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A Redox Imbalance Underlies the Fitness Defect Associated with Inactivation of the Pta-AckA Pathway in *Staphylococcus aureus*

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Abstract

The phosphotransacetylase-acetate kinase (Pta-AckA) pathway is thought to be a vital ATP generating pathway for *Staphylococcus aureus*. Disruption of the Pta-AckA pathway during overflow metabolism causes significant reduction in growth rate and viability, albeit not due to intracellular ATP depletion. Here we demonstrate that toxicity associated with inactivation of the Pta-AckA pathway resulted from an altered intracellular redox environment. Growth of the *pta* and *ackA* mutants under anaerobic conditions partially restored cell viability. NMR metabolomics analyses and ¹³C₆-glucose metabolism tracing experiments revealed the activity of multiple pathways that promote redox (NADH/NAD⁺) turnover to be enhanced in the *pta* and *ackA* mutants during anaerobic growth. Restoration of redox homeostasis in the *pta* mutant by overexpressing L-lactate dehydrogenase, partially restored its viability under aerobic conditions. Together our findings suggest that during overflow metabolism the Pta-AckA pathway plays a critical role in preventing cell viability defects by promoting intracellular redox homeostasis.

Graphical abstract

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Supporting information

The Supporting Information is available free of charge on the ACS Publications website. Supplementary figures demonstrating that the inactivation of the Pta-AckA pathway impairs growth of *S. aureus*.

Author Contributions

D.D.M., M.R.S., V.C.T., R.P. and K.W.B. designed the experiments, analyzed the data and wrote the manuscript; D.D.M., M.R.S. and V.C.T. performed the experiments.

Notes

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Keywords

Pta-AckA pathway; metabolomics; redox imbalance; NMR; *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a versatile human pathogen responsible for a variety of infections that range from folliculitis to life-threatening diseases such as severe sepsis, endocarditis and bacteremia.^{1–3} *S. aureus* infections represent an enormous challenge to physicians because of the emergence and dissemination of multidrug-resistant strains in the health care setting.^{1, 2} The ability of this bacterial pathogen to survive and efficiently colonize diverse host environments is based on its proficiency to optimize virulence factor production and adjust its metabolism to rapid environmental changes.^{4–9}

When grown under aerobic conditions, *S. aureus* primarily metabolizes glucose to acetate through the Pta-AckA pathway. In a recent study, we observed that inactivation of the Pta-AckA pathway, resulted in poor growth and reduced viability at the exponential phase. Although carbon flux through the Pta-AckA pathway is used to generate ATP, the aerobic growth and viability defects associated with the *pta* and *ackA* mutants could not have resulted from ATP depletion as these strains surprisingly exhibited increased levels of intracellular ATP, presumably through increased glycolytic flux and redirection of carbon into the TCA cycle.¹⁰ One possibility for the altered growth and viability defects associated with the *pta* and *ackA* mutants may arise from an altered intracellular redox environment resulting from inflated concentrations of NAD⁺ and NADH.¹⁰

In the present study, we address this hypothesis by comparing the growth characteristics and metabolic changes in the *pta* and *ackA* mutants relative to their isogenic wild-type strain following cultivation under aerobic and anaerobic conditions. We demonstrate that although growth defects due to *pta* and *ackA* mutations persisted under both aerobic and anaerobic growth, cell viability relative to the wild type strain could be partially restored under fermentative growth. Metabolic differences associated with fermentative growth of the *pta* and *ackA* mutants were determined by NMR spectroscopy and the observed metabolic changes support a role for the normalization of cellular redox status in the restoration of cell viability in the *pta* and *ackA* mutants. Finally, confirming a strong relationship between cellular redox status and cell viability, the increased cell death in the *pta* mutant under

aerobic growth could be partially restored to the wild-type levels by increasing NADH turnover following overexpression of the *ldh1* gene in this mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The construction of the *ackA* and *pta* mutants in *S. aureus* strain UAMS-1 was described previously.¹⁰ The plasmid, pMRS110, containing the *ldh1* gene under the control of the cadmium-inducible promoter P_{cadC} was constructed by amplifying 1-kb region from the *S. aureus* UAMS-1 chromosome using the primers, PstI-RBS-ldh1-f (GTCTCTGCAGCATAAGGAGGAATTTGTAATGAACAAATTTAAAGGGAACAAAGT) and ldh1-r (GGGGTAAGGTTTTACAATTTTGGGAATGG), and inserting the resulting DNA fragment into the PstI and SmaI sites of the shuttle vector, pBK123.¹¹

