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Staphylococcus epidermidis Polysaccharide Intercellular Adhesin Production Significantly Increases during Tricarboxylic Acid Cycle Stress

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Staphylococcal polysaccharide intercellular adhesin (PIA) is important for the development of a mature biofilm. PIA production is increased during growth in a nutrient-replete or iron-limited medium and under conditions of low oxygen availability. Additionally, stress-inducing stimuli such as heat, ethanol, and high concentrations of salt increase the production of PIA. These same environmental conditions are known to repress tricarboxylic acid (TCA) cycle activity, leading us to hypothesize that altering TCA cycle activity would affect PIA production. Culturing *Staphylococcus epidermidis* with a low concentration of the TCA cycle inhibitor fluorocitrate dramatically increased PIA production without impairing glucose catabolism, the growth rate, or the growth yields. These data lead us to speculate that one mechanism by which staphylococci perceive external environmental change is through alterations in TCA cycle activity leading to changes in the intracellular levels of biosynthetic intermediates, ATP, or the redox status of the cell. These changes in the metabolic status of the bacteria result in the attenuation or augmentation of PIA production.

Staphylococcus aureus and *S. epidermidis* infections cause a considerable amount of morbidity in humans and animals. Although the types and severity of diseases produced by these opportunistic pathogens vary, both are important causes of infections associated with indwelling medical devices (e.g., catheters) (27). Catheter-associated infections usually involve a two-step process leading to the formation of a bacterial biofilm. The first step in biofilm formation involves attachment of the organism to an uncoated plastic surface, or a plastic surface coated with host proteins (39). The second step involves the accumulation of bacteria on top of the bacteria adhering to the plastic surface, a step requiring the production of polysaccharide intercellular adhesin (PIA). PIA is a β-1,6-linked polysaccharide that is strongly associated with the staphylococcal cell surface and mediates cell-to-cell adhesion (22).

Synthesis of PIA requires the enzymes encoded within the intercellular adhesion (*ica*) operon (*icaADBC*) (13). Regulation of the *ica* operon involves at least one DNA binding protein (IcaR) and the alternative sigma factor σ^B (Fig. 1). IcaR binds immediately 5′ to the *icaA* transcriptional start site and acts as a transcriptional repressor of the *ica* operon (4, 14). Compelling evidence also indicates that σ^B is involved in the production of PIA, although this involvement is likely indirect as the *ica* operon lacks a σ^B consensus recognition sequence (15, 28). Recent data from the Mack laboratory provide strong evidence that σ^B affects PIA production by controlling the expression on *icaR* (16). In addition to these regulatory elements, staphylococci regulate the production of PIA in response to nutrient availability, environmental signals, and stress-inducing stimuli. Expression of the *ica* operon is increased during growth in oxygen-limiting (7), nutrient-replete (9, 23), or iron-limiting conditions (8). Additionally, stress-inducing stimuli such as heat (29), ethanol (5), and high concentrations of salt (15) increase *ica* expression and PIA production. These observations highlight a recurrent theme in pathogenesis: the regulation of many virulence genes is controlled by environmental factors (26).

S. aureus and *S. epidermidis* both possess a complete tricarboxylic acid (TCA) cycle, but lack a glyoxylate bypass (34; G. A. Somerville unpublished observations). TCA cycle activity is essential for the complete catabolism of nonpreferred carbon sources and the subsequent generation of reducing potential and biosynthetic intermediates. The activity of TCA cycle enzymes is affected by the nutritional status of the cell and a variety of stress-inducing stimuli. *S. aureus* represses TCA cycle activity when grown in nutrient-replete conditions (3, 32) or under conditions of low oxygen availability (35). In addition, the enzymatic activity of several TCA cycle enzymes requires iron (i.e., aconitase, succinate dehydrogenase complex, and fumarase); hence, during growth under conditions of low iron availability TCA cycle activity is dramatically reduced (30, 37). TCA cycle activity can also be disrupted by certain stress-inducing stimuli such as heat (36) and ethanol (17; I. Chatter-

