of 1400, 800 and 600 bp (Baba et al. 2006). Kanamycin resistance was removed by the helper plasmid pCP20, which exploits the flanking repeated site (FRT) at either end of the resistance gene enabling homologous recombination (Cherepanov and Wackernagel 1995). Successful removal of the kanamycin cassette was confirmed by PCR amplification of the respective knockout gene yielding a 110-bp band (scar). Removal of the heat labile ampicillin-resistance-affording pCP20 was by culturing overnight at 42 °C and verified when no growth occurred on TSA containing either 50 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin. All successful kanamycin cassette-excised *E.coli* W3110 strains are listed in Table 1.

Growth with various carbon sources

Four sets of test tubes containing 4 ml minimal medium (60 mM K_2HPO_4, 30 mM KH_2PO_4, 1 mM $MgSO_4$, 76 mM $(NH_4)_2SO_4$ and 0.1 % *v*/*v* SL10 trace elements) supplemented with either 30 mM acetate, 30 mM propionate, 20 mM D-lactate or 20 mM L-lactate were inoculated with overnight TSB-grown cells (100–150 μl). The test tubes were incubated at 37 °C and 200 rpm; optical density was measured at 600 nm using a Jenway 6300 spectrophotometer at 24-h intervals. Control experiments were conducted in which no carbon source was included in the medium.

Synthetic leachate composition

Cirne et al. (2007) reported that biological leachate (BL) exiting the first phase of a two-phase grass-silage fed AD can contain acetate, DL-lactate and butyrate in a 1:20:1 ratio. Although no measurement for ethanol was made, Nizami and Murphy (2011) reported that the grass silage fed to an AD had acetate and ethanol in a 1:3.5 ratio. Based on these measurements, a synthetic leachate comprised of acetate, butyrate, ethanol and DL-lactate in a 1:5:5:25 ratio was prepared and used in resting cell studies.

Table 1 Bacteria, plasmids and phages used in the study

Bacterium, plasmid or phage	Description	Reference or source
Bacterium		
W3110	*E.coli* wild-type strain from which various JW derivatives were created.	J.Lederberg, Laboratory of Molecular Genetics and Informatics, The Rockefeller University, NY
IMD Wd	JW2121 *dld*759(del)::*kan E.coli* Δ*dld* single mutant	Keio Collection (Baba et al. 2006)
IMD Wl	JW3580 *lldD*789(del)::*kan E.coli* Δ*lldD* single mutant	Keio Collection (Baba et al. 2006)
IMD Wh	JW1375 *ldhA*744(del)::*kan E.coli* Δ*ldhA* single mutant	Keio Collection (Baba et al. 2006)
IMD Wg	JW2946 *glcD*753(del)::*kan E.coli* Δ*glcD* single mutant	Keio Collection (Baba et al. 2006)
IMD Wy	JW0300 *ykgF*750(del)::*kan E.coli* Δ*lldD* single mutant	Keio Collection (Baba et al. 2006)
IMD Wl Δ*kan*^{R}	IMD Wl with *kan* cassette removed	This study
IMD Wld	*E.coli* Δ*lldD dld* double mutant	This study
IMD Wld Δ*kan*^{R}	IMD Wld with *kan* cassette removed	This study
IMD Wldh	*E.coli* Δ*lldD dld ldhA* triple mutant	This study
IMD Wldg	*E.coli* Δ*lldD dld glcD* triple mutant	This study
IMD Wldg Δ*kan*^{R}	IMD Wldg with *kan* cassette removed	This study
IMD Wldgy	*E.coli* Δ*lldD dld glcD ykgF* quadruple mutant	This study
IMD Wldgy Δ*kan*^{R}	IMD Wldgy with *kan* cassette removed	This study
Plasmid		
pCP20	Extracted from the *E.coli* BT340 strain which was acquired from CGSC.	(Cherepanov and Wackernagel 1995)
pUC19	Transformation protocol positive control	New England Biolabs
Phage		
P1vir stock	Generalised transduction of *E.coli* strains	*E.coli* Genetic Stock Centre, Yale University

Oxygen consumption by resting cells

Erlenmeyer flasks (2 l) containing 800 ml minimal medium supplemented with acetate (30 mM) as the carbon source was inoculated with 20 ml of overnight TSB-grown *E.coli* cells. The flasks were incubated at 37 °C and 200 rpm and removed when the OD_{600} was 1.4–1.6. The cultures were centrifuged at 9000 rpm for 7 min, the supernatant discarded and the cells were washed in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, separated by centrifugation and resuspended in the same buffer (20 mg wet cells ml^{-1}). The cells were stored at 4 °C for 48 h before use.