S. aureus strains were grown in tryptic soy broth (TSB) without dextrose (BD Biosciences) supplemented with 0.25% glucose (Sigma-Aldrich) or on TSB containing agar. *S. aureus* cultures for both aerobic and anaerobic conditions were inoculated to 0.06 optical density at 600 nm (OD₆₀₀) units from overnight cultures (grown in tryptic soy broth (TSB) without dextrose (BD Biosciences)), incubated at 37°C, and aerated at 250 rpm with a flask-to-medium ratio of 10:1. Bacterial growth was assessed by measuring the optical density at 600 nm or by determining the number of colony forming units (cfu) ml⁻¹. Chloramphenicol was purchased from Fisher Scientific and was used at a final concentration of 10 µg/ml.

Measurement of extracellular glucose, acetic acid, and D, L- lactate

Aliquots of bacterial cultures (1 ml) were centrifuged for 3 min at 14,000 rpm at 4°C. The supernatants were removed and stored at -20°C until use. Acetate, glucose, and D- and L- lactate concentrations were determined using kits purchased from R-Biopharm according to the manufacturer's protocol.

Determination of intracellular ATP concentrations

Intracellular ATP concentrations were determined using the BacTiter-Glo kit (Promega). The kit was used according to the manufacturer's directions and as previously described.¹⁰ Metabolite concentrations were normalized to the number of viable-cell counts.

NMR metabolomics sample preparation

Samples for one-dimensional (1D) ¹H NMR and two-dimensional (2D) ¹H-¹³C heteronuclear single quantum coherence (HSQC) experiments were prepared from six and three replicate 50-mL cultures, respectively. *S. aureus* wild type strain UAMS-1, and mutant strains UAMS-1-*ackA*, and UAMS-1-*pta* were grown aerobically or anaerobically in TSB containing 0.25% glucose for 1D ¹H NMR experiments or 0.25% [¹³C₆] glucose (Sigma-Aldrich) for 2D ¹H-¹³C HSQC experiments. Bacterial cells were harvested during the exponential growth phase (3 h) after reaching an optical density of 10 OD₆₀₀ units. Samples were kept on ice through the entire preparation protocol. Bacterial cultures were centrifuged at 4°C for 7 min at 4,100 rpm and then washed with ice cold 50 mM phosphate buffer at pH 7.1. Enzymatic activity were quenched by suspending the cells in 700 µL of ice cold ethanol

[60% ethanol, 40% D₂O (Isotec)].¹² The cells were then lysed using lysing matrix B tubes in a FastPrep instrument (Qbiogene). The lysates were centrifuged at 4°C for 5 min at 14,000 rpm to remove the cell debris. The samples were then lyophilized and suspended in 600 µL of 99.8% D₂O phosphate buffer at pH 7.1 (uncorrected) containing 50 mM of (trimethylsilyl) propionic-2, 2, 3, 3-D₄ acid sodium salt (TMSP-D₄) for 1D ¹H NMR experiments or 500 mM of TMSP-D₄ for 2D ¹H-¹³C HSQC experiments. Samples were then transferred to 5 mm NMR tubes for data collection.