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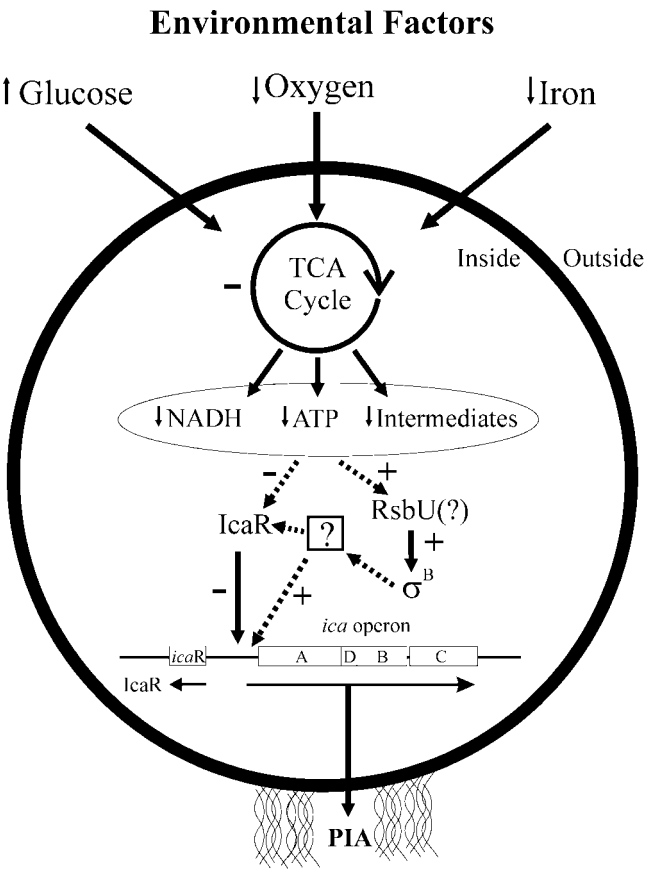

FIG. 1. Proposed model for the regulation of the *ica* locus. Plus symbols indicate a positive influence, and minus symbols indicate a negative influence. The dashed lines indicate proposed connections.

jee and M. Herrmann, personal communication). The similarity between the environmental and nutritional factors influencing TCA cycle activity and PIA production led us to hypothesize that altering TCA cycle activity would affect PIA production. In the present study, we tested the hypothesis that increased PIA production is associated with decreased TCA activity.

MATERIALS AND METHODS

Bacterial strains, materials, and growth conditions. *S. epidermidis* strain SE1457 is an *agr*-positive, biofilm-forming strain (23, 40). *S. epidermidis* strains were grown in tryptic soy broth containing 0.5% or 0.3% (wt/vol) glucose (TSB) (BD Biosciences, Sparks, Md.) or in TSB containing 0.001% phenol red. All bacterial cultures, unless stated otherwise, were inoculated 1:100 from an overnight culture into TSB, incubated at 37°C, and aerated at 160 rpm. A flask-to-medium ratio of 4:1 was used to produce less aeration, whereas, a flask-to-medium ratio of 7:1 was used for greater aeration. Bacterial growth was assessed by measuring the optical density at 600 nm or by determining the CFU ml^{-1}. Barium fluorocitric acid (Sigma Chemical, St. Louis, MO) was dissolved in 2 N HCl, neutralized with 10 N NaOH, centrifuged to remove the BaOH, and the volume was increased with H_2O to the appropriate concentration. After removal of the BaOH, the fluorocitric acid was used to specifically inhibit the TCA cycle enzyme aconitase. Anti-PIA antisera were generated against purified PIA by Sigma-Genosys.

Free amino acid concentrations in TSB. The concentrations of free amino acids in TSB were determined with a Beckman model 6300 amino acid analyzer (Scientific Research Consortium, Inc., St. Paul, MN). The μM concentrations of free amino acids in TSB were as follows: Ser, 1,098; Gly, 140; Ala, 720; Glu, 1,840; Gln, 546; Thr, 1,134; Met, 1,136; Ile, 1,556; Arg, 2,014; His, 598; Pro, 184; Leu, 6,426; Lys, 3,360; Val, 2,072; Phe, 2,654; Trp, 482; Asp, 670; Asn, 782; Tyr, 442; and Cys, 190.

Measurement of acetate and glucose in culture supernatants. Aliquots of bacteria (1.5 ml) were centrifuged for 5 min at 20,800 × *g* at 4°C, and supernatants were removed and stored at −20°C until use. Acetate and glucose concentrations were determined with kits purchased from R-Biopharm, Inc. (Marshall, MI) and used according to the manufacturer's directions.