The cell suspension (20 ml) was stirred for at least 1 h before the organic acid was added. An Orion 5-Star Plus Dissolved Oxygen (DO) probe was submerged into the centre of the stirred cell suspension and clamped into position. Care was taken to ensure the stir bar did not interfere with the electrode. Data points were recorded at 5-s intervals and automatically logged.

Results

Substrate utilisation in wild-type *E. coli* W3110

To establish a baseline measurement of organic acid utilisation, the rate of O_2 consumption was measured after suspended cells of *E. coli* W3110 were supplied with organic compounds normally found in biological leachate: acetate, propionate, D-lactate, L-lactate, ethanol and butyrate. It is evident from Fig. 1a that 1 mM L-lactate elicited a greater O_2 consumption than either 0.1 mM acetate or 1 mM D-lactate, and this was reflected in the initial rate of O_2 consumption for each substrate (2.86, 2.00 and 1.01 mg l^{-1} min^{-1} for L-lactate, acetate and D-lactate, respectively). A combination of acetate (0.1 mM) and D-lactate (1 mM) resulted in an initial O_2 consumption rate of 2.36 mg l^{-1} min^{-1}, which was comparable to that of acetate only, although the overall O_2 consumption was greater (4.91 mg l^{-1} compared to 2.5 mg l^{-1}). The rate of O_2 consumption for a combination of acetate and L-lactate (2.97 mg l^{-1} min^{-1}) was comparable to L-lactate only; the overall O_2 consumption was also similar for these substrate combinations. Although no discernible response curve was observed when either ethanol or butyrate was supplied to acetate- and propionate-grown IMD W cells (data not shown), it is quite evident that based on O_2 consumption, it would be impossible to distinguish the acetate and propionate concentrations in biological leachate without disabling lactate catabolism.

Gene knockouts

E.coli possesses two lactate dehydrogenases Dld and LldD; it was hypothesised that the creation of a double *dld-lldD*