NMR data collection

1D and 2D NMR data collection and analysis were performed as described previously.^{13, 14} Briefly, all NMR spectra were collected on a Bruker Avance DRX 500 MHz spectrometer equipped with a 5-mm triple-resonance (¹H, ¹³C, ¹⁵N), Z-axis gradient cryoprobe. Automated data collection utilized a Bruker ATM unit for automatic tuning and matching, a BACS-120 sample changer and Bruker Icon NMR software. The 1D ¹H NMR spectra were collected using excitation sculpting for water suppression.¹⁵ Six replicates were acquired at 298.15 K with 128 scans, 16K data points, 16 dummy scans, a relaxation delay of 1.5 s, a spectral width of 5000 Hz, and a total acquisition time of approximately 7 minutes. 2D ¹H-¹³C HSQC NMR spectra were collected at 298.15 K with 128 scans and a relaxation delay of 1.5 s. The spectra were collected with 2K data points and a spectrum width of 5,000 Hz in the direct dimension, and 64 data points and a spectrum width of 17,605.6 Hz in the indirect dimension, and a total acquisition time of approximately 4 hours. The 2D ¹H-¹³C HSQC NMR spectra were processed in NMRPipe and analyzed with NMRViewJ version 9.^{16, 17}

Multivariate Statistical Analysis

The 1D ¹H NMR spectra were processed in our MVAPACK software suite (<http://bionmr.unl.edu/mvapack.php>).¹⁸ For principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA), all spectra were processed with a 1.0 Hz exponential apodization function prior to being Fourier transformed, automatically phased and normalized using our phase-scatter correction (PSC),¹⁹ referenced to TMSP-D₄ (0.0 ppm), and scaled using Pareto scaling. For PCA, spectra were binned using an adaptive intelligent binning algorithm that minimizes splitting signals between multiple bins.²⁰ Spectral regions containing noise or solvent signals were removed manually.²¹ For OPLS-DA, full-resolution spectra were used to build the model after alignment with the icoshift algorithm²² implemented in MVAPACK. OPLS-DA models were calculated using one predictive and one orthogonal component and were cross-validated using a Monte Carlo leave-*n*-out (MCCV) procedure.²³ The R^2_y (degree of fit) and Q^2 (predictive ability) metrics of 0.99236 and 0.97135 for UAMS-1, 0.98905 and 0.96931 for UAMS-1-*ackA*, and 0.95065 and 0.87454 for the UAMS-1-*pta*, respectively, indicated high-quality OPLS-DA models. Model validation by CV-ANOVA²⁴ indicated reliable models with p values of 3.1×10^{-3} , 2.6×10^{-4} , and 7.3×10^{-5} for the UAMS-1, UAMS-1-*ackA* and UAMS-1-*pta* data, respectively. For all strain comparisons, response permutation tests for OPLS-DA model validation also returned p values of less than 0.01. The OPLS-DA scores plots and back-scaled loadings were calculated using the entire NMR spectrum (no binning). In this manner, the back-scaled loadings plot resembles a traditional 1D ¹H NMR spectrum,

where peaks (metabolites) abundant under anaerobic conditions are positive and peaks (metabolites) abundant under aerobic conditions are negative.

Metabolite identification

The metabolites significantly contributing to class separation in the OPLS-DA scores plot were identified using the Chenomx NMR Suite 8.0. Simply, the experimental 1D ^1H NMR spectra were assigned using the Chenomx software and then the back-scaled loadings plot was overlaid with the assigned 1D ^1H NMR spectrum to assign the major features in the back-scaled loadings plot.

Metabolite identification using 2D NMR datasets was performed as described previously.^{13, 14} Briefly, the 2D ^1H - ^{13}C HSQC NMR spectra were referenced to TMSP-D₄, and lists of chemical shifts were submitted to the Human Metabolome Database (HMDB),²⁵ and the Platform for RIKEN Metabolomics PRIME,²⁶ for metabolite identification. An error tolerance of 0.08 ppm and 0.25 ppm, for ^1H and ^{13}C , respectively, was used to assign the metabolites.

