

# Lack of glyoxylate shunt dysregulates iron homeostasis in *Pseudomonas aeruginosa*

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## Abstract

The *aceA* and *glcB* genes, encoding isocitrate lyase (ICL) and malate synthase, respectively, are not in an operon in many bacteria, including *Pseudomonas aeruginosa*, unlike in *Escherichia coli*. Here, we show that expression of *aceA* in *P. aeruginosa* is specifically upregulated under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and under iron-limiting conditions. In contrast, the addition of exogenous redox active compounds or antibiotics increases the expression of *glcB*. The transcriptional start sites of *aceA* under iron-limiting conditions and in the presence of iron were found to be identical by 5' RACE. Interestingly, the enzymatic activities of ICL and isocitrate dehydrogenase had opposite responses under different iron conditions, suggesting that the glyoxylate shunt (GS) might be important under iron-limiting conditions. Remarkably, the intracellular iron concentration was lower while the iron demand was higher in the GS-activated cells growing on acetate compared to cells growing on glucose. Absence of GS dysregulated iron homeostasis led to changes in the cellular iron pool, with higher intracellular chelatable iron levels. In addition, GS mutants were found to have higher cytochrome c oxidase activity on iron-supplemented agar plates of minimal media, which promoted the growth of the GS mutants. However, deletion of the GS genes resulted in higher sensitivity to a high concentration of H<sub>2</sub>O<sub>2</sub>, presumably due to iron-mediated killing. In conclusion, the GS system appears to be tightly linked to iron homeostasis in the promotion of *P. aeruginosa* survival under oxidative stress.

## INTRODUCTION

*Pseudomonas aeruginosa* is a well-known opportunistic Gram-negative bacterium involved in human cystic fibrosis (CF) [1–5]. The surfactant from the lower respiratory airways of CF patients presents an altered fatty acid profile [2]. Specifically, palmitic acid, the major fatty acid in the surfactant, and mono-unsaturated oleic acid are found in higher concentrations in the surfactant of CF patients compared to non-CF patients, which has been attributed to the increased phospholipid and phosphatidylcholine levels found in the former [2]. Interestingly, *P. aeruginosa* grown in the sputum of CF patients showed a decreased expression of genes related to glucose transport and metabolism [3]. In addition, micro-array analysis of *P. aeruginosa* isolated from the lungs of CF patients showed that genes involved in fatty acid and lipid metabolism, and the glyoxylate shunt (GS), are upregulated, indicating that lipids are the main carbon source of *P. aeruginosa* in the lungs of CF patients [4]. Indeed, bacterial growth in the presence of fatty acids, acetate and ethanol as sole carbon sources requires the GS. Importantly, the GS is a major metabolic pathway for

the growth of *Mycobacterium tuberculosis* within its host [6].

The GS consists of two specific enzymes: isocitrate lyase (ICL), encoded by the *aceA* gene, and malate synthase (MS), encoded by *aceB* or *glcB* [7, 8]. ICL catalyses the conversion of isocitrate to glyoxylate and succinate, and MS condenses acetyl-CoA and glyoxylate to malate [8]. The GS is a bypass of the tricarboxylic acid (TCA) cycle, which can supplement intermediates of the TCA cycle or precursors for the synthesis of amino acids, helping bacteria utilize C<sub>2</sub> compounds more efficiently by avoiding the two CO<sub>2</sub>-producing steps of the TCA cycle [9]. In *E. coli*, the glyoxylate produced by ICL can be converted to malate by two MS: malate synthase A (MSA), encoded by *aceB*, and malate synthase G (MSG), encoded by *glcB* [10]. Importantly, the GS is not present in humans and therefore it can be used as a target in the development of antimicrobial agents [11]. The GS has been extensively studied in several bacterial species such as *M. tuberculosis* and *E. coli*. In *M. tuberculosis*, the causative agent of tuberculosis, ICL is essential for the establishment of long-term persistence in mice and for defence

Received 5 November 2017; Accepted 31 January 2018

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Keywords: glyoxylate bypass; iron; oxidative stress; gene expression; bacteria; TCA cycle.

Abbreviations: DPD, 2,2'-dipyridyl; GS, glyoxylate shunt; ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; MS, malate synthase; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

against oxidative stress related to antibiotic treatment [6, 12, 13]. Expression of *icl1*, one of two *M. tuberculosis* ICL genes, increased 4- to 16-fold in the presence of antibiotics such as rifampicin, streptomycin and isoniazid [13]. In fact, deletion of the *icl* gene resulted in a 100- to 1000-fold increase in *M. tuberculosis* susceptibility to these antibiotics [13]. In contrast, disruption of the GS-coding genes *aceA* or *aceB* increased tolerance of *E. coli* to gentamicin [14].

Iron functions as a co-factor for many iron-sulfur (Fe-S) cluster-containing enzymes involved in various cellular processes, including central carbon metabolism, electron transport and response to reactive oxygen species [15]. *E. coli* contains around  $10^5$ – $10^6$  atoms of iron per cell, which is similar to the iron content in other bacteria [15]. In general, bacteria require an external iron concentration of around  $10^{-7}$ – $10^{-5}$  M for optimal growth. However, the solubility of ferric iron at pH 7 is much lower, at approximately  $10^{-17}$  M [15]. Moreover, within the host, the concentration of free extracellular iron is usually kept at very low levels ( $10^{-18}$  M) as a defence mechanism against invading pathogens [15]. Thus, acquiring adequate amounts of iron is essential to maintain the function of certain metabolic pathways, and for the virulence of pathogens in the host. For instance, iron limitation affects oxygen transfer and the formation of virulence factors in *P. aeruginosa* [16]. To overcome iron starvation imposed by the host, bacteria have evolved iron acquisition mechanisms, including the use of ferric iron-chelating compounds called siderophores [17]. Importantly, the genes involved in the biosynthesis of the siderophores pyoverdine and pyochelin are upregulated when *P. aeruginosa* is growing in CF sputum [3].

Despite the importance of iron for the growth of *P. aeruginosa* in the host, little is known about the link between the GS and iron homeostasis in this bacterium. Therefore, the objective of this study was to determine the effect of iron on the regulation of metabolic carbon flux in *P. aeruginosa* at the transcriptional and translational levels using quantitative RT-PCR (qRT-PCR) and enzyme activity assays. Physiologies associated with the intracellular iron pool were further analysed in this study, to determine whether deletion of the GS genes would affect both iron homeostasis and the sensitivity of *P. aeruginosa* to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. This research provides a better insight into the role of GS on iron homeostasis under iron-limiting conditions in pathogenic bacteria.

## METHODS

### Bacterial strains and culture conditions

The wild-type *P. aeruginosa* MPAO1 strain and the derived mutants *aceA* (PA2634) and *glcB* (PA0482), purchased from the Washington University Genome Center, were used in this study. The primers used in this study are listed in Table 1. *P. aeruginosa* was grown at 37 °C in Luria-Bertani (LB) or M9 minimal medium supplemented with D-(+)-glucose (0.2%) (M9G) in a shaking incubator at 220 r.p.m. M9 medium was supplemented as required with different

**Table 1.** Primers used in this study

Primers	Sequence 5'-3'
qRT-PCR	
534R	ATTACCGCGGCTGCTGGCA
b341	CCTACGGGAGGCAGCAG
PA2634_qRT_F	TGCGAAGAAATCTCCGAGTCC
PA2634_qRT_R	TCGGTTTCGATCCAGAGCAG
PA0482_qRT_F	GTGATCTACCGCAACTGGCT
PA0482_qRT_R	GAGGATCGCATCGTTGGTCA
PA0519_qRT_F	CTGGAACCGAAGCAGATCGT
PA0519_qRT_R	TTGACGGATGAACCTCGGGGTG
PA0523_qRT_F	TGGGAACAGAACAACTGCGT
PA0523_qRT_R	TGGAGGAAGGTGTTGAAGGCC
PA3875_qRT_F	ACGGCACCAAGCTTCTTCTAC
PA3875_qRT_R	TTGTAGTCCAGGGCGTGTTC
5'-RACE	
PA2634_RACE_sp1	TCTTCGATCAGGCCGAAAC
PA2634_RACE_sp2	TGGTGGTCTTCAGGTGCTTC
PA2634_RACE_sp3	GCGCTTCTTCTTTAGCG
oligo dT-anchor primer	GACCACGCGTATCGATGTCGAC TTTTTTTTTTTTTTTV-3', V=A,C or G
PCR anchor	GACCACGCGTATCGATGTCGAC

carbon sources: sodium acetate (0.5%) and palmitic acid (0.2%). When necessary, 2,2'-dipyridyl (DPD) was added to the final concentration described in the figure legend. Growth was monitored by measuring the OD<sub>595</sub> of liquid cultures using a BioPhotometer (Eppendorf, Germany).