PIA immunoblot assay. The relative amount of PIA produced was determined essentially as described (38). Briefly, equal numbers of *S. epidermidis* cells (0.51 A_{600} units) were harvested by centrifugation, and the PIA was extracted in 0.5 M EDTA (pH 8.0) by boiling for 5 min. Aliquots of PIA were applied to a nitrocellulose membrane and blocked with 5% skim milk overnight. The nitrocellulose membrane was incubated for 2 h with anti-PIA antiserum and, subsequently, for 2 h with an anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate. The presence of PIA was detected by the addition of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. The integrated density values of bands on autoradiographs were determined with the TotalLab software (Nonlinear Dynamics Ltd.)

Determination of soluble NAD^+ and NADH concentrations. Intracellular NAD^+ and NADH concentrations were determined with an enzymatic cycling assay as described (1). Briefly, aliquots (5 ml) were harvested at 2, 3, and 4 h postinoculation by passing the bacterial culture through a 0.45 μM nitrocellulose filter (Millipore). Depending upon which dinucleotide was being measured, either 0.2 M KOH (NAD^+) or 0.2 M HCl (NADH) (1 ml) was added to 15-ml tubes containing the membranes, the tubes were vortexed, and immediately placed in a boiling water bath for 10 min. Cooled lysates were centrifuged for 5 min at 15,000 × *g* at 4°C, cell-free lysates (400 μl) were transferred to microcentrifuge tubes, and the pH was adjusted to 7.0 with 0.2 M KOH or 0.2 M HCl (volumes were normalized with phosphate-buffered saline). Cycling buffer (800 μl) (4.4 ml of 0.623 M bicine σ, 4.4 ml of 0.0026 M methylthiazotetrazolium [MTT], 4.4 ml of 0.026 M EDTA, 3.5 ml of 0.0104 M phenazine ethosulfate [PES], and 0.8 ml ethanol) was added, and the mixtures were equilibrated at room temperature in the dark for 5 min. Quadruplicate aliquots (200 μl) were transferred into 96-well microtiter plates and the reactions were started by the addition of 40 μl of 1.3 mg/ml yeast alcohol dehydrogenase (Sigma). The reaction was followed by monitoring the change in absorbance at 570 nm (A_{570}) every 30 s for a total of 10 min. The intracellular concentrations of NAD^+ and NADH were determined by comparing the change in A_{570} of the samples to the change in absorbance for known concentrations of NAD^+ and NADH (2, 4, 16, 64, and 128 μM). All assays were performed in quadruplicate from three independent cultures.

Enzymatic activity assays. Aconitase and isocitrate dehydrogenase enzymatic activity assays were performed essentially as described (32); except that bacterial suspensions were lysed with a FastPrep instrument using FastProtein Blue kit (Qbiogene, Carlsbad, CA). Protein concentrations were determined by the method of Lowry et al. (20).

Statistical analysis. Statistical significance was assessed with Student's *t* test. To determine if a correlation existed between two parameters, a Pearson's correlation coefficient (ρ) was calculated.

RESULTS

"Deoptimization" of PIA production. *S. epidermidis* growth conditions have been optimized to permit maximal PIA production in the laboratory setting (e.g., nutrient-rich media). The same conditions that enhance PIA production repress TCA cycle activity. Hence, in order to determine if TCA cycle activity affected PIA production, it was necessary to increase TCA cycle function. To do this, strain SE1457 was grown under PIA inducing conditions (TSB containing 0.5% glucose, aerated at 160 rpm, and with a flask-to-medium ratio of 4:1 [41]) and TCA cycle function was assessed by determining the concentration of acetate in the culture medium throughout the growth cycle (Fig. 2). Acetate catabolism is an indictor of TCA cycle activity, because the catabolism of acetate requires a fully functioning TCA cycle (31, 33). Using PIA-inducing growth conditions, glucose remained in the culture medium until the bacterial culture had reached the stationary phase; this coin-