Relative metabolite concentration changes

2D ^1H - ^{13}C HSQC NMR spectra were used to measure relative metabolite concentration changes by comparing HSQC peak intensities between spectra collected for the wild-type strain UAMS-1 and the mutant strains UAMS-1-*ackA* and UAMS-1-*pta*. The calculation of relative metabolite concentration changes was performed as described previously.^{13, 14} Briefly, a 2D ^1H - ^{13}C HSQC NMR spectrum was collected for each of the three biological replicates obtained for each group (e.g., UAMS-1). The 2D ^1H - ^{13}C HSQC spectra were all referenced to TMSP-D₄. Each individual 2D ^1H - ^{13}C HSQC NMR spectrum was then normalized to the sum of peak intensities for *that* specific spectrum. The peak intensities were then scaled, from 1 to 100, across the entire HSQC dataset, for each individual peak (e.g., ATP HSQC peak: ^{13}C 98.6 ppm, ^1H 6.10 ppm). An average peak intensity was calculated for each of these HSQC peaks per group. If multiple HSQC peaks were assigned to a single metabolite, then an average of all the HSQC peaks assigned to a metabolite were reported. A paired Student's t-test was utilized to determine the statistical significance of metabolite concentration changes between aerobic and anaerobic growth conditions.

RESULTS

Anaerobic fermentation partially restores viability of the *pta* and *ackA* mutants

Growth of the *pta* and *ackA* mutants under anaerobic (fermentative) conditions provided an important basis for understanding the physiological defects associated with inactivation of the Pta-AckA pathway. Similar to their aerobic growth characteristics, fermentation by both *pta* and *ackA* mutants was accompanied by impaired growth rates (Fig 1A, S1), decreased acidification (Fig S1) and a reduction in the levels of excreted acetate relative to the wild-type strain (Fig 1C, S1). Further, an increase in glucose uptake from the culture media was also evident for both the *pta* and *ackA* mutants under aerobic and fermentative growth (Fig 1B). Yet surprisingly, both the *pta* and *ackA* mutants exhibited a partial restoration of viability (increase in colony forming units (cfu) per unit OD₆₀₀) relative to the wild-type

strain under fermentative conditions (Fig 1D), despite a decrease in their intracellular ATP levels (Fig 1E). These observations not only suggest a role for fermentative metabolism in increasing survival of the *pta* and *ackA* mutants, but also discount perturbations in intracellular ATP as a source of the observed differences in viability of these mutants. Although both aerobic and anaerobic-fermentation resulted in poor growth of the *pta* and *ackA* mutants, only the latter fermentative growth conditions led to restoration of cell viability (Fig 1D). This suggests that the relative decrease in growth rate exhibited by the *pta* and *ackA* mutants to that of the wild-type strain does not result from increased cell death following inactivation of the Pta-AckA pathway.

Redox homeostasis in the *pta* and *ackA* mutants is restored under fermentative conditions

To further address the differences in viability observed under different oxygen tensions, we utilized 1D ^1H NMR to compare the exponential phase metabolomes of the *pta* and *ackA* mutants to its isogenic wild-type strain grown under both aerobic and fermentative conditions. Following acquisition of the 1D ^1H NMR spectra, the table of integrals was used for principal component analysis (PCA). Although the PCA showed significant separation between the wildtype strain and corresponding *pta* and *ackA* mutants under both aerobic and fermentative conditions, a less pronounced separation was observed following fermentation, suggesting that fermentative metabolism (as opposed to aerobic metabolism) may be less prone to perturbations associated with inactivation of the Pta-AckA pathway (Fig 2A). We reasoned that metabolic compensations in the *pta* and *ackA* mutants relative to the wild-type strain may have augmented its survival during fermentation. To identify these metabolic differences, we performed orthogonal partial least squares discriminant analysis (OPLS-DA), which relies on class membership, where the variation between classes are represented in the predictive component on the X-axis (Pp) and all other variations are represented in the orthogonal component Y-axis (Po). Multiple metabolites including branched-chain amino acids (valine/isoleucine/leucine), ethanol, lactate, alanine, acetate, betaine, glutamate, aspartate, glycerol, formate and homoserine allowed discrimination of the *pta* and *ackA* mutant metabolomes from that of their isogenic wild-type strain under fermentative conditions (Fig 2B and C). However, among these metabolites only lactate, betaine, formate and aspartate appeared to be common to and in excess in both *pta* and *ackA* mutants relative to the wild type strain (Fig 2B and C). Although these latter metabolites appear to be unrelated, metabolic pathways involved in their biosynthesis or degradation are potential contributors to cellular redox status during fermentation.