### Gene expression analysis by qRT-PCR

Cells grown in M9G media to exponential phase (OD<sub>600</sub> ~0.3 control cultures and OD<sub>600</sub> ~0.5 iron-supplemented cultures) were harvested. Exponentially grown cells (OD<sub>600</sub> ~0.4 in LB) were treated with 1 mM (v/v) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 1 mM (w/v) menadione sodium bisulfite (MDs), 0.2 mM (w/v) phenazine methosulfate (PMS), 2 mM (w/v) pyocyanin (PYO), 0.2 mM (w/v) plumbagin (PL), 1 μM (w/v) ampicillin (Amp) and 0.05 μM (w/v) norfloxacin (Nor) for 15 min. Total RNA was isolated from 5 ml cultures using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. cDNA, synthesized from 1 μg of RNA with primers specific for the target gene (Table 1), was used as a template for the qRT-PCR. The PCR mixture contained 10 μl of iQ SYBR Green Supermix (Bio-Rad, USA), 1 μl of each primer (0.5 μM) and 2 μl of 100-fold diluted cDNA in a total volume of 20 μl. PCR was performed at 95 °C for 3 min, followed by 42 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C as described previously [18]. To normalize the expression of each gene, expression of 16S rDNA was quantified using primers 534R and b341 [18]. Results from triplicate experiments are presented.

### Transcriptional start site (TSS) mapping by 5' RACE

The TSS of the *aceA* gene was determined by 5' RACE using a 5'/3' RACE kit (Roche Diagnostics, Canada) following

the manufacturer's instructions. RNA was prepared as described for the qRT-PCR assay. First-strand cDNA was synthesized from 2 µg of total RNA using reverse transcriptase and the *aceA*-specific PA2634\_RACE\_sp1 primer at 55 °C for 1 h. To remove unincorporated nucleotides, first-strand cDNA was purified using a Gel/PCR Kit (Genenmed, South Korea). Next, a poly A-tail was added to the 3' end of the first-strand cDNA using a terminal transferase at 37 °C for 20 min. A first round of PCR amplification was performed using dA-tailed cDNA, oligo dT-anchor primer and another *aceA*-specific PA2634\_RACE\_sp2 primer in a 20 µl reaction. PCR was performed at 94 °C for 2 min followed by 35 cycles of 15 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C, with a final elongation step at 72 °C for 7 min. The first-round PCR product, diluted 20-fold, was used as a template in the second-round PCR with the nested PA2634\_RACE\_sp3 primer and the PCR anchor primer. PCR cycling conditions were the same as in first-round PCR, except that the annealing temperature was 63 °C. Finally, the amplified products were cloned into pGEM-T easy vector (Promega, USA). Inserts were confirmed by DNA sequencing performed by Macrogen (South Korea).

#### **Analysis of the enzymatic activities of GS-related enzymes**

Enzymatic activities were measured using the ICL Assay Kit (MyBioSource, USA), the Isocitrate Dehydrogenase (IDH) Activity Colorimetric Assay Kit (BioVison, USA) and the Succinate Dehydrogenase (SDH) Activity Colorimetric Assay Kit (BioVison, USA), following the manufacturer's instructions. Briefly, cells grown overnight in LB were washed, diluted 100-fold and inoculated into fresh M9G media. Cells in exponential phase or stationary phase were harvested, washed, resuspended in the corresponding assay buffer and sonicated. Following removal of cellular debris by centrifugation, the protein concentration in the supernatant was determined with a Bradford protein assay. To detect enzymatic activities, 10 µl of the ICL assay sample in a 200 µl reaction, 10 µl of the IDH assay sample in a 100 µl reaction and 30 µl of the SDH assay sample in a 100 µl reaction were used. Changes in absorbance were measured over time at  $A_{340}$  for the ICL assay,  $A_{450}$  for the IDH assay and  $A_{600\text{nm}}$  for the SDH assay.

#### **Analysis of intracellular iron content using inductively coupled plasma-optical emission spectroscopy (ICP-OES)**

Cells grown overnight in LB were washed, diluted 100-fold and grown to stationary phase in 300 ml of M9G media at 37 °C. Harvested cells were washed twice with 1 mM EDTA, resuspended in 100 ml of 20 mM ice-cold Tris-HCl buffer (pH 7.4), washed once with the same buffer without EDTA, resuspended in 3 ml of 20 mM Tris-HCl (pH 7.4), and sonicated. Cellular debris was removed by centrifugation at 11 000 r.p.m. for 30 min followed by filtration. Protein concentration was determined with a Bradford protein assay for sample normalization. The metal content in the supernatant was determined with a 730 series ICP-OES (Agilent, USA).

#### **Chrome azurol S (CAS) and pyoverdine assay**

Siderophore activity in culture supernatants was analysed using CAS assay. Briefly, 0.5 ml of diluted culture supernatant mixed with 0.5 ml of CAS shuttle solution was incubated at 37 °C for 30 min. Using M9 medium as reference,  $A_{630}$  was determined using a UV/Vis Spectrophotometer (Mecasys, South Korea). Siderophore activity (%) was defined as follows:  $[(A_{630} \text{ of the reference} - A_{630} \text{ of sample}) / A_{630} \text{ of reference}] \times 100$  [19]. The amount of pyoverdine produced was determined at  $A_{405}$  using 10-fold diluted culture supernatants in 100 mM Tris-HCl (pH 8.0), and was normalized by  $A_{600\text{nm}}$  [20].

#### **Analysis of intracellular chelatable iron pool**

Starter cultures of the wild-type and mutant strains grown overnight in LB were washed, diluted 100-fold and used to inoculate M9G media. When cultures reached stationary phase the cells were harvested, washed twice with PBS, followed by the addition of 20 µM Phen Green SK, dipotassium salt (PG SK) (Life Technologies, USA) and incubation at 37 °C for 30 min. Next, the cells were collected by centrifugation and the fluorescence intensity of the cell-free supernatants was measured to correct the intensities. Harvested cells were washed twice with PBS to remove any extracellular dye, and the baseline fluorescence intensity (a.u.) was measured. Fluorescence intensities at different time points were monitored using an excitation wavelength of 485 nm and an emission of 535 nm after adding DPD, a cell-permeable iron chelator [21, 22].

#### **Detection of cytochrome oxidase c activity by Nadi assay**

Spotted cells on M9G agar plates were incubated at 37 °C for 24 h. A 1:1 mixture of 1 %  $\alpha$ -naphthol in 95 % ethanol and 1 % *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride was poured onto the agar plate and removed after 15 s. Cytochrome c oxidase activity was visualized based on the formation of indophenol blue.

#### **Sensitivity tests against oxidative stress**

To determine the sensitivity of *P. aeruginosa* to oxidative stress, 20 µl of 1 M H<sub>2</sub>O<sub>2</sub> were added to paper disks on M9G agar plates containing approximately 10<sup>8</sup> cells ml<sup>-1</sup>. After 24 h of incubation at 37 °C, the bacteria-free zone around the disk was measured. For the sensitivity assays using 1 mM DPD-containing M9G plates, 10-fold serial dilutions of 10<sup>6</sup> cells ml<sup>-1</sup> were spotted onto the plate and incubated at 37 °C for 24 h.