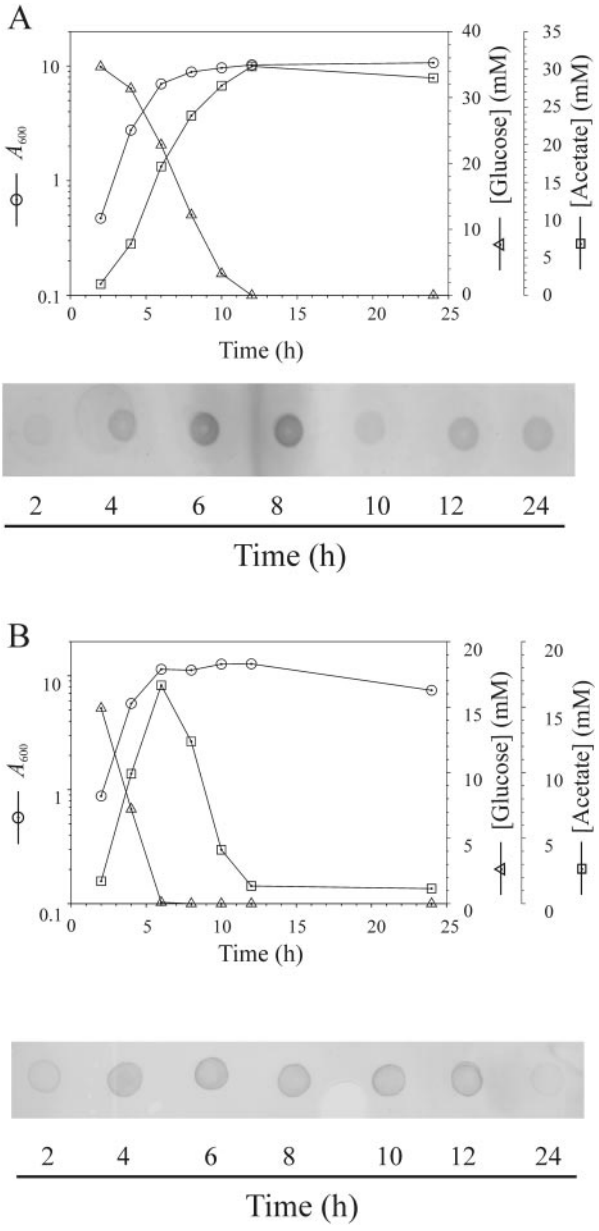

FIG. 2. Influence of glucose on growth, catabolism, and PIA production. (A) Strain SE1457 was grown in TSB containing 0.5% (wt/vol) glucose, aerated at 160 rpm, and with a flask-to-medium ratio of 4:1. At 2-h intervals for the first 12 h of growth and after 24-h samples were removed and culture medium was assayed for the concentrations of glucose and acetate. At these same time intervals, an equivalent number of bacteria were harvested, PIA was extracted from the whole cells, and the relative amounts of PIA were determined using a PIA immunoblot assay (representative results are shown beneath each graph). (B) Strain SE1457 was grown in TSB containing 0.3% (wt/vol) glucose, aerated at 160 rpm, and with a flask-to-medium ratio of 7:1. The collection of samples was performed as described for panel A. The results are representative of independent experiments performed at least twice.

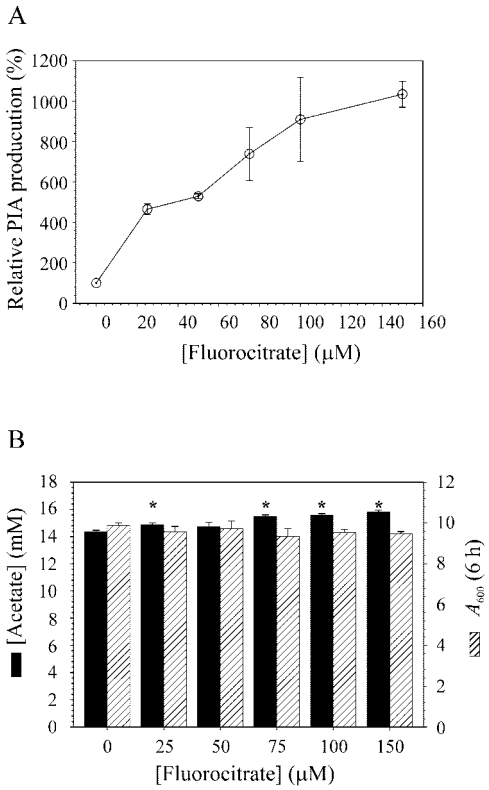

FIG. 3. TCA cycle stress induces PIA production. (A) Bacterial cultures were incubated with increasing concentrations of fluorocitrate, grown for 24 h, and the relative amounts of cell-associated PIA were determined by a PIA immunoblot assay. (B) After 6 h of growth (early post-exponential-growth phase), the concentrations of acetate in the culture medium and the cell densities were determined. The data are presented as the means and standard errors of the means (SEM) of triplicate determinations from two independent experiments. Asterisks in panel B indicate a significant difference relative to the untreated control (0 μM fluorocitrate).