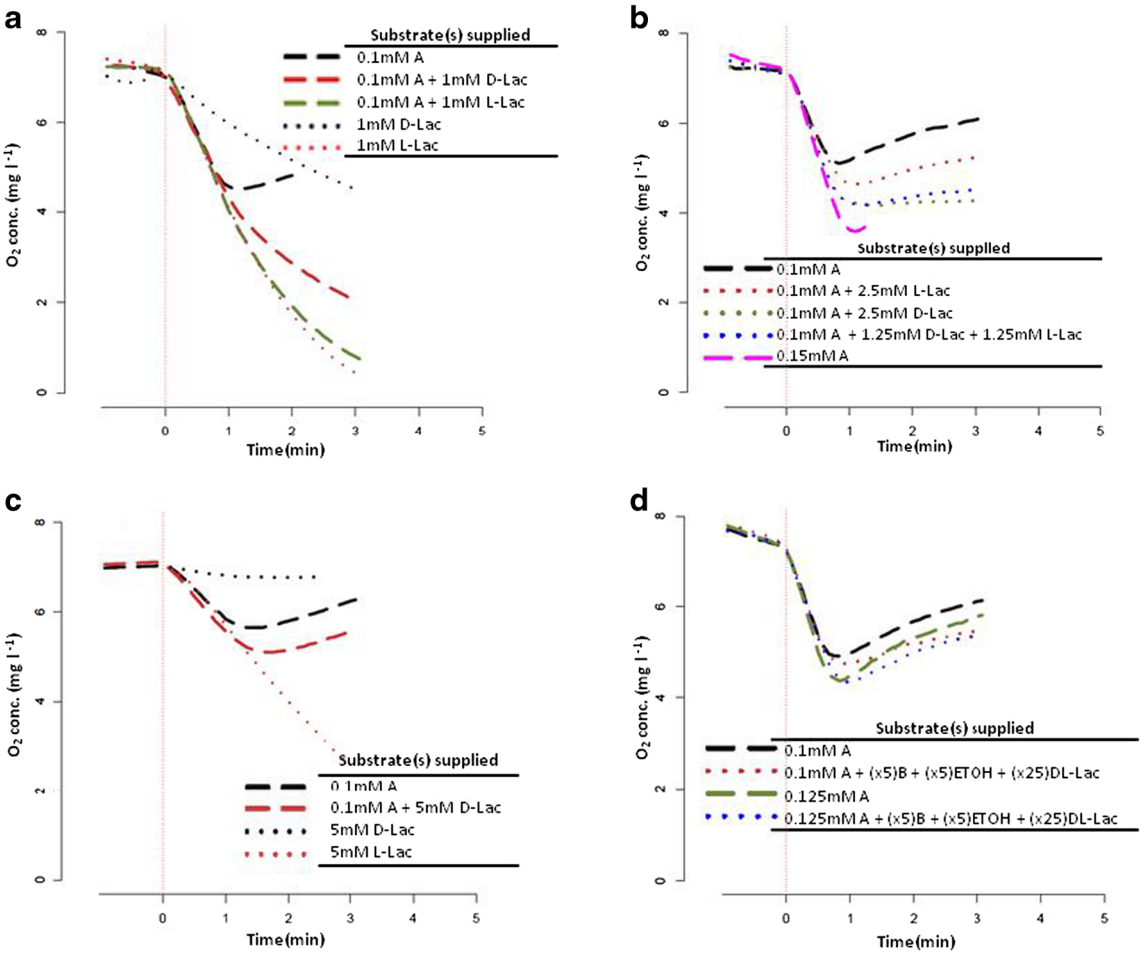

Fig. 1 The O_2 response curves of acetate-grown **a** IMD W, **b** IMD Wld, **c** IMD Wldg and **d** IMD Wldgy cells supplied with various A (acetate), D-Lac (D-lactate), L-Lac (L-lactate), B (butyrate), ETOH (ethanol) and DL-Lac (DL-lactate) concentrations. The data presented are from a series of single experiments; the experiments were replicated and the maximum variability between replicate response curves was 8.6 % for total O_2 consumption and 6.9 % for rate of O_2 consumption

knockout mutant strain (*E. coli* IMD Wld) would produce an *E.coli* strain incapable of aerobically respiring either D- or L-lactate. Successful deletion of these genes by P1 phage transduction was confirmed by the appearance of 110-bp scar residues after PCR (Fig. 2).

The growth of *E. coli* IMD Wld and W3110 supplied with minimal medium containing either acetate, propionate, D-lactate or L-lactate as a carbon source was assessed at 24 and 48 h by measuring OD_{600} (Table 2). The observation that IMD Wld had no discernible reduction in its max OD_{600} values for D- and L-lactate when compared to W3110 indicated that the removal of Dld and LldD had not removed IMD Wld's ability to aerobically respire either D- or L-lactate. The ability of acetate-grown IMD Wld to respire acetate and combinations of acetate and L-lactate, D-lactate and DL-lactate is shown in Fig. 1b. It is clear that the O_2 uptake from the various combinations of substrates was at least that of acetate only, for example, 0.1 mM acetate plus 2.5 mM D-lactate gave an initial O_2 consumption rate of 2.71 mg l^{-1} min^{-1} which is greater than that of 0.1 mM acetate only (2.26 mg l^{-1} min^{-1}). Thus, this mutant would not be able to distinguish between acetate and lactate catabolism. It is possible that constitutively expressed fermentative D-lactate dehydrogenase (LdhA) was responsible for the ability of IMD Wld to use D- with a higher preference than L-lactate for growth as it can facilitate the conversion of D-lactate to pyruvate, albeit at a much reduced rate (0.01 %) in comparison to the forward reaction (Tarmy and Kaplan 1968). Therefore, a triple mutant, Wldh in which *dld*, *lldD* and *ldhA* were knocked out, was created, and which was observed to behave in the same way as IMD Wld (data not shown); thus, another enzyme must be present that enables the cell to catabolise D-lactate.