## **RESULTS**

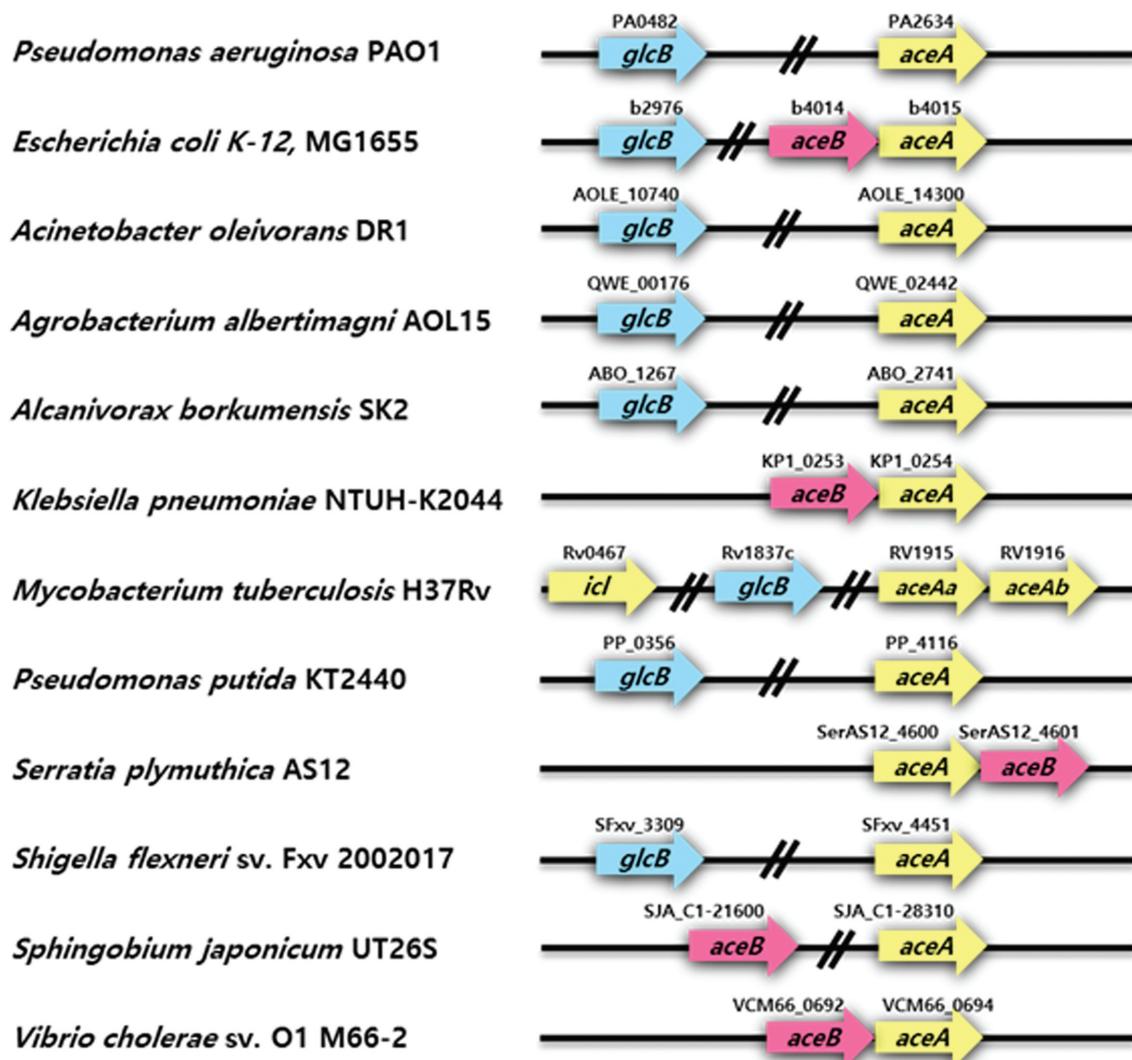
#### **Genomic distribution of GS systems in various bacteria**

The distribution of the genes encoding the ICL and MS enzymes of the GS system was investigated. In *E. coli*, the MSA encoded by *aceB* in the *aceBAK* operon is the predominant enzyme for the metabolism of the glyoxylate produced during the catabolism of acetate [8, 10, 23]. The other

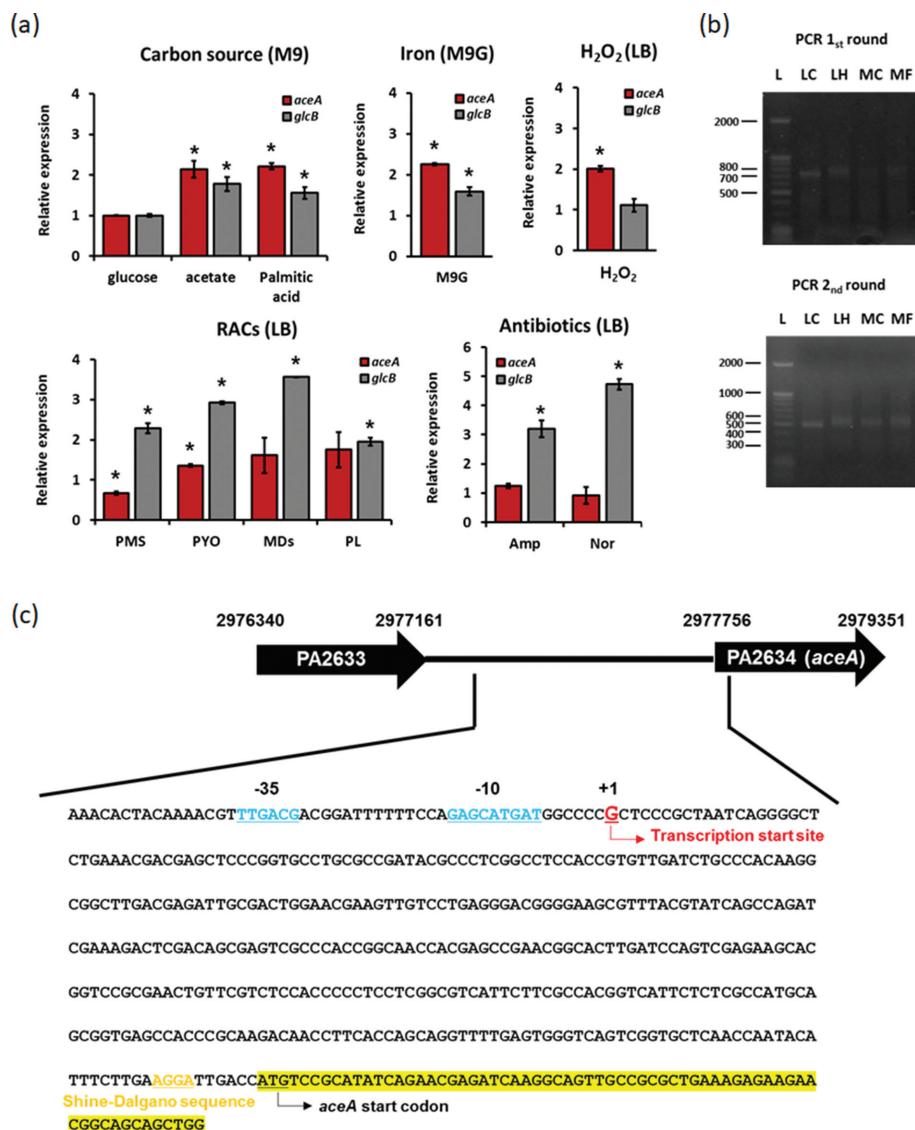
malate synthase, MSG, encoded by *glcB* in the *glcDEFGB* operon is involved in the metabolism of glycolate and glyoxylate [8, 10, 23]. Although *E. coli* possesses two MS for the metabolism of glyoxylate, most bacteria have only one of these two isoenzymes (Fig. 1). BLASTP analysis showed that the ICL of *P. aeruginosa* PAO1 (531 aa, encoded by *aceA*, PA2634) shared 27 % sequence identity with the ICL of *E. coli* (434 aa, encoded by *aceA*, b4015). On the other hand, the MS of *P. aeruginosa* PAO1 (725 aa, encoded by *glcB*, PA0482) shared 59 % sequence identity with MSG of *E. coli* (723 aa, encoded by *glcB*, b2976), but only 24 % sequence identity with MSA (533 aa, encoded by *aceB*, b4014). Around 67 % of the bacteria investigated showed an interspersed distribution of the genes of the GS system in their genomes, suggesting that they might be independently regulated (Fig. 1).

