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Two NAD-independent L-lactate dehydrogenases drive L-lactate utilization in *Pseudomonas aeruginosa* PAO1

Yujiao Wang,[1,2†] Dan Xiao,[1†] Qiuyuan Liu,[1] Yipeng Zhang,[1] Chunxia Hu,[1] Jinkai Sun,[1] Chunyu Yang,[1] Ping Xu,[3] Cuiqing Ma[1] and Chao Gao[1*]

[1]*State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China.*

[2]*Department of Laboratory Medicine, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan 250014, People's Republic of China.*

[3]*State Key Laboratory of Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China.*

Summary

***Pseudomonas aeruginosa* often establishes a chronic infection in the airways of patients with cystic fibrosis (CF). L-Lactate is the most abundant carbon source in the CF sputum, and L-lactate utilization may be important for *P. aeruginosa* to survive in the lungs of CF patients. In this study, the key enzymes involved in L-lactate utilization by *P. aeruginosa* PAO1 were characterized using the synthetic CF sputum medium (SCFM). A highly conserved membrane-bound NAD-independent L-lactate dehydrogenase (L-iLDH) encoded by *lldD* (PA4771) and a novel flavin-containing membrane-bound L-iLDH encoded by *lldA* (PA2382) were both found to contribute to L-lactate utilization by *P. aeruginosa* PAO1. In addition, an *lldD* and *lldA* double mutant was incapable of growing in a medium containing L-lactate as the sole carbon source. This study clarifies the mechanism and importance of L-lactate catabolism, and demonstrates the first *Pseudomonas* spp. expressing two L-lactate-oxidizing enzymes.**

Received 30 November, 2016; accepted 28 May, 2018. *For correspondence. E-mail jieerbu@sdu.edu.cn; Tel. (+86) 531 883 640 03; Fax (+86) 531 883 694 63. †These authors contributed equally to this work.

Introduction

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium that normally grows in the soil and in aqueous environments (Stover *et al.,* 2000; Gellatly and Hancock, 2013). It also thrives in various sites in the human body and causes serious infections, such as bacteremia in victims of severe burns, urinary-tract infections (Lyczak *et al.,* 2000; Stover *et al.,* 2000) and chronic respiratory infections in patients with cystic fibrosis (CF) (Folkesson *et al.,* 2012; Varga *et al.,* 2015). The complex nutritional composition of the sputum of CF patients (hereafter, CF sputum) offers sufficient carbon and energy sources to support robust growth of chronically colonizing pathogens (Palmer *et al.,* 2005, 2007). Among the various nutritional components of CF sputum, L-lactate is the most abundant and is likely generated by bacteria and human cells during aerobic respiration as well as anaerobic glycolysis (Borregaard and Herlin, 1982; Bensel *et al.,* 2011).

In previous research, L-lactate utilization was found to be associated with the infection processes and pathogenicity of several pathogenic microbes (Smith *et al.,* 2001, 2007; Exley *et al.,* 2005a,b, 2007). For example, L-lactate metabolism stimulates oxygen consumption by *Neisseria gonorrhoeae*, and this increased consumption leads to the impairment of oxygen-dependent bactericidal mechanisms (Britigan *et al.,* 1988). *Staphylococcus aureus* resists the action of the broad-spectrum antimicrobial nitric oxide (NO•) produced by the host through the production, excretion and reutilization of L-lactate (Richardson *et al.,* 2006, 2008; Fuller *et al.,* 2011). *P. aeruginosa* can use L-lactate as the sole carbon source (Gao *et al.,* 2012a; Jiang *et al.,* 2014). The L-lactate utilization mechanism of *P. aeruginosa* remains to be investigated.

In this study, two independent L-lactate oxidizing enzymes were identified in *P. aeruginosa* PAO1 – the conserved NAD-independent L-lactate dehydrogenase (L-iLDH) encoded by *lldD* (PA4771) and a novel flavin-containing membrane-bound L-iLDH encoded by *lldA* (PA2382). Both L-iLDHs contribute to L-lactate utilization by *P. aeruginosa* PAO1. Furthermore, using an *in vitro* co-culture assay of the wild-type and *lldD* and *lldA* double mutant strains, we found that L-lactate catabolism contributes to the competence of *P. aeruginosa* PAO1 in synthetic CF sputum medium (SCFM).