cided with the accumulation of acetate in the medium. These same conditions also prevented the depletion of acetate from the culture medium, suggesting that TCA cycle activity was inhibited in the post-exponential-growth phase (Fig. 2A). Decreasing the glucose concentration to 0.3% and increasing the aeration in the bacterial culture (flask-to-medium ratio of 7:1) resulted in the complete catabolism of glucose and acetate from the culture medium (Fig. 2B), suggesting the TCA cycle was functioning. Importantly, altering the growth conditions to favor TCA cycle activity reduced PIA production (Fig. 2A and B), but did not eliminate PIA production.

Fluorocitrate significantly enhances PIA production. Altering the growth conditions to favor TCA cycle function facilitated acetate catabolism and reduced PIA production (Fig. 2B), consistent with our hypothesis that reduced PIA production is associated with increased TCA cycle activity. To test this hypothesis further, we incubated *S. epidermidis* strain SE1457 with a TCA cycle specific inhibitor, fluorocitrate, and determined the amount of cell-associated PIA. Fluorocitrate is a highly toxic compound when metabolized to 4-hydroxy-*trans*-aconitate by the TCA cycle enzyme aconitase (19, 25). The toxicity is due to very tight, noncovalent binding of 4-hydroxy-*trans*-aconitate to aconitase (19). As the concentration of fluorocitrate in the culture medium was increased, the amount of PIA produced increased ($\rho = 0.91$) (Fig. 3A). Of importance, the concentrations of fluorocitrate used in these experiments

did not affect the ability of *S. epidermidis* to catabolize glucose, the growth rate, or the growth yields at 6 h (data not shown and Fig. 3B). However, increasing concentrations of fluorocitrate caused partial TCA cycle inhibition, resulting in significant ($P \leq 0.05$) increases in the post-exponential-growth-phase (6-h) accumulation of acetate in the culture medium, an indication of altered TCA cycle function (Fig. 3B). In other words, the increased concentrations of acetate in the culture medium demonstrated that fluorocitrate was entering into the bacteria and partially inhibiting TCA cycle function, but the absence of effects on glucose catabolism and growth demonstrate that low levels of fluorocitrate are not inducing a stress response. While the increase in the concentration of acetate in the culture medium at 6 h was statistically significant, in our experience, these minor differences in acetate concentrations are not biologically significant, suggesting that another TCA cycle-derived product was responsible for the increased PIA production. Taken together, these data demonstrate that small perturbations in the TCA cycle can significantly increase PIA production.

Fluorocitrate alters the intracellular redox status. The addition of fluorocitrate to the growth medium increased the exponential-growth-phase synthesis of PIA per A_{600} unit (Fig. 4A). The TCA cycle provides biosynthetic intermediates (α-ketoglutarate, succinyl-coenzyme A [CoA], and oxaloacetate), ATP, and reducing potential; therefore, altering TCA cycle activity alters the metabolic status of the cell. TSB is a nutrient-rich medium containing high concentrations of free amino acids and peptides (Materials and Methods); hence, minor alterations in TCA cycle activity are unlikely to affect the intracellular availability of biosynthetic intermediates during the exponential phase of growth. The majority of the ATP derived from TCA cycle activity comes from the oxidation of NADH during respiration. Thus, if an alteration in TCA cycle activity resulted in a decreased concentration of ATP, then the likely cause would be a change in the redox status of the bacteria. For these reasons, we chose to examine the effects of fluorocitrate on the concentrations of unbound NAD^+ and NADH (Fig. 4B and C). As expected, the addition of 100 μM fluorocitrate to the culture medium significantly decreased the intracellular concentrations of free NAD^+ and NADH. Surprisingly, this effect occurred early in the growth cycle, suggesting the TCA cycle was active during the early exponential growth phase. The early induction of TCA cycle activity differs somewhat from that found in *S. aureus*, which induces the TCA cycle late in the exponential growth phase when readily catabolizable carbon sources become limiting (32).