### Regulation of the GS genes under stress conditions

The GS is required for the growth of *M. tuberculosis*, *Corynebacterium glutamicum* and *E. coli* on fatty acids and acetate. Therefore, the GS genes are upregulated under these conditions. Besides, deletion of the GS genes in these bacteria results in either a limited or lack of growth on these substrates [6–8]. To study the role of the GS in *P. aeruginosa* growth on acetate or fatty acids as sole carbon sources, the relative expression levels of *aceA* and *glcB* were determined using qRT-PCR. The expression levels of these two genes in wild-type *P. aeruginosa*, grown in M9 minimal media supplemented with acetate or palmitic acid as sole carbon sources, were 1.5- to 2-fold higher than in *P. aeruginosa* grown on glucose (Fig. 2a), suggesting that the GS genes also play a role in acetate or fatty acid metabolism in *P. aeruginosa*.



**Fig. 1.** Schematic organization of the glyoxylate shunt (GS) genes in various bacteria. The GS consists of two specific enzymes: isocitrate lyase (*aceA*) and malate synthase (*aceB*, *glcB*).



**Fig. 2.** Expression and transcriptional start site (TSS) analysis of *aceA* in wild-type *P. aeruginosa* under various stress conditions. (a) Growth under different carbon sources: the relative expression levels of genes in cells grown with 0.5 % acetate or 0.2 % palmitic acid were compared to those in cells grown on 0.2 % glucose. Presence or absence of iron: the relative expression levels of genes in cells grown in M9G media, with no iron, were compared to those in cells grown in M9G media containing 0.1 mM FeCl<sub>3</sub>. H<sub>2</sub>O<sub>2</sub>, RACs and antibiotics: the relative expression levels of genes in treated cells were compared to those in untreated cells. Error bars indicate SD. Significant differences with a *p*-value of less than 0.05 based on *t*-test comparisons are marked with a single asterisk (\**P*<0.05). Reagents were used at the following final concentrations: 1 mM H<sub>2</sub>O<sub>2</sub>, 0.2 mM PMS, 2 mM PYO, 1 mM MDs, 0.2 mM PL, 1 μM Amp and 0.05 μM Nor. (b) PCR products of the *aceA* transcripts. Lane L, ladder; lane LC, LB control; lane LH, LB treated with 1 mM H<sub>2</sub>O<sub>2</sub> for 15 min; lane MC, M9G control; lane MF, M9G with 0.1 mM FeCl<sub>3</sub>. (c) The identical TSS of the *aceA* gene under all tested conditions was confirmed by sequencing.

The GS has been linked to bacterial virulence and to the response to environmental stress in different bacteria [13, 14]. Therefore, we investigated the expression of the GS genes under various environmental stresses [including iron starvation, H<sub>2</sub>O<sub>2</sub>, redox-active compounds (RACs) and antibiotics] in *P. aeruginosa*. M9G minimal medium was used in these experiments as the iron-limiting condition. Cells grown in M9G media showed an increased

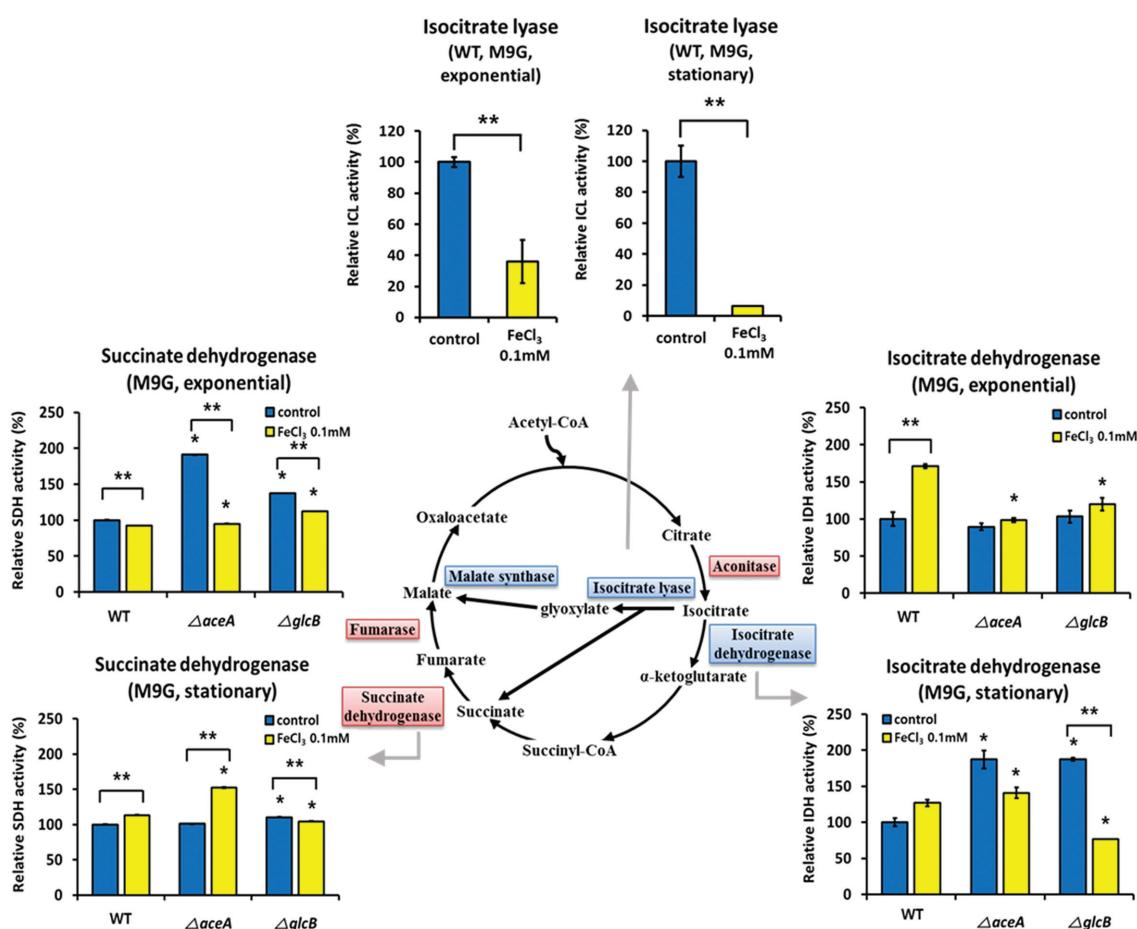
expression of both GS genes compared to cells grown in FeCl<sub>3</sub>-supplemented M9G media, containing sufficient iron to fulfil the iron demand of the cells (Fig. 2a). On the other hand, treatment of *P. aeruginosa* with H<sub>2</sub>O<sub>2</sub> resulted in a 2-fold increase in *aceA* expression while expression of *glcB* stayed at basal levels (Fig. 2a). Conversely, the addition of exogenous RACs and antibiotics increased *glcB* expression without affecting the expression of *aceA*.

(Fig. 2a). This is in contrast to the increased expression levels of both *aceA* and *glcB* observed in response to Amp and Nor, but not tetracycline and kanamycin, in *Acinetobacter* species [24].