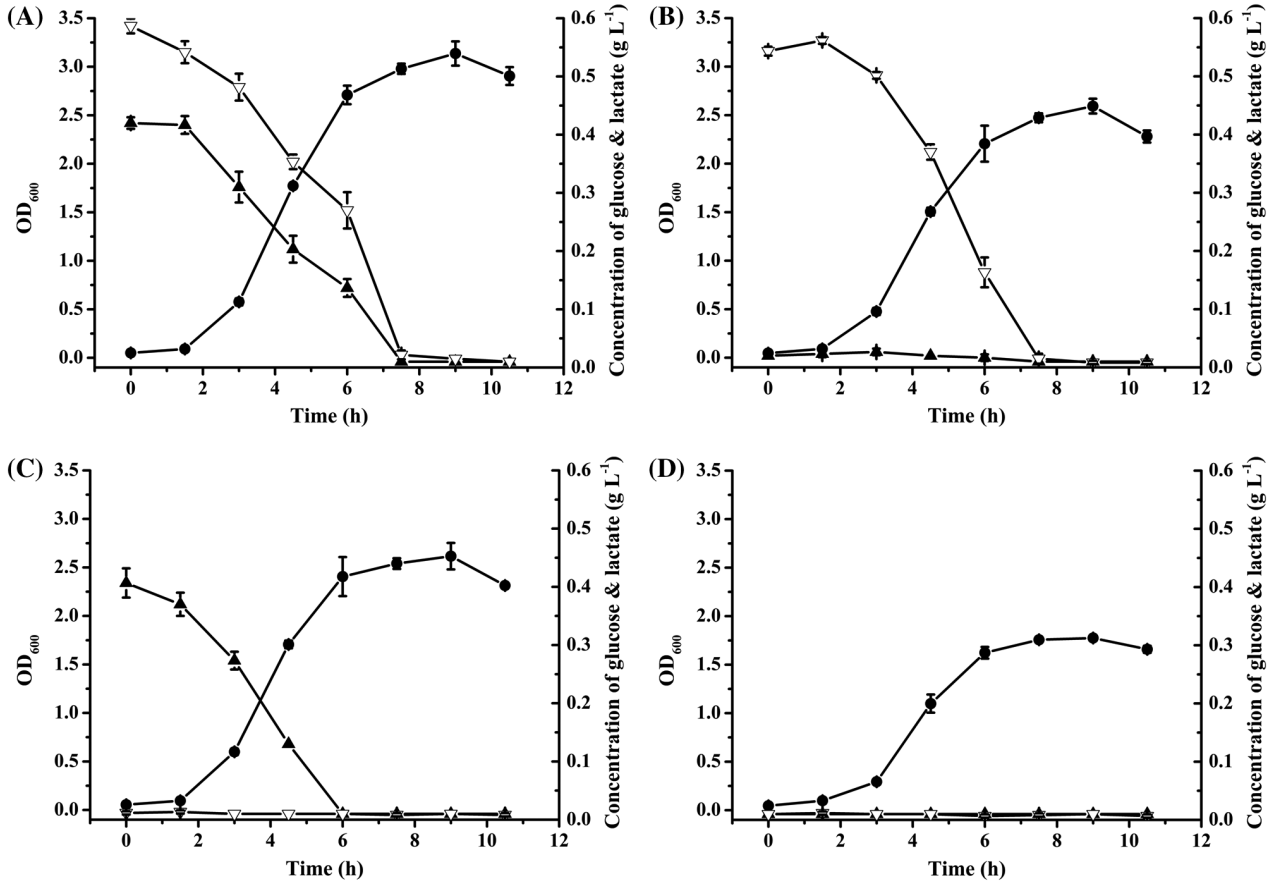
Results

L-Lactate enhances the growth of P. aeruginosa PAO1

In order to examine whether L-lactate in CF sputum influences the behaviour of *P. aeruginosa* PAO1, we compared the growth curves of *P. aeruginosa* PAO1 in SCFM and SCFM without L-lactate or/and glucose (Fig. 1). As shown in Fig. 1, *P. aeruginosa* PAO1 showed decreased growth in SCFM lacking glucose and L-lactate (maximum OD_{600} of 1.77). In SCFM with only L-lactate or glucose, the maximum OD_{600} of the strain was 2.59 ± 0.08 and 2.62 ± 0.14 respectively (Fig. 1B and C). As expected, the maximum OD_{600} of *P. aeruginosa* PAO1 in SCFM with L-lactate and glucose (3.14) (Fig. 1A) was much higher than that in the SCFM without L-lactate and glucose (Fig. 1D). The robust growth of *P. aeruginosa* PAO1 in SCFM paralleled the rapid consumption of L-lactate. The concentration of L-lactate dropped from 0.59 g L^{-1} to 0.27 g L^{-1} after 6 h of growth and was exhausted after 9 h, when the strain reached maximum biomass (Fig. 1A). These results suggested that L-lactate enhances the growth of *P. aeruginosa* PAO1 in SCFM.

P. aeruginosa PAO1 harbours two L-iLDHs

After inspecting the *P. aeruginosa* PAO1 genome sequence, putative enzymes involved in lactate catabolism were identified (Table 1). Three adjoining genes, *lldP* (PA4770, encoding a lactate permease), *lldD* (PA4771,



	A		B		C		D	
Medium composition	L-Lac[a]	Glu[b]	L-Lac	Glu	L-Lac	Glu	L-Lac	Glu
	+	+	+	-	-	+	-	-
The maximum OD_{600}	3.14 ± 0.12		2.59 ± 0.08		2.62 ± 0.14		1.77 ± 0.01	

Fig. 1. The impacts of L-lactate and glucose on the growth of *P. aeruginosa* PAO1 in SCFM.
Pseudomonas aeruginosa PAO1 was cultured in normal SCFM with L-lactate and glucose (A), with only L-lactate (B), with only glucose (C) and without L-lactate and glucose (D) respectively. Cell density (filled circle), L-lactate (hollow inverted triangle) and glucose (filled triangle) in mediums were assayed. All error bars represent the standard deviations of at least three independent experiments. **a.** L-Lactate; **b.** glucose.