***S. epidermidis* TCA cycle activity.** Growth of strain SE1457 in the presence of fluorocitrate altered the redox status of the bacteria early in the exponential phase of growth (Fig. 4B and C), indicating the TCA cycle was active early in the growth cycle. Consistent with this hypothesis, the nonstoichiometric accumulation of acetate (1 glucose = 2 acetate) in the culture medium (Fig. 2) suggested that glucose carbons were being diverted from substrate-level phosphorylation. These data, in addition to the absence of lactate in the culture medium (data not shown), led us to postulate that strain SE1457 was diverting acetyl-CoA into the TCA cycle. Enzymatic assays of isocitrate dehydrogenase and aconitase (Fig. 5) confirmed that *S. epidermidis* had a low level of TCA cycle activity early in the

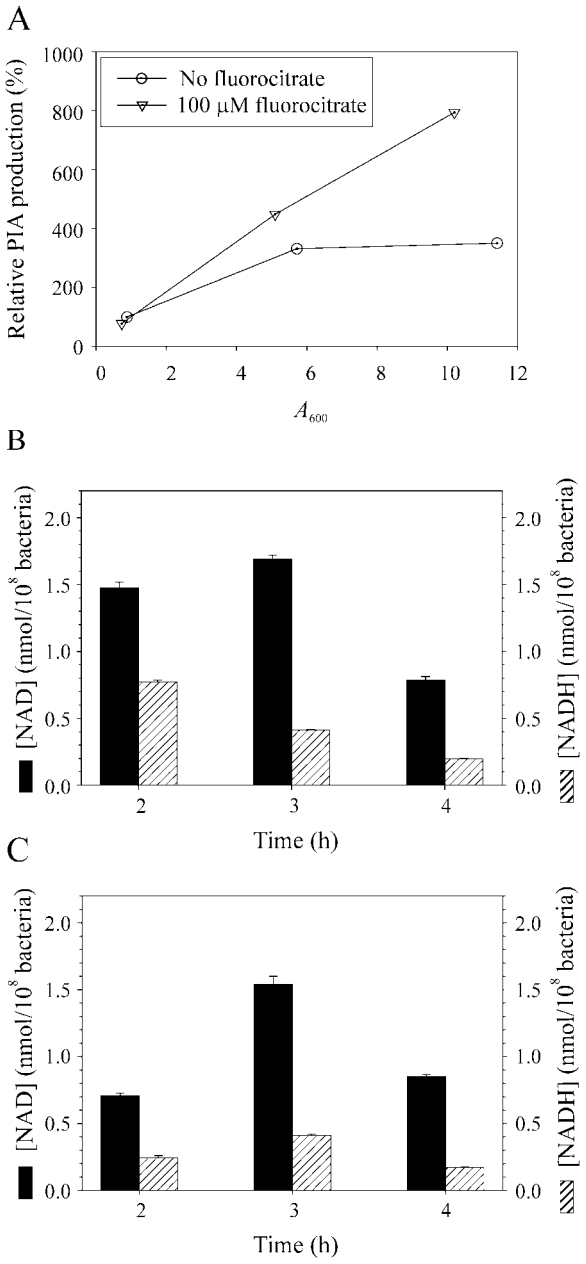

FIG. 4. Fluorocitrate increased PIA production and altered the exponential-growth-phase redox status of strain SE1457. (A) Incubation of strain SE1457 with 100 μM fluorocitrate increased the amount of PIA produced per A_{600} unit. The data are presented as the percent increases in PIA accumulation relative to the culture containing no fluorocitrate at 2 h (first time point). The results are representative of independent experiments performed at least twice. The symbols in the graph correspond to 2, 3, and 4 h of growth. (B) The free NAD and NADH concentrations were determined from exponential-growth-phase bacterial cultures grown in the absence of fluorocitrate. (C) Bacterial cultures were grown in the presence of 100 μM fluorocitrate, and the concentrations of free NAD and NADH were determined during the exponential-growth phase. The results are presented as the means and SEM of quadruplicate determinations for three independent experiments.