TSS mapping of the *aceA* gene was carried out by 5' RACE under the above-mentioned upregulated conditions ( $H_2O_2$ -induced oxidative stress and iron starvation). The sequencing results showed that the TSS was the same under all tested conditions (Fig. 2b), and it was localized 393 bp upstream of the start codon. This position was very close to that predicted by a promoter-predicting program (<http://www.fruitfly.org>), with only one nucleotide of difference. Putative –10 (GAGCATGAT) and –35 (TTGACG) regions with a spacing region of 14 bp were identified (Fig. 2c). Overall, our data strongly suggest that the GS is involved in the defence of *P. aeruginosa* cells against environmental stresses such as iron starvation and oxidative stress.

### Analysis of the enzymatic activity of GS-related enzymes

Next, we measured the activity of ICL to determine whether the observed transcriptional upregulation of *aceA* under iron starvation resulted in changes at the protein level. As shown in Fig. 3, the ICL activity in *P. aeruginosa* grown in the presence of iron was low, regardless of the growth phase. In contrast, a 2.3-fold increase in *aceA* expression and a 2.7-fold increase in ICL activity were observed during the exponential phase of *P. aeruginosa* grown under iron-limiting conditions, in M9G media (Figs 2a and 3). Remarkably, ICL activity increased 15-fold in stationary phase, suggesting a growth phase-dependent regulation of ICL. The reason for the upregulation of *aceA* under iron-limiting conditions is currently unclear. Interestingly, previous reports have shown an increase in both gene expression and enzymatic activity of ICL, in *Alteromonas macleodii* and SAR11 in marine environments with very low iron solubility [25, 26].



**Fig. 3.** Analysis of iron-dependent carbon flux through the TCA cycle and the GS using GS mutants. Error bars indicate SD. Significant differences with a *p*-value of less than 0.05 based on *t*-test comparisons are marked with a single or a double asterisk (\**P*<0.05, comparison of enzyme activities between different strains grown under identical conditions; \*\**P*<0.05, comparison of enzyme activities for a given strain grown under different conditions).

Since ICL competes with IDH for their common substrate isocitrate, the metabolic flux between the GS and the TCA cycle is controlled by the activities of these two enzymes in *E. coli* [27]. For instance, the activity of *A. macleodii* IDH is lower under iron-limiting conditions [25], and therefore we studied the activity of *P. aeruginosa* IDH under those conditions. The highest enzymatic activity of IDH was observed during the exponential growth phase, in iron-supplemented media, in the wild-type *P. aeruginosa* strain. Significant differences were not observed between the two GS mutants, regardless of the presence of iron in the media. However, both mutants showed lower IDH activity than the wild-type strain when growing in iron-supplemented media. During stationary growth phase, IDH activity was lower in the wild-type strain compared to the exponential phase, but it was generally higher in the GS mutants. Only the *glcB* mutant showed a significant downregulation of IDH activity upon iron supplementation. Both GS mutants showed higher IDH activities than the wild-type strain during stationary phase, except for the *glcB* mutant grown under iron-supplemented conditions. This suggests that deletion of the GS genes redirects the carbon flux to the TCA cycle during the stationary phase (Fig. 3).

SDH is an important enzyme for the study of iron-dependent metabolic fluxes, since it contains an Fe-S cluster and it is an enzyme of the respiratory chain. Therefore, we next studied the activity of SDH during growth under different iron conditions. We hypothesized that the growth of *P. aeruginosa* in the presence of iron would lead to high SDH activity. Indeed, during the stationary phase, in iron-supplemented media, higher SDH activity was observed in the wild-type and *aceA* mutant strains (Fig. 3). However, during the exponential phase, iron addition decreased SDH activity in all the strains tested (Fig. 3). A previous study had shown a differential regulation of *sdhCDAB* in *Helicobacter* by the Ferric-uptake regulator (Fur) depending on the growth phase [15, 28]. The different response of SDH activity to iron addition dependent on the growth phase observed here in *Pseudomonas* could also be related to a differential regulation by Fur. In any case, the ICL enzymatic results support our findings that metabolic flux to the GS decreases when there is sufficient iron in the media.

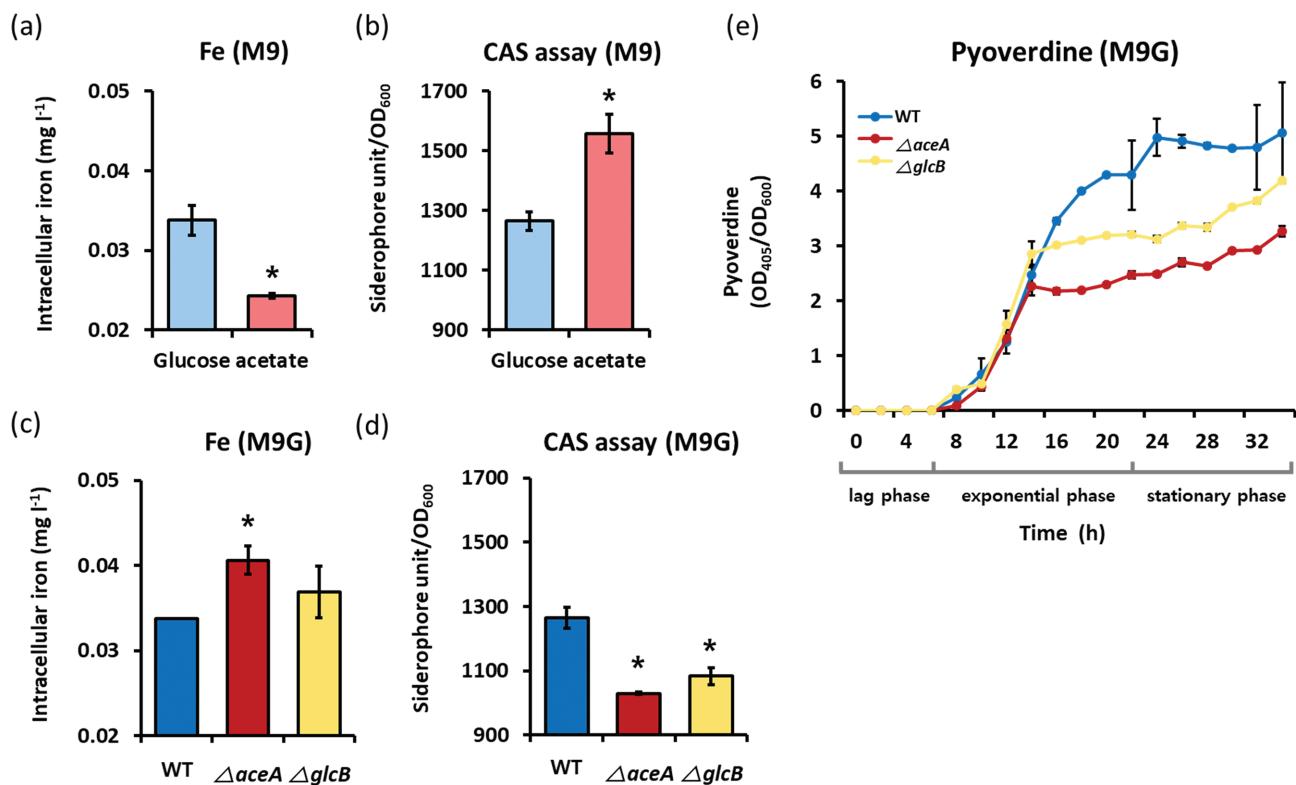
#### Intracellular iron pool and iron demand analysis

We next compared the intracellular and chelatable iron content in *P. aeruginosa* grown on glucose or acetate as carbon sources. We found that when acetate was used as the sole carbon source, the intracellular iron content was significantly lower while the synthesis of siderophores increased (Fig. 4a, b). There was a negative correlation between intracellular iron content and siderophore synthesis, suggesting that the acetate-triggered GS could result in a decreased intracellular content, which would increase iron demand. To further investigate this, we determined the effect of deletion of the GS genes on the intracellular iron pool, the amount of cellular iron and synthesized siderophore, as an indicator of the iron requirements for cellular metabolism.