Table 1. The putative proteins involved in the lactate metabolism in *P. aeruginosa* PAO1.

Locus tag	Gene symbol	Product	Protein description	Protein ID	Characteristic domain
PA2382	*lldA*	L-iLDH	L-Lactate dehydrogenase	NP_251072.1	FMN-binding domain
PA4770	*lldP*	L-Lactate permease	Translocator	NP_253458.1	An integral membrane protein
PA4771	*lldD*	L-iLDH	L-Lactate dehydrogenase	NP_253459.1	FMN-binding domain
PA4772	*dldD*	D-iLDH	Ferredoxin	NP_253460.1	FAD-binding domain; 4Fe-4S dicluster domain

encoding an L-iLDH) and *dldD* (PA4772, encoding a D-iLDH), constitute a putative lactate utilization operon that has been annotated in many different *Pseudomonas* strains (Wang *et al.,* 2014). Unexpectedly, gene *lldA* (PA2382), which encodes another putative L-lactate dehydrogenase, was found in the *P. aeruginosa* PAO1 genome (Table 1).

The genes encoding LldD and LldA were amplified by PCR and cloned into the expression vector pETDuet-1 respectively. Then, the proteins were overexpressed in *E. coli* C43 (DE3) with an N-terminus 6-histidine tag. The specific L-iLDH activities in the crude cell extracts of the *E. coli* C43 (pETDuet-1-*lldD*) and *E. coli* C43 (pETDuet-1-*lldA*) were 1.35 ± 0.02 and 2.57 ± 0.07 μmol min^{-1} mg^{-1} respectively (Table 2). Finally, LldD and LldA were purified by affinity chromatography using a His-Trap column, and confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 11.25% gel (see Supporting Information Fig. S1).

The rate of dehydrogenation of L-lactate catalysed by LldD and LldA followed Michaelis–Menten kinetics (Table 3). The apparent K_m and V_{max} of purified LldD towards L-lactate were 335.78 ± 12.21 μM and 0.19 ± 0.00 μmol min^{-1} mg^{-1} respectively. In comparison, the apparent K_m and V_{max} for L-lactate of LldA were 1102.94 ± 79.32 μM and 0.87 ± 0.04 μmol min^{-1} mg^{-1}, which were much higher than those of LldD. These results indicated that the substrate affinity for L-lactate of LldD was higher than those of LldA, whereas the maximum catalytic rate for L-lactate of LldA was higher than that of LldD.