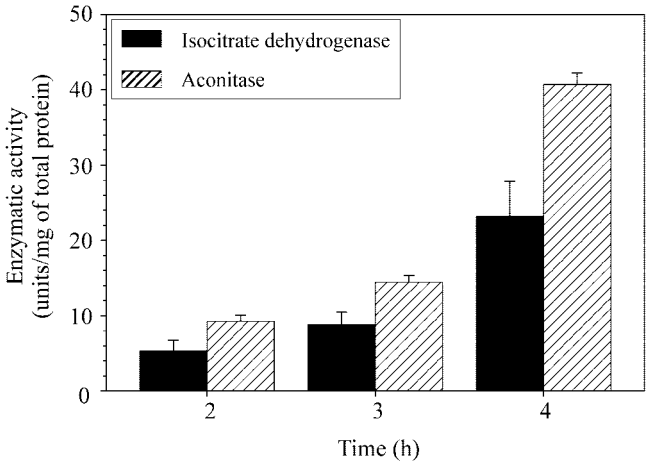

FIG. 5. Aconitase and isocitrate dehydrogenase activity during the exponential phase of growth. Aconitase and isocitrate dehydrogenase activities were determined in triplicate from three independent cultures during the exponential phase of growth. The results are presented as the means and SEM.

exponential growth phase. As stated, these same growth conditions are known to repress TCA cycle activity in *S. aureus* (32). These data strongly suggest that the early exponential growth phase difference in the intracellular concentration of NAD^+ and NADH was due to an early induction of TCA cycle activity.

DISCUSSION

A common mechanism by which staphylococci induce disease is through the formation of a biofilm on an indwelling medical device (6, 12). One important step in the formation of a staphylococcal biofilm is production of the exopolysaccharide PIA (39). Numerous studies have demonstrated that *S. epidermidis* regulates the production of PIA in response to nutrient availability, environmental signals, and stress-inducing stimuli (4, 8, 9, 15, 23, 29), leading to considerable speculation about the nature of *ica*/PIA regulatory elements. Recent data have led to the hypothesis that PIA production might be limited, even in the presence of the Ica proteins, due to a shortage of PIA precursors and/or energy (9). These observations and the similarity of environmental stimuli affecting PIA production to those that affect TCA cycle activity led us to examine the role of the TCA cycle in PIA production.

The metabolism of PIA production. Staphylococcal PIA is a β(1-6)-linked *N*-acetylglucosamine polymer (24) of at least 130 residues (21); hence, production of PIA creates a need for UDP-activated *N*-acetylglucosamine (11). The synthesis of UDP-*N*-acetylglucosamine is a multistep process that begins with the transamination of the glycolytic intermediate fructose 6-phosphate (Fig. 6). Growth of *S. epidermidis* in TSB medium containing glucose will produce abundant levels of fructose 6-phosphate through the glycolytic (Embden-Meyerhof-Parnas) pathway. In the absence of glucose, as occurs during the postexponential growth phase (Fig. 2B), fructose 6-phosphate can be synthesized by gluconeogenesis from the TCA cycle intermediate oxaloacetate (Fig. 6). Gluconeogenesis requires energy and withdraws carbon from the TCA cycle, necessitating anaplerotic reactions at a time when carbon is limiting; thus, it is unlikely that sufficient fructose 6-phosphate can be made to maintain PIA production and growth. This inability to produce sufficient fructose 6-phosphate during the post-exponential-growth phase is exacerbated by the absence of a carbon salvage pathway (i.e., the glyoxylate bypass). In the absence of a glyoxylate bypass, two carbons are lost as CO_2 for every two carbons that enter into the TCA cycle. Therefore, to produce PIA during the post-exponential-growth phase, *S. epidermidis* must overcome a large carbon deficit. Interestingly, the predicted decrease in the availability of fructose 6-phosphate coincides with the post-exponential-growth-phase repression of PIA biosynthetic genes (*ica* operon) (9). Taken together, these observations suggest the post-exponential-growth-phase de-