To validate any effects cellular redox status may have on cell viability of the *pta* and *ackA* mutants, we next tracked metabolic changes within these mutants by 2D ^1H - ^{13}C HSQC NMR following supplementation of the media with labeled $^{13}\text{C}_6$ glucose (see Materials and Methods). Exponentially growing bacteria were collected and used in the 2D ^1H - ^{13}C HSQC NMR experiments to identify metabolic changes associated with inactivation of the Pta-AckA pathway during aerobic and anaerobic growth (Fig S2). Relative changes in the metabolite concentrations were inferred from peak-height differences in the 2D ^1H - ^{13}C HSQC spectra (Table S1). Consistent with the results of PCA and OPLS-DA, the 2D ^1H - ^{13}C HSQC NMR experiments revealed broad similarities in the metabolic changes between the wild-type, *pta* and *ackA* mutant strains during fermentation (Fig 3A). More specifically, a

decrease in the relative concentrations of the TCA cycle intermediates as well as metabolites associated with its activity were observed for all bacterial cultures under anoxic growth conditions (Fig 3A and B). Moreover, in contrast to aerobic growth, where inactivation of the Pta-AckA pathway caused redirection of carbon flux into the TCA cycle as indicated by an increase in intracellular levels of citrate, succinate, α -ketoglutarate, and glutamate,¹⁰ no differences in the relative concentrations of these metabolites between the wild-type, *pta* and *ackA* mutants were detected during fermentation (Fig 3A and B). Furthermore, whereas a marked increase in the intracellular pools of NAD⁺ and NADH was observed following inactivation of the Pta-AckA pathway under aerobic growth, fermentation by the *pta* and *ackA* mutants resulted in a significant decrease in the intracellular concentrations for both of these species (Fig 3A) consistent with inhibition of the TCA cycle activity under anaerobiosis. Additionally, the relative intracellular concentrations of NAD⁺ and NADH were similar between the mutants and the wild-type strain suggestive of a lower energy status for all strains under anoxic conditions (Fig 3A).

In addition to the loss of the TCA cycle activity, previous studies have demonstrated that the redirection of carbon flux into D- and L-lactate generation plays an important role in the maintenance of redox balance in *S. aureus* during anaerobic growth as these pathways are required to replenish the supply of NAD⁺ for glycolysis to proceed.^{27–32} Consistent with these studies, we detected higher levels of intracellular lactate in all bacteria by 2D ¹H-¹³C HSQC NMR during anaerobiosis (Fig 3A and B). However, when compared to the wild-type strain, we observed decreased concentrations of intracellular lactate in the *pta* and *ackA* mutants under fermentative growth (Fig 3A). Although this may appear counterintuitive for both the *pta* and *ackA* mutants that require increased turnover of NADH to NAD⁺ to maintain redox homeostasis, this phenotype did not result from a lack of lactate production. Rather, we observed an increased excretion of both D- and L-lactate by the *pta* and *ackA* mutants relative to the wild-type strain, suggesting that the decreased intracellular levels of lactate reflected increased turnover and export of carbon through the lactate dehydrogenase pathway in these mutants (Fig 1F). Collectively, these results indicate that achieving redox homeostasis may be crucial to increasing viability of the *pta* and *ackA* mutants.

Overexpression of the L-lactate dehydrogenase (Ldh1) increases viability of the *pta* mutant during aerobic growth

Given that the increased activation of fermentative pathways (D- and L-lactate) during anaerobiosis potentially allows *S. aureus* *pta* and *ackA* mutants to maintain redox homeostasis and viability, we argued that a similar correction of the redox status following inactivation of the Pta-AckA pathway under aerobic growth should prevent cell death. To test this hypothesis, we introduced a plasmid overexpressing L-lactate dehydrogenase (pMRS110) into the wild-type strain and the *pta* mutant and monitored cell viability and L-lactate production during aerobic growth. As expected, overexpression of the *ldh1* gene caused a significant increase in the excretion of L-lactate for both the wild-type strain and *pta* mutant (Fig. 4A). However, excreted L-lactate concentrations in the media were higher for the *pta* mutant than the wild-type strain (Fig. 4A), presumably due to increased glycolytic flux associated with disruption of the Pta-AckA pathway. More importantly, while pMRS110 mediated overexpression of the *ldh1* increased viability of the *pta* mutant relative

