

Staphylococcus aureus synthesizes adenosine to escape host immune responses

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Staphylococcus aureus infects hospitalized or healthy individuals and represents the most frequent cause of bacteremia, treatment of which is complicated by the emergence of methicillin-resistant *S. aureus*. We examined the ability of *S. aureus* to escape phagocytic clearance in blood and identified adenosine synthase A (AdsA), a cell wall-anchored enzyme that converts adenosine monophosphate to adenosine, as a critical virulence factor. Staphylococcal synthesis of adenosine in blood, escape from phagocytic clearance, and subsequent formation of organ abscesses were all dependent on *adsA* and could be rescued by an exogenous supply of adenosine. An AdsA homologue was identified in the anthrax pathogen, and adenosine synthesis also enabled escape of *Bacillus anthracis* from phagocytic clearance. Collectively, these results suggest that staphylococci and other bacterial pathogens exploit the immunomodulatory attributes of adenosine to escape host immune responses.

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Abbreviations used: AdsA, adenosine synthase A; CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; ClfA, clumping factor A; MALDI-MS, matrix-assisted laser desorption/ionization–mass spectrometry; RP-HPLC, reversed-phase HPLC.

Staphylococcus aureus is the leading cause of blood-stream, lower respiratory tract, skin, and soft tissue infections (Klevens et al., 2006; Klevens et al., 2007) because of its unique ability to multiply in blood or other host tissues and cause persistent infections (Lowy, 1998). To survive in blood, *S. aureus* must escape a variety of innate immune mechanisms, such as antimicrobial peptides, complement, and phagocytic killing (Foster, 2005; Peschel and Sahl, 2006). An immediate and essential host defense against *S. aureus* is provided by neutrophilic PMNs (neutrophils), which comprise 60–70% of human white blood cells (Voyich et al., 2005). Staphylococci deploy a plethora of mechanisms aimed at subverting innate immune mechanisms, including secretion of factors inhibitory for complement activation and neutrophil chemotaxis (de Haas et al., 2004; Rooijakkers et al., 2005), as well as toxins that lyse neutrophils (Wang et al., 2007), neutralize antimicrobial defensins (Jin et al., 2004), or act as superantigens to inappropriately activate the host's immune system (Jardetzky et al., 1994). In this paper, we report the discovery of a hitherto unknown strategy: synthesis of the immunosuppressive signaling molecule adenosine.

In mammals, adenosine assumes an essential role in regulating innate and acquired immune responses (Thiel et al., 2003). Strong or excessive

host inflammatory responses, e.g., in response to bacterial infection, exacerbate the tissue damage inflicted by invading pathogens (Thiel et al., 2003). Successful immune clearance of microbes therefore involves the balancing of pro- and anti-inflammatory mediators. The cytokines IL-4, IL-10, IL-13, and TGF- β play a role in restricting excessive inflammation, but only adenosine is able to completely suppress immune responses (Németh et al., 2006). The immunoregulatory attributes of adenosine are mediated via four transmembrane adenosine receptors: A₁, A_{2A}, A_{2B}, and A₃ (Haskó and Pacher, 2008). T lymphocytes express the high affinity A_{2A} receptor as well as the low affinity A_{2B} receptor (Thiel et al., 2003). Depending on their activation state, macrophages and neutrophils express all four adenosine receptors, whereas B cells harbor only A_{2A} (Thiel et al., 2003). Engagement of A_{2A} inhibits IL-12 production, increases IL-10 in monocytes (Khoa et al., 2001) and dendritic cells (Panther et al., 2001), and decreases cytotoxic attributes and chemokine production in neutrophils (Cronstein et al., 1986; McColl et al., 2006). Generation of adenosine at sites of

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inflammation, hypoxia, organ injury, and traumatic shock is mediated by two sequential enzymes. Ecto-ATP diphosphophohydrolase (CD39) converts circulating ATP and ADP to AMP (Eltzschig et al., 2003). CD73, expressed on the surface of endothelial cells (Deussen et al., 1993) and subsets of T cells (Thompson et al., 1987; Thompson et al., 1989; Yang et al., 2005), then converts 5'-AMP to adenosine (Zimmermann, 1992).

Although extracellular adenosine is essential for the suppression of inflammation, build-up of excess adenosine is also detrimental. This is exemplified in patients with a deficiency in adenosine deaminase, an enzyme that converts adenosine to inosine (Giblett et al., 1972). Adenosine deaminase deficiency causes severe compromised immunodeficiency syndrome, with impaired cellular immunity and severely decreased production of immunoglobulins (Buckley et al., 1997). As the regulation of extracellular adenosine is critical in maintaining immune homeostasis, perturbation of adenosine levels is likely to affect host immune responses during infection. We report in this paper that *S. aureus* and *Bacillus anthracis*, the causative agent of anthrax, use adenosine synthesis to escape host immune responses.