Table 2. L-iLDH and D-iLDH activities in crude cell extracts of strains expressing LldD and LldA.

Strain	Enzyme activity (μmol min^{-1} mg^{-1})[a]	
	L-iLDH	D-iLDH
E. coli C43 (pETDuet-1-*lldD*)	1.35 ± 0.02	ND[b]
E. coli C43 (pETDuet-1-*lldA*)	2.57 ± 0.07	ND
E. coli C43 (DE3) with empty pETDuet-1	0.01 ± 0.00	0.05 ± 0.00
E. coli C43 (DE3)	0.01 ± 0.00	0.02 ± 0.00

a. Activities of L-iLDH and D-iLDH were examined with 20 mM L-lactate or 20 mM D-lactate. DCPIP was used as the electron acceptor. Results are means ±SD of three parallel replicates.
b. ND: not detected.

Both LldD and LldA are responsible for L-lactate utilization by P. aeruginosa *PAO1*

The *lldD* and *lldA* genes were knocked out individually or in combination in *P. aeruginosa* PAO1 and their respective complement strains were constructed. Disruption of the *lldD* gene impaired the growth of this strain in L-lactate, but the mutant strain could still grow after a long lag phase (about 7.5 h). However, loss of *lldA* had little effect on L-lactate utilization. Furthermore, deletion of both the *lldD* and *lldA* genes completely impaired the L-lactate utilization capacity of the strain. As expected, the complements of both the *lldD* or *lldA* gene successfully restored the ability of PAO1 (Δ*lldD* and Δ*lldA*) to grow in L-lactate (see Supporting Information Fig. S2). In addition, the wild-type PAO1 and mutants grew equally well on D-lactate and pyruvate. Similar results were obtained with growth of the wild-type PAO1 and its derivatives on solid MSM with these three carbon sources (see Supporting Information Fig. S3). These results suggest that both the *lldD* and *lldA* genes encode functional L-iLDHs, which contribute to L-lactate utilization by *P. aeruginosa* PAO1.

LldD and LldA are important for growth of P. aeruginosa *PAO1 in SCFM*

L-Lactate is one of the major carbon sources in the CF lung environment. We assessed the growth of wild-type *P. aeruginosa* PAO1 and its mutant strains in SCFM. As expected, compared with the wild type, which showed a maximum OD_{600} of 3.14 ± 0.01 in the stationary phase (Fig. 2A), mutants lacking *lldD* or *lldA* exhibited only a slight attenuation when grown in SCFM; the final OD_{600} decreased to 2.95 ± 0.04 and 2.98 ± 0.01 respectively (Fig. 2B and C). Furthermore, this growth attenuation was aggravated in PAO1 (Δ*lldD* and Δ*lldA*) which

Table 3. Apparent K_m and V_{max} of LldD and LldA towards L-lactate[a].

Enzyme	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
LldD	335.78 ± 12.21	0.19 ± 0.00
LldA	1102.94 ± 79.32	0.87 ± 0.04

a. The apparent K_m and V_{max} were examined in 50 mM Tris–HCl buffer (pH 7.4) containing various concentrations of L-lactate. DCPIP was used as the electron acceptor. Results are means ±SD of three parallel replicates.

completely incapacitated the utilization of L-lactate, and the maximum OD_{600} of this strain was 2.51 ± 0.02 in the stationary phase (Fig. 2D). However, expression of *lldD* or *lldA* completely restored growth of PAO1 (Δ*lldD* and Δ*lldA*) in SCFM (Fig. 2E and F). These results highlight the fact that these two L-iLDHs in *P. aeruginosa* PAO1, which are encoded by the *lldD* and *lldA* genes respectively, play important roles in growth of this strain in the CF lung environment.