to the control strain (*pta* mutant bearing vector control, pBK123), no differences in the viable cell counts were observed for the wild-type strain carrying either of these plasmids (Fig. 4B). Overall, these results demonstrate that restoration of redox homeostasis can significantly attenuate the negative impact on cell viability associated with inactivation of the Pta-AckA pathway under aerobic growth conditions.

DISCUSSION

During aerobic growth in media containing glucose, when carbon flux towards the TCA cycle is limited due to carbon catabolite repression,^{33–37} *S. aureus* primarily generates acetate by means of the Pta-AckA pathway, a phenomenon known as “acetate overflow”.^{10, 30, 32, 38, 39} We have previously shown that under these conditions the Pta-AckA pathway plays an indispensable role in maintenance of *S. aureus* fitness, as its inactivation results in growth defects and cell death. Although the precise reasons for the observed defects in growth and viability of *pta* and *ackA* mutants are not clearly understood, a role for ATP production (a byproduct of the Pta-AckA pathway) was ruled out earlier.¹⁰ Interestingly, similar observations were also noted in other bacteria, i.e. it was shown that disruption of the *pta* gene in *Escherichia coli*⁴⁰ and inactivation of the *ackA* gene in *Streptococcus mutans*⁴¹ did not cause a substantial loss in the amounts of generated ATP.

How do the *S. aureus* *pta* and *ackA* mutants compensate for the loss of ATP following inactivation of the Pta-AckA pathway? The results of the current study suggest that disruption of this pathway under aerobic conditions increases glycolytic flux and redirects carbon towards the TCA cycle. This was reflected by a higher glucose consumption rate and by increased intracellular concentrations of glycolytic and TCA cycle intermediates. Additionally, transcriptional upregulation of key glycolytic and TCA cycle genes i.e., *pfkA* and *citZ* was also observed in the *pta* and *ackA* mutants.¹⁰ A similar increase in the expression of both TCA cycle and central glycolytic enzymes in the Pta-AckA pathway mutants was reported for *E. coli*.^{42, 43} An increase in carbon flux through both glycolysis and the TCA cycle could compensate for ATP loss following inactivation of the Pta-AckA pathway, since sufficient reducing equivalents generated through these pathways will be converted to ATP by means of oxidative phosphorylation.⁴⁴ In support of this argument, both respiration rates and intracellular NAD⁺ and NADH concentrations were observed to be significantly higher in the *pta* and *ackA* mutants.¹⁰

Both NAD⁺ and NADH are the primary determinants of redox balance in living cells and their intracellular concentrations are crucial for the sustained function of a variety of metabolic pathways. Hence, any compensatory increase in flux through glycolysis and TCA cycle raises the potential risk of a redox imbalance in cells. Indeed, we speculated that the altered redox environment associated with the *pta* and *ackA* mutations might contribute to the observed growth and viability defects in these mutants. Evidence supporting this notion was observed when reduced viability, a characteristic of both *pta* and *ackA* mutant populations, was partially restored to wild-type levels following growth under fermentative conditions. Fermentation by *pta* and *ackA* mutants not only prevented redirection of carbon towards the TCA cycle (as this pathway is inactive under anoxic conditions), but apparently also resulted in a balanced intracellular turnover of NADH to NAD⁺. NMR metabolomics

analysis and metabolite excretion profiles of anaerobically grown *pta* and *ackA* mutants identified multiple possibilities that could aid in the turnover of intracellular pools of NADH and NAD⁺ in these mutants. Specifically, whereas biosynthesis of betaine from betaine aldehyde, and aspartate from glutamate would result in the generation of NADH, the conversion of pyruvate to lactate increases turnover of NADH to NAD⁺. Similarly, excess levels of formate in the cells may be indicative of decreased formate dehydrogenase activity, an adaptive strategy that could potentially limit excess NADH production from NAD⁺. These observations broadly correlate activity of reactions that augment cellular redox homeostasis to increased viability of the *pta* and *ackA* mutants under fermentative conditions.