RESULTS

Adenosine synthase A (AdsA) is required for staphylococcal survival in blood

To identify the staphylococcal genes required for escape from innate immune responses, we examined the ability of *S. aureus* strain Newman to survive in whole blood collected from BALB/c mice or Sprague-Dawley rats by recording bacterial load at timed intervals via the formation of colonies on agar medium (Fig. 1). As expected, the blood of naive mice and rats, which lack antibodies specific for staphylococci (not depicted), were unable to kill *S. aureus* Newman (Fig. 1, A and D). In contrast to the wild-type strain, a variant lacking the structural gene for sortase A (*srtA*) displayed a defect in staphylococcal escape from phagocytic killing ($P < 0.05$; Fig. 1, A and D). Sortase A anchors a large spectrum of different polypeptides in the staphylococcal envelope, using a transpeptidation mechanism and LPXTG motif sorting signal at the C terminus of surface proteins (Mazmanian et al., 2002). To examine these surface proteins for their contribution to staphylococcal survival in blood, we transduced *bursa aurealis* insertions in surface protein genes (Bae et al., 2004) into wild-type strain *S. aureus* Newman and measured the survival of staphylococcal variants in blood (Fig. 1, B and E). Mutations in *cflA* and *sasH* (*Staphylococcus aureus* surface protein), hereafter named *adsA*, displayed consistent survival defects. The phenotype of *cflA* mutants represents an expected result, as the encoded clumping factor A product is known to precipitate fibrin and interfere with macrophage and neutrophil phagocytosis (Palmqvist et al., 2004; Higgins et al., 2006). The contribution of AdsA to pathogenesis is not yet known. AdsA harbors a 5'-nucleotidase domain with the two signature sequences ILHTnDiH-GrL (residues 124–134) and YdamaVGNHEFD (residues 189–201), suggesting that the protein may catalyze the synthesis

of adenosine from 5'-AMP. To further examine the importance of *adsA* in staphylococcal virulence, we complemented the *adsA* gene by cloning the entire *adsA* gene and upstream promoter sequences into expression vector pOS1, generating *padsA*. Transformation of *adsA* mutant staphylococci with *padsA* restored their ability to survive in mouse or rat blood, indicating that the observed virulence defect is indeed caused by the absence of *adsA* expression (Fig. 1, C and F; see Fig. 3 C for confirmation of AdsA expression). *S. aureus* survival was also examined in the blood of human volunteers. Similar to mouse blood, the number of *adsA* mutant staphylococci was reduced as compared with wild-type *S. aureus* Newman, and this defect was restored by transformation of *adsA* mutants with *padsA* (Fig. 1 G).

AdsA is required for staphylococci virulence and abscess formation

To investigate the contribution of *adsA* to invasive staphylococcal disease, BALB/c mice were infected by intravenous inoculation with 10^7 CFU of wild-type *S. aureus* Newman or its isogenic *adsA* variant. Animals were killed 5 d after infection and both kidneys were removed. The right kidney was homogenized, and staphylococcal load was enumerated by plating on agar and colony formation (Fig. 2 A). The left kidney was fixed with formalin, embedded in paraffin, thin sectioned, and analyzed for histopathology (Fig. 2 B). As expected, wild-type *S. aureus* Newman formed abscesses in kidneys with a mean bacterial load of 3×10^7 CFU per gram of tissue. In contrast, *adsA* mutant staphylococci were unable to form abscesses and displayed a >10 -fold reduction in bacterial load as compared with the wild type ($P < 0.03$; Fig. 2, A and B).

Staphylococcal strains that cause community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) have been characterized by pulsed-field gel electrophoresis and DNA sequencing. Currently, the major CA-MRSA clone is USA300 (McDougal et al., 2003), the predominant cause of skin and soft tissue infections as well as bacteremia (Carleton et al., 2004). To assess the contribution of *adsA* toward virulence of USA300, we isolated an isogenic *adsA* mutant using phage transduction and *S. aureus* strain LAC (USA300; Bae et al., 2004). BALB/c mice were infected by intravenous injection of 10^7 CFU. 5 d after challenge, staphylococci were enumerated in homogenized kidney tissue and histopathology was visualized in hematoxylin-eosin-stained thin sections (Fig. 2 C). Similar to *S. aureus* Newman, we observed a 1-log reduction in CFUs recovered from the kidneys of animals infected with the *adsA* mutant of *S. aureus* USA300. Further, we observed fewer abscesses and smaller lesions in the kidneys of mice infected with the *adsA* variant (Fig. 2 D and Fig. S1). Collectively, these results suggest a significant contribution of *adsA* toward virulence of two clinical isolates, *S. aureus* strains Newman and USA300.