Our results revealed that the intracellular iron content of the *aceA* mutant was higher and the iron demand lower than those of the wild-type strain (Fig. 4c, d). This suggests that deletion of the GS genes dysregulates the intracellular iron pool. To analyse growth phase-dependent changes on iron demand, the amount of synthesized pyoverdine, one of the two siderophores of *P. aeruginosa*, was monitored over time. The amount of pyoverdine produced by the wild-type strain and the GS mutants was similar up to mid-exponential phase. The differences in the amount of pyoverdine synthesized steadily increased up to early stationary phase (Fig. 4e). These results suggest that deletion of the GS genes not only affects the intracellular iron pool, but also the cellular chelatable iron pool. To further test this possibility, we measured the chelatable intracellular iron, which is a small fraction of the intracellular iron that comprises two types of iron: that associated with low molecular weight and affinity chelators [21, 22], and that loosely attached to proteins [21, 22]. To measure the intracellular chelatable iron, a fluorescent dye, PG SK, was used. The fluorescence of this cell-permeable dye decreases upon binding to iron. Therefore, the baseline fluorescence intensity (a.u.) was first measured, followed by measurement of fluorescence quenching after adding the cell-permeable iron chelator, DPD. The relative fluorescence intensity of the  $\Delta aceA$  ( $14.21\% \pm 3.71$ ) and  $\Delta glcB$  ( $67.72\% \pm 18.36$ ) strains greatly increased compared to that of the wild-type strain ( $2.69\% \pm 0.24$ ) (Table 2). Taken together, these results demonstrate that deletion of GS dysregulates iron homeostasis by modulating the intracellular iron content, iron demand and the chelatable iron pool.

#### Effect of deletion of GS genes on the respiratory chain and sensitivity to oxidative stress

The growth curves of PAO1 cells in the presence or absence of iron showed that deletion of the GS genes resulted in a higher growth rate in M9G media, possibly because of the higher intracellular iron content of the mutant strains (Fig. 5a). Nevertheless, the addition of iron resulted in a greatly enhanced cellular growth indicating that cells grown in M9G media were iron deprived (Fig. 5a). In a previous study, we had shown that paraquat (PQ) treatment of *P. aeruginosa* GS mutants induced a metabolic shift towards aerobic denitrification that renders them more resistant to PQ-induced superoxide stress in LB media [29]. To study whether the GS mutants use aerobic denitrification under iron-limiting conditions, the expression of denitrification genes, including *nirS* (PA0519), *norC* (PA0523) and *narg* (PA3875), was analysed. However, a shift to aerobic denitrification in the GS mutants was not observed under M9G iron-limiting conditions, suggesting that the increased growth of these mutants under this condition was not due to aerobic denitrification (Fig. 5b). Using transcriptome analysis, we had previously shown that expression of the *bo3* oxidase gene (*cyo*, PA1317-PA1321) in *P. aeruginosa* treated with 1 mM PQ increased 4–10-fold, whereas in GS mutants treated with PQ the upregulation was just 1.2–4.0-fold [29]. In addition, comparison of the wild-type and mutant strains



**Fig. 4.** Changes in intracellular total iron pool, iron demand and chelatable iron pool in GS mutants. (a, c) Total intracellular iron content in wild-type (a) and GS mutant (c) cells grown on glucose or acetate as sole carbon source was measured using ICP-OES, and normalized to protein concentration. Data are presented as the mean of three independent cultures. (b, d) Siderophore synthesis by wild-type (b) and GS mutant (d) cells grown on glucose or acetate as sole carbon source was measured. Error bars indicate SD. Significant differences with a *p*-value of less than 0.05 based on *t*-test comparisons are marked with a single asterisk. (e) Synthesis of pyoverdines in wild-type,  $\Delta aceA$  and  $\Delta glcB$  grown in culture broth with shaking at 220 r.p.m. was measured over time.

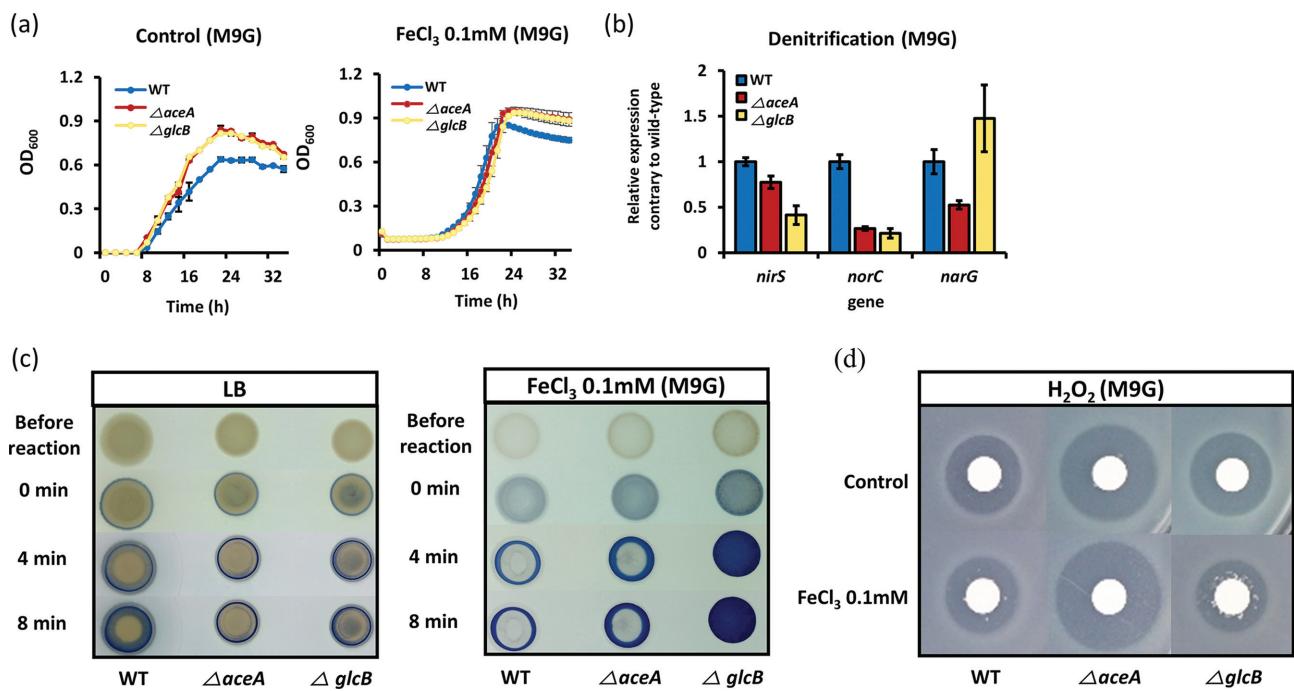
showed a 0.5~1.2-fold decrease in the expression of two cytochrome c oxidases (*cbb3* oxidase 1, *ccol*, PA1552-PA1554; *cbb3* oxidase 2, *cco2*, PA1555-PA1557) in the mutants, indicating that deletion of the GS might have altered the respiratory strategy of the cells [29]. In the current study, these results were corroborated using the Nadi assay which showed that wild-type cells grown on LB agar plates had higher cytochrome c oxidase activity than GS mutants (Fig. 5c). In contrast, significant differences were not observed in the cytochrome c oxidase activity of these strains grown on M9G agar plates (Fig. 5c), while in the presence of iron, both GS mutants generated a more visible colour change than the wild-type strain (Fig. 5c). Therefore, deletion of the GS genes resulted in an increased activity of

cytochrome c oxidase, a major terminal oxidase, under normal laboratory conditions.