Confirmatory evidence linking the altered intracellular redox status and decreased viability following inactivation of the Pta-AckA pathway could be established only after overexpressing L-lactate dehydrogenase in the *S. aureus pta* mutant. *S. aureus* possesses two L-lactate dehydrogenases (Ldh1/2) and two D-lactate dehydrogenases (SACOL2535 and SACOL2574).^{30, 45, 46} However, these enzymes are generally not active under aerobic conditions.³¹ Overexpression of *ldh1* in the *pta* mutant using an inducible promoter partially restored its viability to wild-type levels under aerobic conditions. Interestingly, this approach did not restore the decreased growth rate of the *pta* mutant (data not shown) suggesting redoxindependent regulation of growth following inactivation of the Pta-AckA pathway. An alternate possibility is that restoration of redox homeostasis in the *pta* mutant by overexpression of *ldh1* may decrease the intracellular pools of ATP leading to defects in growth rate.

In conclusion, it has often been argued that in glucose-rich environments, carbon flux through the Pta-AckA pathway is a rapid means by which *S. aureus* generates ATP to support fast growth under aerobic conditions. Although this may be true in principle, our data clearly suggests that the Pta-AckA pathway may have an alternate role in maintaining redox homeostasis by diverting carbon away from the TCA cycle. The inability to achieve this balance results in growth defects and cell death due to redox toxicity. Given that multiple bacterial species not only possess the Pta-AckA pathway, but also exhibit similar physiological phenotypes following its inactivation, we argue that the ability to maintain redox homeostasis by directing carbon through the Pta-AckA pathway may be a universal phenomenon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Inactivation of the Pta-AckA pathway affects growth characteristics and alters viability and energy status of *S. aureus* during aerobic and anaerobic growth

(A) Growth rate μ (hour^{-1}) of the wild type strain UAMS-1, and mutant strains UAMS-1-*ackA*, and UAMS-1-*pta* grown aerobically or anaerobically in TSB containing 0.25% glucose determined after 3 hours of growth. (B) The concentrations of glucose consumed from the culture medium per OD₆₀₀ determined for strains UAMS-1, UAMS-1-*ackA* and UAMS-1-*pta* after 3 hours of growth. (C) The concentrations of accumulated acetic acid in the culture medium per OD₆₀₀ determined for strains UAMS-1, UAMS-1-*ackA* and UAMS-1-*pta* after 3 hours of growth. (D) Number of viable cells per OD₆₀₀ unit determined for strains UAMS-1, UAMS-1-*ackA*, and UAMS-1-*pta* after 3 h of growth. (E) Intracellular ATP concentrations determined for strains UAMS-1, UAMS-1-*ackA*, and UAMS-1-*pta* after 3 h of growth. (F) The concentrations of accumulated D- and L-lactic acids in the culture medium per OD₆₀₀ determined for strains UAMS-1, UAMS-1-*ackA* and UAMS-1-*pta* after 3 hours of growth. The results are presented as the means plus standard errors of the mean of duplicate determinations for at least three independent experiments. Statistical significance between the wild-type strain and *pta* and *ackA* mutants (*) and *pta* and *ackA* mutants grown aerobically and anaerobically (**) was determined by Student's *t* test ($P < 0.001$).

Figure 2. Disruption of the Pta-AckA pathway has relatively smaller impact on *S. aureus* metabolome during anaerobiosis