Differences in abscess formation and recovery of CFUs from the kidneys of infected mice may stem from enhanced bacterial clearance in the blood stream, causing fewer bacteria to reach peripheral organ tissues. Alternatively, *adsA* could

play a direct role in the formation of abscesses and infectious foci. To discern between these possibilities, BALB/c mice were infected by intravenous inoculation with 10^7 CFU and peripheral blood was sampled at timed intervals by cardiac puncture. In agreement with observations of enhanced clearance of *adsA* mutant staphylococci in vitro, significantly fewer CFUs of *adsA* mutant staphylococci were retrieved 90 min after infection as compared with the wild-type parent strain *S. aureus* Newman ($P < 0.05$). Transformation of the *adsA* mutant strain with *padsA* restored its ability to survive in blood after intravenous challenge (Fig. 2 E). Although we cannot rule out the possibility that *adsA* also contributes specifically to abscess formation, these data suggest that the reduced virulence of *adsA* mutant staphylococci results from their decreased survival in blood.

5'-Nucleotidase activity is the mediator of the virulence attributes of AdsA

Given that AdsA harbors a 5'-nucleotidase signature sequence, we asked whether AdsA can synthesize adenosine from AMP. Cell-wall peptidoglycan of *S. aureus* wild type, *adsA*, and the *adsA:padsA* strain was degraded with lysostaphin (Schindler and Schuhardt, 1964), and cell-wall extracts were incubated with radiolabeled [14 C]AMP. The relative abundance of

AdsA in the staphylococcal envelope was assessed by immunoblotting (Fig. 3 C), and the production of adenosine was monitored by TLC (Fig. 3 A). Lysostaphin extracts of *adsA* mutant staphylococci displayed reduced adenosine synthase activity, which could be restored to wild-type levels when *adsA* mutants were transformed with *padsA*, a plasmid-encoded wild-type allele (Fig. 3 A). AdsA harbors 5'-nucleotidase signature sequences, domains critical for the catalytic activity of mammalian enzymes (Zimmermann, 1992). To assess the role of adenosine synthase activity toward staphylococcal virulence, the YdamaVGNHEFD signature sequence (AdsA residues 189–201) was removed to generate AdsA*. Immunoblotting revealed the expression of AdsA* in the envelope of *adsA* mutant staphylococci that had been transformed with *padsA** (Fig. 3 C). Expression of AdsA* restored neither the defects in adenosine synthase activity (Fig. 3 A) nor the increased clearance of *adsA* mutant staphylococci in blood (Fig. 3 C). Incubation of staphylococci with adenosine 5'-(α , β -methylene) diphosphate (Knöfel and Sträter, 2001), a known 5'-nucleotidase inhibitor, reduced adenosine production and survival of wild-type *S. aureus* Newman in blood (Fig. S2). Collectively, these experiments suggest that AdsA adenosine synthase activity may be responsible for the virulence attributes of this surface protein.