Lord (1972) described an *E.coli* glycolate oxidoreductase that oxidised D- and L-lactate to pyruvate, and compared to the natural substrate, the oxidation rates were at 114 and 16 %, respectively. *E.coli's* glycolate oxidase (Glc) is expressed when grown on glycolate, acetate and any derived metabolite that leads to isocitrate formation, which could possibly include propionate (Pellicer et al. 1999; Pellicer et al. 1996). Thus, the creation of an *E.coli dld*, *lldD* and *glc* knockout mutant was expected to remove the D-lactate activity observed in IMD

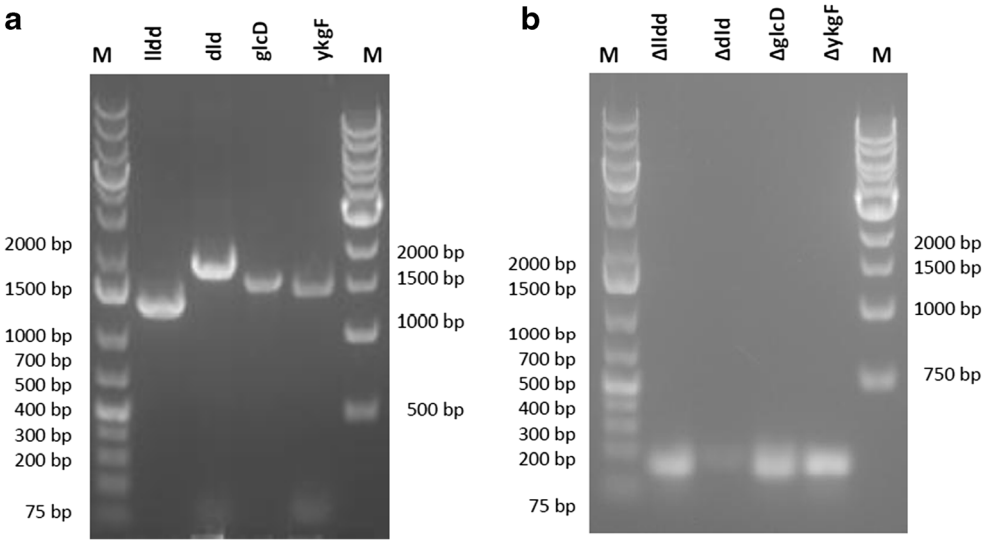

Fig. 2 **a** PCR amplifications of W3110's native *lldd*, *dld*, *glcD* and *ykgF* genes. Four bands that corresponded to *lldd*, *dld*, *glcD* and *ykgF*'s native 1190, 1715, 1499 and 1427 bp gene lengths were observed. **b** PCR amplifications of the Δ*lldd*, Δ*dld*, Δ*glcD* and Δ*ykgF* scar residues from IMD Wldgy. A single 110-bp scar residue band infers successful gene removal. *M* Marker

Wld. Pellicer et al. (1996) reported that the deletion of any one of the three GlcD, GlcE and GlcF subunits resulted in the enzyme being inactivated. Thus, the *glcD* gene was knocked out and the resulting triple mutant's growth rates and O_2 utilisation measured. *E. coli* IMD Wldg's 24 and 48 h OD_{600} measurements were comparable to IMD Wld for all organic acids except D-lactate, which did not support IMD Wldg growth (Table 2). The O_2 uptake rate of L-lactate (5 mM) by acetate-grown IMD Wldg was 1.29 mg l^{-1} min^{-1} (Fig. 1c), which was higher than that of 0.1 mM acetate (0.93 mg l^{-1} min^{-1}). The total O_2 consumption was also greater with L-lactate (4.31 mg l^{-1}) compared to acetate (1.35 mg l^{-1}). There was no apparent O_2 consumption when D-lactate was added to the cells, but a combination of D-lactate and acetate gave a slightly increased initial rate and overall O_2 consumption compared with acetate only (1.30 mg l^{-1} min^{-1} and 1.90 mg l^{-1}, respectively). When the initial rate and total O_2 consumption values of acetate-grown IMD Wldg cells were compared to those of IMD Wld, it was apparent that the removal of the Glc had caused the expression of a hitherto unknown enzyme which possessed stereospecific L-lactate dehydrogenase activity to be upregulated.