(**A**): 2D LDA scores plots comparing the metabolic fingerprints of aerobically grown wild type (red), UAMS-1-*ackA* (purple), and UAMS-1-*pta* (black) vs. anaerobically grown wild type (yellow), UAMS-1-*ackA* (green), and UAMS-1-*pta* (cyan). Dendrogram was generated from PCA scores using a Mahalanobis distance matrix with *p* values for the null hypothesis reported at each node. (**B and C**) OPLS-DA back-scaled loadings plot comparing anaerobically grown UAMS-1-*pta* (**B**) and UAMS-1-*ackA* (**D**) vs. UAMS-1 wild-type strain. Peak intensities reflect the contribution of that peak to class separation, an up orientation represents a metabolite increased under anaerobic growth, and conversely a down orientation represents a metabolite increased under aerobic growth. The coloring of the backscaled loadings plot is based on the scale factor applied during variable scaling prior to model training. Metabolite labels correspond to: (1) branched chain amino acids (valine/isoleucine/

leucine), (2) ethanol, (3) lactate, (4) alanine, (5) acetate, (6) betaine, (7) glutamate, (8) aspartate, (9) glycerol, (10) formate, and (11) homoserine.

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Figure 3. Oxygen availability alters catabolic fate of glucose in the *pta* and *ackA* mutants
(A) The heat map generated from normalized peak intensities in 2D ^1H - ^{13}C HSQC NMR spectra with the dendrogram representing a hierarchical clustering that compares UAMS-1, UAMS-1-*ackA*, and UAMS-1-*pta* *S. aureus* grown aerobically or anaerobically. The color scale ranges from 0 (red) (less intense) to 1 (green) (intense) and strain colored asterisks denotes statistical significance at the 95% confidence comparing aerobic vs. anaerobic conditions. A metabolite that was not observed under either aerobic or anaerobic growth conditions was assigned a value of zero. Please see Table S1 for a complete listing of the normalized peak intensities. **(B)** Metabolic network summarizes the metabolic perturbations observed comparing UAMS-1, UAMS-1-*ackA*, and UAMS-1-*pta* under aerobic vs. anaerobic conditions. Up arrows indicate a relative increase in metabolite concentration, and a down arrow indicates a relative decrease in the metabolite concentration for aerobic compared to anaerobic conditions. The asterisk above each arrow denotes statistical significance at the 95% confidence level ($p < 0.05$). Abbreviations are as follows: acetyl-phosphate (Acetyl-P); (1,3)BP-G, 1,3-bisphosphoglycerate; F6-P, fructose 6-phosphate; Glu, glutamate; Gln, glutamine; FB-P, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G6-P, glucose 6-phosphate; G1-P, glucose 1-phosphate; GlcN-6-P, glucosamine-6-phosphate; GlcN-1-P, glucosamine-1-phosphate; GA3-P, glyceraldehyde 3-phosphate; GlcNAc-1-P, N-acetyl-glucosamine-1-phosphate; GlcNAc-6-P, N-acetyl-glucosamine-6-phosphate; 3P-G, 3-phosphoglycerate; 2P-G, 2-phosphoglycerate; 6P-GL, 6-phosphonoglucono-lactone; PEP, phosphoenolpyruvate; Ru5P, ribulose 5-phosphate; UDP-G, uridine diphosphate glucose; UDP-GlcNAc, uridine diphosphate N-

acetylglucosamine;R5P, ribose-5-phosphate; Ru5P, ribulose 5-phosphate; X5-P, xylulose 5-phosphate.

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Figure 4. Overexpression of the *ldh1* gene attenuates negative impact on viability of the *pta* mutant during aerobic growth

(A) Relative concentrations of accumulated L-lactic acid in the culture medium per OD₆₀₀ determined for strains UAMS-1, UAMS-1-*ackA* and UAMS-1-*pta* containing vector plasmid (pBK123) or *ldh1* expressing plasmid (pMRS110) after 3 hours of growth. **(B)** Relative number of viable cells per OD₆₀₀ unit determined for strains UAMS-1, UAMS-1-*ackA*, and UAMS-1-*pta* containing vector plasmid (pBK123) or *ldh1* expressing plasmid (pMRS110) after 3 h of growth. The results are presented as the means plus standard errors of the mean of duplicate determinations for at least three independent experiments. Statistical significance between strains containing vector plasmid (pBK123) and *ldh1* expressing plasmid (pMRS110) (**A and B**) and between the wild-type strain and *pta* and *ackA* mutants (**B**) was determined by Student's *t* test (*, **, *P* < 0.001).