L-Lactate metabolism participate in competence of P. aeruginosa *PAO1 in SCFM*

To better understand whether L-lactate catabolism contributes to *P. aeruginosa* PAO1 competence in the CF lung environment, we conducted an *in vitro* dual-bacterial co-culture assay. Wild-type PAO1 and PAO1 (Δ*lldD* and Δ*lldA*) were adjusted to the same OD_{600} and then co-inoculated into SCFM at the same volume. The co-cultures were transferred to fresh SCFM every 12 h. Both wild-type PAO1 and PAO1 (Δ*lldD* and Δ*lldA*) can grow on solid MSM with pyruvate or L-lactate whereas PAO1 (Δ*lldD* and Δ*lldA*) can only grow on solid MSM with pyruvate. The relative fitness of wild-type PAO1 and PAO1 (Δ*lldD* and Δ*lldA*) can be calculated based on the bacterial colonies that can grow on solid MSM with pyruvate or L-lactate as the sole carbon source. As shown in Fig. 3, over 12 generations of serial subculturing, wild-type PAO1 became the major constituent in the co-culture and PAO1 (Δ*lldD* and Δ*lldA*) failed to compete with the wild type. The results also implied that L-lactate catabolism contributes to competitive ability of *P. aeruginosa* PAO1 in the CF lung environment.