Table 2 The optical density readings of W3110, IMD Wld, IMD Wldg and IMD Wldgy grown on SL10 minimal media supplemented with different organic acids

Strain	30 mM Acetate	30 mM Propionate	20 mM D-lactate	20 mM L-lactate
OD_{600} after 24 h				
W3110	1.336	0.399	1.446	1.124
IMD Wld	0.902	0.184	0.685	0.989
IMD Wldg	1.227	0.139	0.006	0.978
IMD Wldgy	1.033	0.664	0.000	0.040
OD_{600} after 48 h				
W3110	0.927	0.994	0.995	0.727
IMD Wld	1.262	0.716	1.352	1.241
IMD Wldg	1.471	0.803	0.032	1.274
IMD Wldgy	1.221	1.468	0.016	0.103

Pinchuk et al. (2009) and Chai et al. (2009) described the previously unknown L-lactate dehydrogenases LlddEFG and LutABC, belonging to *Shewanella oneidensis* and *Bacillus subtilis*, respectively. Both shared homology with *E.coli*'s uncharacterised *ykgEFG* genes and complementation experiments demonstrated that *llddEFG* and *ykgEFG* could restore the ability of knockout mutants to grow on D- and L-lactate, strongly suggested that *ykgEFG* codes for a previously uncharacterised lactate dehydrogenase which is only expressed under certain conditions (Pinchuk et al. 2009). Therefore, a quadruple knockout, Wldgy (Δ*lldD dld glcD ykgF*), was created (Fig. 2b) and was incapable of growing on L- or D-lactate (Table 2). This mutant was grown on acetate and the resuspended cells' O_2 consumption was measured in synthetic leachate (SL) comprising of acetate, butyrate, ethanol and DL-lactate at a 1:5:5:25 ratio. Figure 1d shows that rate and consumption of O_2 with 0.1 mM acetate only were 2.79 mg l^{-1} min^{-1} and 2.09 mg l^{-1}, respectively, which were identical to those for synthetic leachate (2.70 mg l^{-1} min^{-1} and 2.25 mg l^{-1}). The same observation was made when 0.125 mM acetate was used either alone or as part of the SL. These data demonstrate that all of the key genes involved in lactate catabolism had been inactivated and the mutant responded in the same fashion as the wild type when supplemented with acetate.

Discussion

Current whole cell BOD sensors are not selective thus cannot be applied to the determination of the acetate and propionate concentrations in AD leachate. Nevertheless, it has been suggested

that a biosensor using *E. coli* could be developed, since the key enzymes citrate synthase (GltA) and 2-methylcitrate synthase (PrpC) are inducible and selective for acetate and propionate, respectively (Man et al. 1995; Textor et al. 1997). However, Cirne et al. (2007) reported that biological leachate (BL) exiting the first phase of a two-phase grass-silage fed AD can contain a concentration of lactate that is 20-fold higher than that of acetate. As *E. coli* can readily catabolise lactate, it could not be used to estimate the concentration of acetate and propionate in BL if used in conjunction with an O_2 probe, which is a common device employed in these applications. Therefore, as a first step to developing a specific biosensor to monitor acetate concentrations in AD leachate, the lactate catabolic enzymes must be inactivated. In this study, four genes coding for enzymes involved in lactate catabolism in *E. coli* W3110: *lldD*, *dld*, *glcD* and *ykgF* were knocked out rendering the strain incapable of utilising lactate yet still able to grow and metabolise other organic acids such as acetate. Furthermore, we have confirmed *ykgF* encodes an L-lactate dehydrogenase and demonstrated that it is only expressed when the other pathways for lactate metabolism in the cell are inactive. Current biosensors used with oxygen probes are often immobilised to enable stability, longevity and ease of use (Hikuma et al. 1981; Liu and Mattiasson 2002), and so it remains to optimise the mutant strain for in situ acetate/propionate measurements.

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Conflict of interest The authors declare that they have no competing interests.

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