Iron is known to be an important factor in the regulation of the cellular response against oxidative stress [30]. The amount of intracellular chelatable iron is directly related to defence against oxidative stress because it can react with  $H_2O_2$ , generating highly reactive oxygen species such as hydroxyl radicals [30]. The increase in intracellular chelatable iron in the GS mutants suggested that these mutants might be more sensitive to  $H_2O_2$ -induced oxidative stress compared to the wild-type strain (Table 2). Indeed, the sensitivity of the GS mutants to high  $H_2O_2$  concentrations was greater than that of the wild-type strain, with  $\Delta aceA$

**Table 2.** Measurement of the intracellular chelatable iron pool

Strain	Reduced fluorescence in supernatant (%/30 min)	Baseline fluorescence intensity (a.u.)	Maximal fluorescence after 2,2'-DPD (a.u.)	Increased fluorescence (a.u.)	Relative increase (%)
WT	5.85±0.39	10362.12±79.78	11990.86±22.73	1628.74±95.79	2.69±0.24
$\Delta aceA$	6.55±1.63	11659.29±322.94	21828.31±136.88	10169.02±405.01	14.21±3.71
$\Delta glcB$	2.93±0.75	11342.84±822.60	33898.69±973.50	22555.85±1471.68	67.72±18.36



**Fig. 5.** Enhanced growth of GS mutants resulted from respiratory changes and implies an increased sensitivity to oxidative stress. (a) Growth of wild-type and GS mutants was measured at OD<sub>600</sub>. (b) Analysis of the expression of the denitrification genes in cells grown in M9G media. To confirm whether growth in M9G media induced denitrification, the expression levels of *nirS*, *norC* and *narG* in the GS mutants were compared to those in the wild-type. (c) The activity of the cytochrome c oxidase was visualized using the Nadi reaction. Colonies on LB, M9G or 0.1 mM FeCl<sub>3</sub>-supplemented M9G agar plates were treated with Nadi reagents for 15 s. The degree of colour change indicates the activity of cytochrome c oxidase. (d) M9G agar plates were inoculated with 10<sup>8</sup> cells ml<sup>-1</sup> of the wild-type strain or the GS mutants. Then, 10 µl of 1 M H<sub>2</sub>O<sub>2</sub> were added to a paper disk on the M9G agar plate and the inhibition zones were measured after 24 h of incubation at 37 °C.

presenting the highest sensitivity, which further increased upon iron addition (Fig. 5d). The inhibition zone of  $\Delta glcB$  was larger than that of the wild-type strain regardless of the presence or absence of iron (Fig. 5d). Taken together, these results indicate that deletion of GS can result in enhanced growth in iron-supplemented media as a result of a higher cytochrome c oxidase activity, an energy-efficient respiratory terminal oxidase. However, it can also render cells more sensitive to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress.

#### Analysis of the GS under DPD-induced iron-deficiency stress

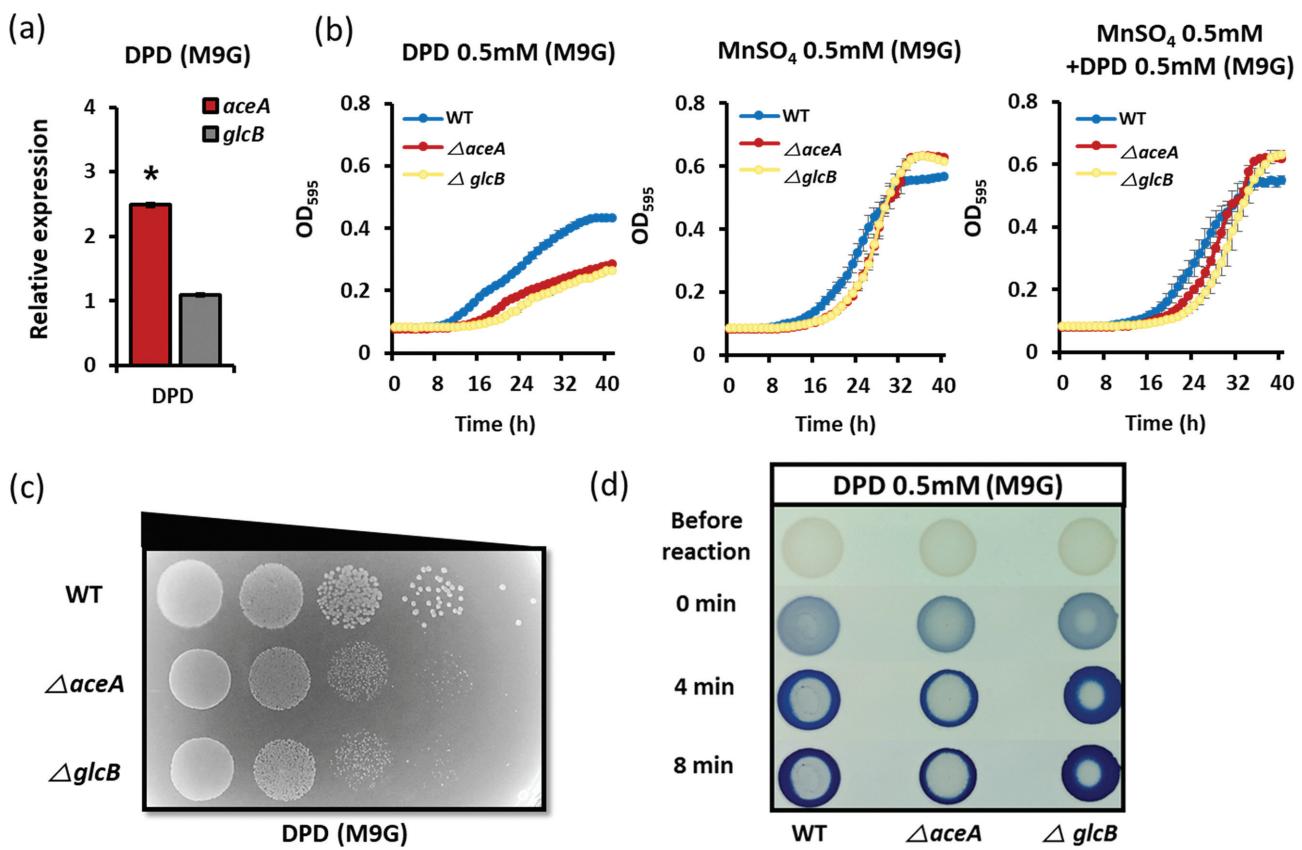
To better understand the link between iron and the GS, iron-deficiency was created by DPD treatment. A 2.5-fold increase in the expression of *aceA* was observed in wild-type cells grown in DPD-containing M9G compared to cells grown in M9G media (Fig. 6a). Manganese is not a major element during normal unstressed cellular metabolism. However, under strict iron-deficient conditions, manganese acts as a substitute for iron, facilitating cell survival [31, 32]. In *E. coli*, when iron is present in abundance, Fe<sup>2+</sup>-metallated Fur can repress transcription of *mntH*, which encodes a manganese importer that is activated by OxyR, by binding to its promoter region. However, under iron-deficient conditions, Fur can no longer bind to *mntH* or interfere with its

transcription, suggesting that manganese can partially replace iron in the cell [31, 32]. Both GS mutants showed more severe growth defects than the wild-type strain under iron-deficient conditions (Fig. 6b, c). A slightly longer lag phase was observed when cells were grown at high concentrations of manganese.

Interestingly, the DPD-induced growth defect was recovered by manganese treatment, indicating that manganese can override the stress caused by iron deficiency (Fig. 6b). In contrast to what was observed during growth on iron-supplemented media, DPD treatment resulted in a higher cytochrome c oxidase activity in the wild-type strain than in  $\Delta aceA$ , but lower than in the  $\Delta glcB$  strain, which presented the highest cytochrome c oxidase activity of all three strains (Fig. 6d). Therefore, our results show that the GS mutants are more sensitive to iron deprivation, demonstrating a more severe growth defect in both culture broth and agar plates, and that manganese can act as an iron substitute under iron-deficient conditions.