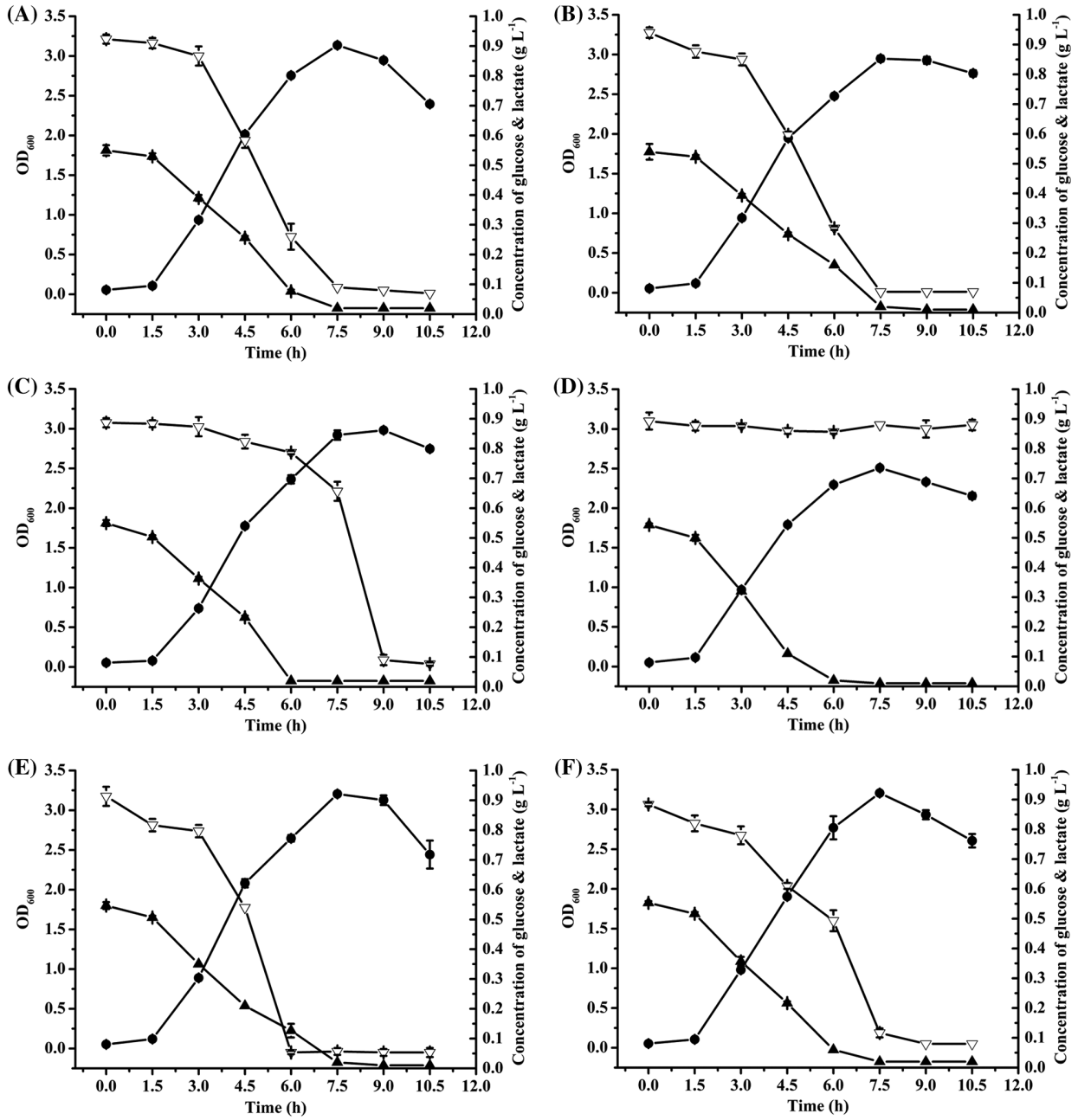
Discussion

In CF sputum, L-lactate is one of the utilizable carbon sources for *P. aeruginosa* (Palmer *et al.,* 2007). We observed that *P. aeruginosa* PAO1 grew much better when L-lactate was added to SCFM. Therefore, identification of the enzymes responsible for L-lactate utilization by *P. aeruginosa* PAO1 was the primary focus of this study. Two independent L-iLDHs, LldD (PA4771) and LldA (PA2382), which catalysed L-lactate oxidation, were identified. Both LldD and LldA belong to the FMN-dependent α-hydroxyacid-oxidizing enzyme family, whose members contain an FMN-binding domain. However, it is possible that LldD evolved separately from LldA. LldD of *P. aeruginosa* PAO1 showed strikingly high amino acid sequence identity to L-iLDHs from *E. coli* K12 (83.1%) and most *Pseudomonas* strains, including *Pseudomonas putida* KT2440 (88.5%) and *Pseudomonas stutzeri* SDM (85.7%.) (Gao *et al.,* 2012b; Wang *et al.,* 2014). By contrast, LldA of *P. aeruginosa* PAO1 showed < 50% amino acid sequence identity to L-iLDHs from the strains mentioned previously, but exhibited 65.4% homology to the L-iLDH from *Neisseria meningitidis* (Erwin and Gostchlich, 1996). Phylogenetic analyses also showed that these enzymes belong to two distinct clusters of the FMN-dependent α-hydroxyacid-oxidizing enzyme family (see Supporting Information Fig. S4 and Table S1).

The function and physiological significance of these two L-iLDHs in *P. aeruginosa* PAO1 were also investigated. Mutants lacking either LldD or LldA could still grow in medium containing L-lactate as the sole carbon source, whereas the *lldD* and *lldA* double mutant failed to grow in this medium, indicating that both enzymes are involved in L-lactate catabolism. Combining the results of this study and the previously reported features of L-lactate utilization by *P. aeruginosa*, we propose here a model for L-lactate catabolism in *P. aeruginosa* PAO1. L-Lactate may enter cells via the action of lactate permease, encoded by *lldP.* Two systems for L-lactate utilization exist in *P. aeruginosa* PAO1, comprising two independent membrane-bound L-iLDHs, LldD and LldA. *lldD* is located close to *dldD* and *lldP*, and together they constitute a putative *lldPDE* operon. The transcription of genes in this operon is regulated by the upstream transcription repressor LldR (Gao *et al.,* 2012a). *lldA* is located outside of the *lldPDE* operon; however, the mechanism regulating the transcription of this gene remains unclear. Both LldD and LldA can catalyse the oxidation of L-lactate to pyruvate and transfer electrons to the putative native electron acceptor quinone and then to the electron transport chain. Finally, the electrons are delivered to oxygen to generate ATP via oxidative phosphorylation, providing energy for growth.

Microaerobic respiration is the predominant mode of *P. aeruginosa* growth in lung of CF patients (Alvarez-Ortega and Harwood, 2007). *P. aeruginosa* colony biofilms can develop steep oxygen gradients and be divided into metabolic subpopulations under oxic or anoxic conditions. Because the L-lactate utilization process is oxygen dependent, the wild-type PAO1 had small but detectable growth advantage over PAO1 (Δ*lldD* and Δ*lldA*) when cultured under low oxygen conditions (see Supporting Information Fig. S5). In addition, interaction with human airway epithelial cells can induce the expression of L-lactate utilization related genes, such as *lldP* (Frisk *et al.,* 2004). Thus, the L-lactate catabolism might take place *in vivo* and play roles in pathogenic process of *P. aeruginosa.*

The L-iLDH genes are often present as a single copy in bacterial genomes such as those of *P. putida* and *P. stutzeri.* However, some bacteria harbour more than one L-iLDH-encoding gene. For example, *C. jejuni* NCTC 11168 possesses two independent L-iLDH enzymes encoded by *cj0075c-73c* and *cj1585c* (Thomas *et al.,*