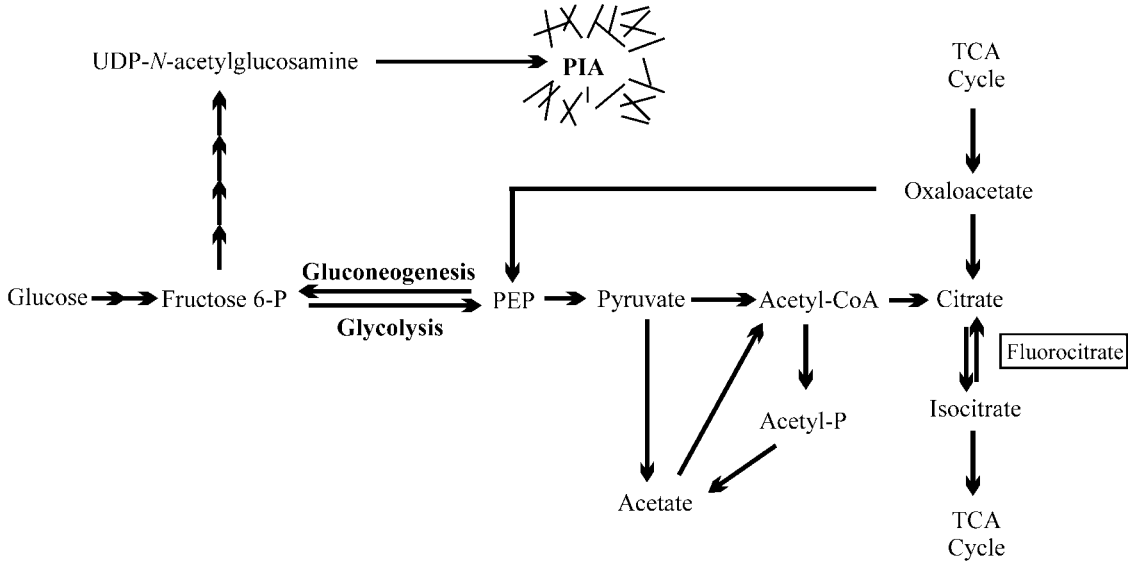

FIG. 6. Proposed carbon flux during PIA synthesis. The enzymatic reaction inhibited by fluorocitrate is indicated.

crease in PIA accumulation (Fig. 2B) occurs through a synchronization of metabolism and *icaR*-dependent regulation of *ica* transcription. This suggestion is supported by the fact that addition of fluorocitrate to the culture medium correlates with a significant increase in the synthesis of PIA (Fig. 3A and 4A), demonstrating a close association between PIA production and metabolism.

The role of the TCA cycle in PIA production. Increased PIA production is associated with decreased TCA activity, whether it is the result of slowing the TCA cycle by fluorocitrate (Fig. 3A) or by reduced culture aeration (Fig. 2A and B), leading us to hypothesize that impairment of TCA cycle function shunts metabolites toward PIA production. In support of this hypothesis, the addition of fluorocitrate to the culture medium increases the concentration of acetate in the culture medium (Fig. 3B), demonstrating carbon is diverted from the TCA cycle. The diversion of carbon to PIA is suggested by an increase in the amount of PIA produced per A_{600} unit when *S. epidermidis* is grown in the presence of fluorocitrate (Fig. 4A). Additionally, the shunting of metabolites away from the TCA cycle during growth in a medium containing fluorocitrate is indicated by an increase in the strength of the correlation between the decrease in glucose concentration (Fig. 2B) and the increase in PIA spot density (Fig. 3A) relative to the untreated culture medium (untreated medium, $\rho = -0.907$; 100 μM fluorocitrate-containing medium, $\rho = -0.996$). Taken together, these data suggest that one effect of TCA cycle stress is the shunting of carbon away from the TCA cycle and toward PIA production.

In addition to biosynthetic intermediates, the TCA cycle provides the reducing potential (NADH) to drive ATP synthesis, suggesting that TCA cycle stress will alter the redox status of *S. epidermidis*. As anticipated, incubation of *S. epidermidis* with fluorocitrate decreases the intracellular concentration of NADH and NAD^+ (Fig. 4B and C). Surprisingly, this alteration occurred early in the exponential phase of growth (Fig. 3B and C), suggesting the TCA cycle was active early in the growth cycle (Fig. 5). These data lead us to speculate that events early in the growth cycle of *S. epidermidis*, such as the availability of NAD^+ or NADH, determine the amount of PIA that will be produced. Furthermore, we predict a regulatory protein is responding to the redox status of the bacteria to affect *ica* transcription or PIA production. We note that several NADH sensor proteins have been identified in other organisms (e.g., Rex [2], NmrA [18], and CcpA [10]). Our laboratories are actively investigating these possibilities.

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