#### DISCUSSION

To fully understand the survival mechanisms of pathogenic bacteria within host cells, it is important to identify the metabolic processes utilized by bacteria to metabolize



**Fig. 6.** Effect of iron deficiency on GS mutants. (a) Relative expression levels of the GS genes in the wild-type strain grown in M9G media compared to those in cells grown in M9G media containing 0.1 mM DPD. Significant differences with a *p*-value of less than 0.05 based on t-test comparisons are marked with a single asterisk. (b) GS mutants showed a more severe growth defect under iron-deficiency conditions. Growth was restored by the addition of manganese. (c) Sensitivity to iron deficiency was measured using 1 mM DPD on M9G agar plates. (d) Nadi assay using DPD-treated M9G agar plates.

the nutrients available inside the host cell. For instance, CF sputum, a major infection site of *P. aeruginosa*, has an altered composition characterized by high levels of fatty acids such as palmitic acid and oleic acid [2]. Thus, in the CF lung, the GS, required for growth on fatty acids and acetate, would be more important than the TCA cycle. Our bioinformatics analysis showed that the genes encoding the GS enzymes are located distant from each other in the genome of many bacteria (Fig. 1), suggesting that these genes might be differentially regulated under various stresses. In that sense, we have shown that the GS likely contributes to the bacterial response against environmental stress, at least for all the stresses tested in the present study. We have shown that *aceA* protects against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and iron-deficiency stress by specifically upregulating its own expression under these conditions (Fig. 2a).

Many studies have investigated the function and regulation of ICL during stress defence in *E. coli* [14, 33–39]. However, regulation of the GS in *P. aeruginosa* has been little studied. In *E. coli*, the proteins responsible for the regulation of the

*aceBAK* operon include FadR, IclR, the integration host factor (IHF) and the catabolite repressor/activator (Cra, previously known as FruR). Specifically, IclR, whose gene is adjacent to the *aceBAK* operon, negatively regulates the operon by binding to specific operator sequences known as IclR boxes, thus interfering with binding of the RNA polymerase [33]. FadR, a regulator of fatty acid metabolism, is indirectly involved in the regulation of the GS by activating the expression of the IclR repressor [34–36]. IHF causes a bend in the DNA by binding to specific sequences, and activates the expression of the *aceBAK* operon by binding to one of the two IHF binding sites present in the upstream region of the *aceBAK* promoter [37]. Cra controls the GS by binding to the promoter of the gene encoding IDH and of the *aceBAK* operon [38, 39].

Fur, a major regulator of the iron acquisition system, also represses genes in *E. coli* (*cyoA*, *fumC*, *gpmA*, *purR* and *sodA*) with functions not related to iron acquisition, including respiration, the TCA cycle, glycolysis, purine metabolism and redox stress resistance [15]. In addition, Fur is indirectly involved in the induction of several genes such as

*acnA*, *fumA*, *fumB*, *sdhCDAB* and *sodB* [15]. Besides, Fur mediates indirect positive regulatory effects by controlling other transcriptional factors such as the small RNAs (sRNAs) RhyB in *E. coli*, *Vibrio cholerae* and *Shigella*, NrrF in *Neisseria meningitidis* and *N. gonorrhoeae*, and PrrF1 and PrrF2 in *P. aeruginosa* [40–44]. The iron-regulated PrrFs sRNAs in *P. aeruginosa* are known to regulate proteins involved in the TCA cycle such as the aconitase and citrate synthase, and to interact with the CrcZ sRNA, which is involved in post-transcriptional regulation of the cognate gene [45, 46]. In addition, PrrFs have been associated with quorum sensing. Production of the *Pseudomonas* quinolone signal decreased significantly in a  $\Delta$ prrF1,2 mutant compared to the wild-type as a result of increased degradation of anthranilate [47]. The *E. coli* RhyB is a regulator negatively controlled by Fur, involved in the negative regulation of enzymes of the TCA cycle such as the aconitase (*acnA*), fumarase (*fumA*) and succinate dehydrogenase (*sdhCDAB*), by binding and inducing degradation of their mRNAs [41]. RsbU in *Staphylococcus aureus* controls the central metabolism. Specifically, RsbU is a regulator of acetate catabolism whose activation by Fur results in downregulation of the TCA cycle during iron starvation [48]. Transcriptome analysis and beta-galactosidase assays have shown that iron limitation can increase the expression of *cyoABCDE* in *P. aeruginosa* [49, 50]. Further analyses of Fur and other regulators, along with several known sRNAs, are necessary to understand the mechanism of the GS system under iron limitation in *P. aeruginosa*.

In the present study, enzymatic activities were also measured to determine whether regulation of the expression of *aceA* by iron resulted in changes at the protein level and shifted the carbon flux through the TCA cycle. Our results showed a decreased metabolic flux to the GS under iron supplementation conditions (Fig. 3). Recent reports have shown that ICL is upregulated in *Alteromonas macleodii* and the SAR11 clade living in marine environments where iron availability is very low [25, 26]. It has been speculated that the iron limitation in seawater might have resulted in the development of a metabolic strategy to reduce the expression levels of Fe-S cluster-containing enzymes of the TCA cycle (aconitase, succinate dehydrogenase and fumarase) or the respiratory chain [15, 25, 26, 51–54]. Changes in the respiratory chain are especially relevant, because most of the cellular iron (94 %) is allocated to components of the respiratory chain, such as succinate-Q reductase, NADH-Q reductase, cytochrome oxidase complexes and cytochrome  $b_1$ , in *E. coli* [55]. *P. aeruginosa* can selectively use five terminal oxidases during aerobic respiration depending on the composition of its environment [56–60]. First, the electron donor donates electrons to ubiquinone, which are then transported to quinol oxidases such as the  $bo_3$  oxidase (Cyo) and the cyanide-insensitive oxidase (CIO). Electrons transported from the ubiquinone to the  $bc_1$  complex, rather than to quinol oxidases, are delivered to cytochrome c and passed on to cytochrome c oxidases such as  $aa_3$  oxidase (Aa3),  $cbb_3$  oxidase 1 (Cbb3-1) and  $cbb_3$  oxidase 2 (Cbb3-2) [56, 61].

Under iron-starvation conditions, Aa3 and Cytochrome c are upregulated [61], which suggests that Cytochrome c might be able to accept electrons from ubiquinone rather than from the iron-containing cytochrome  $bc_1$  complex or the soluble cytochrome c [61]. It is worth noting that glyoxylate induces phenotypic tolerance by inhibiting cellular respiration with acetyl-coenzyme A diversion through the GS [62]. Our data demonstrate that deletion of the GS genes results in dysregulated cellular iron homeostasis by modulation of the intracellular total iron pool and iron demand (Fig. 4c, d). We had previously shown that the *aceA* mutant of *P. aeruginosa* undergoes a metabolic shift towards aerobic denitrification upon PQ treatment, becoming more resistant to PQ-induced superoxide stress [29]. However, in the present study, the *aceA* mutant showed increased sensitivity to oxidative stress induced by a high concentration of H<sub>2</sub>O<sub>2</sub> (Fig. 5d). This could have been due to the distinct mechanisms of action of H<sub>2</sub>O<sub>2</sub> and superoxide, and to differences in the media and the concentrations of the reagents used [29]. The high concentration of H<sub>2</sub>O<sub>2</sub> used could have affected the cells by directly interacting with the intracellular iron pool, which would not have given them time to change their metabolic strategies.

Taken together, our results demonstrate that the genes of the GS in *P. aeruginosa* are differentially regulated under stress conditions and that *aceA* plays a role in the defence of *P. aeruginosa* against iron starvation, by regulating the intracellular iron pool and the composition of the respiratory chain. Additional research is needed to determine the correlation between *aceA* and the respiratory chain, which would ultimately help to better understand the metabolic strategies used by pathogenic bacteria to survive within the host.

#### Funding information

This work was supported by a grant (NRF-2017R1A2B4005838 to WP) of the National Research Foundation of Korea (NRF). WP and BS were supported by a Korea University grant (K1625751).

#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### Ethical statement

No ethics or consent approval was required for the research in this study.

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