	A	B	C	D	E	F
Strain	**WT**	**Δ*lldD***	**Δ*lldA***	**Δ*lldD*&Δ*lldA***	***lldD* complement**	***lldA* complement**
Ability of L-lactate utilization	+	+	+	-	+	+
The maximum OD_{600}	**3.14 ± 0.01**	**2.95 ± 0.04**	**2.98 ± 0.01**	**2.51 ± 0.02**	**3.21 ± 0.02**	**3.21 ± 0.01**

Fig. 2. Comparison of the growth of *P. aeruginosa* PAO1 and its derivatives in SCFM.
Pseudomonas aeruginosa PAO1 (A), *P. aeruginosa* PAO1 (Δ*lldD*) (B), *P. aeruginosa* PAO1 (Δ*lldA*) (C), *P. aeruginosa* PAO1 (Δ*lldD* and Δ*lldA*) (D), *P. aeruginosa* PAO1 (Δ*lldD* and Δ*lldA*) *lldD* complement (E) and *P. aeruginosa* PAO1 (Δ*lldD* and Δ*lldA*) *lldA* complement (F) were cultured with SCFM respectively. Cell density (filled circle), L-lactate (hollow inverted triangle) and glucose (filled triangle) in mediums were assayed. All error bars represent the standard deviations of at least three independent experiments.

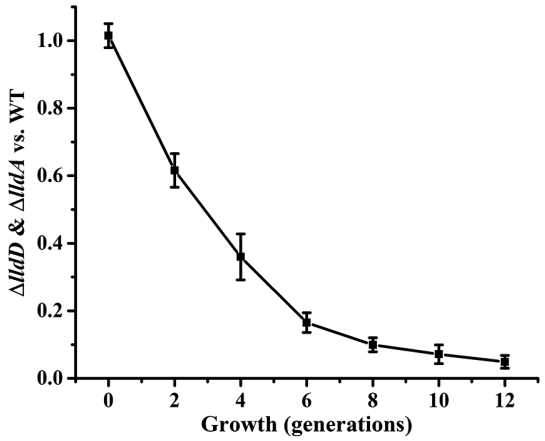


Fig. 3. L-Lactate catabolism ability is important for competitive capacity of the *P. aeruginosa* PAO1 in the SCFM.
For co-culture samples, iso-volumetric of 1 OD_{600} wild-type PAO1 and 1 OD_{600} PAO1 (Δ*lldD* and Δ*lldA*) were mixed. The mixed cultures were inoculated into the SCFM and subculture for every 12 h. The relative fitness of wild-type PAO1 and PAO1 (Δ*lldD* and Δ*lldA*) was calculated based the bacterial colonies that can grow on solid MSM with pyruvate or L-lactate as the sole carbon source. All error bars represent the standard deviations of at least three independent experiments.

2011). *N. meningitidis* contains at least one unidentified L-iLDH in addition to the product of *lldA* (Erwin and Gotschlich, 1993, 1996). In this study, we observed attenuated growth of *lldD* and *lldA* double mutant in SCFM compared with that of the wild-type strain. Furthermore, when co-cultured with wild-type PAO1, the growth of PAO1 (Δ*lldD* and Δ*lldA*) decreased with each subculture in SCFM, suggesting that the ability to catabolize L-lactate might play an important role in the ability of *P. aeruginosa* PAO1 to compete and survive in the CF lung environment. L-Lactate has been reported to accumulate in inflammatory conditions (Peter *et al.,* 2015). We speculate that these pathogens harbouring more than one L-iLDH-encoding gene may be benefit for them to compete with other bacteria, because the former might use L-lactate as a carbon and energy source more efficiently than other bacterial strains *in vivo.*

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information

Table S1 List of protein sequences information included in the phylogenetic analyses.

Table S2 Strains, plasmids, and primers used in this study.

Fig. S1. SDS-PAGE analysis of purified LldD and LldA.

Fig. S2. Comparison of the growth of *P. aeruginosa* PAO1 and its derivatives in liquid MSM with different carbon sources.

Fig. S3. Comparison of the growth of *P. aeruginosa* PAO1 and its derivatives on solid MSM with different carbon sources.

Fig. S4. Phylogenetic analyses of LldD and LldA.

Fig. S5. Growth of *P. aeruginosa* PAO1 and PAO1 (Δ*lldD* & Δ*lldA*) in SCFM under different oxygen conditions.