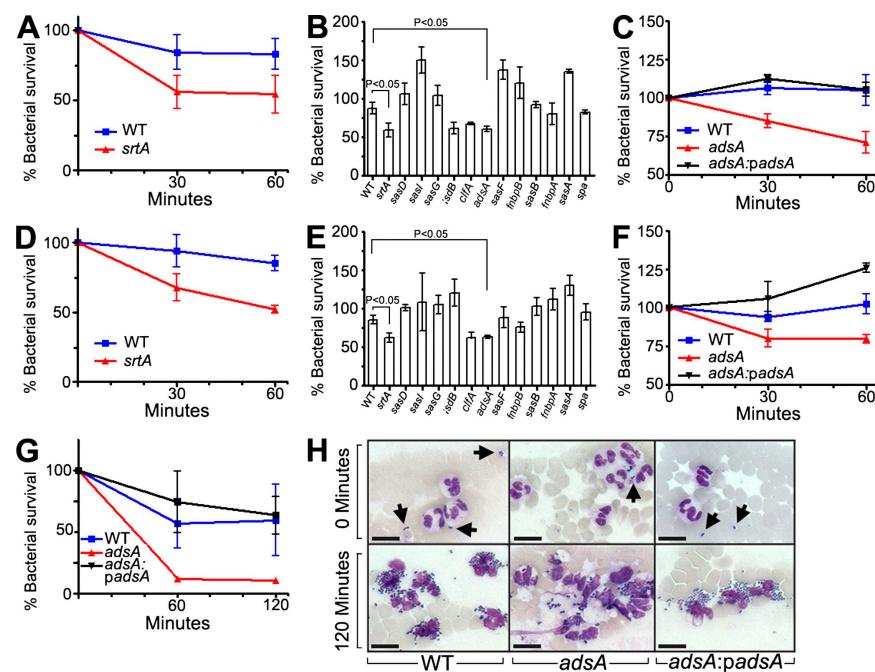


Figure 1. AdsA, a cell wall-anchored surface protein, is required for staphylococcal survival in blood. Comparison of the survival of 10^5 CFU of wild-type *S. aureus* Newman (WT) or the isogenic *srtA* variants in 1 ml of blood from (A) BALB/c mice or (D) Sprague-Dawley rats. Data are the means, and error bars represent \pm SEM from three independent analyses. To assess the relative contribution of sortase A-anchored cell-wall surface proteins for staphylococci survival in blood, isogenic mutants with transposon insertions in the indicated genes were incubated in blood from mice (B) or rats (E) for 60 min. Expression of *padsA* rescues staphylococcal survival of an *adsA* mutant in blood from mice (C), rats (F), or human volunteers (G). 10^5 CFU of staphylococci were incubated with 1 ml of human blood for 0 or 120 min, and Giemsa-stained samples were viewed by microscopy (H). At 0 min, only extracellular staphylococci were detected (arrows), whereas after 120 min of incubation staphylococci were mostly associated with neutrophils. The abundance of *adsA* mutants was reduced compared with wild-type staphylococci or *adsA* mutants harboring the complementing plasmid (*padsA*). Data are representative of three independent analyses with blood from three different human donors. Bars, 10 μ m.

To characterize the enzymatic activity of AdsA, we expressed a soluble affinity-tagged recombinant form of *S. aureus* AdsA (residues 1–400) in *Escherichia coli*. Purified AdsA, removed of its affinity tag (Fig. 3 D), cleaved [¹⁴C]AMP to generate adenosine (Fig. 3 E). Maximal activity ($K_m = 44$ nM) was observed in the presence of 5 mM MgCl₂ or 5 mM

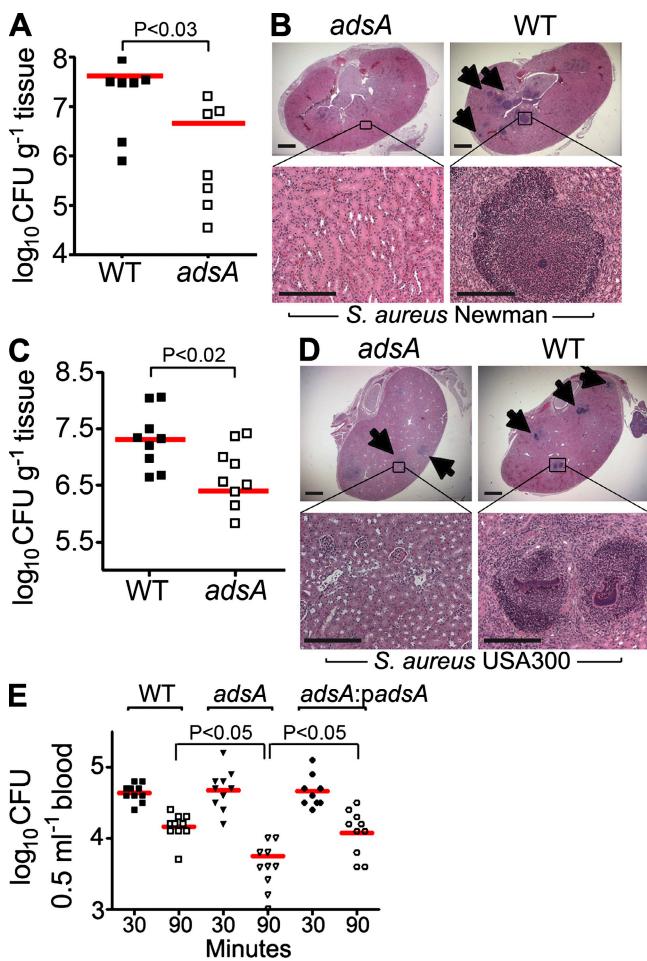


Figure 2. AdsA is a virulence factor that enables staphylococcal replication and abscess formation in vivo. Staphylococcal burden in kidneys after intravenous challenge of BALB/c mice ($n = 10$) with (A) 10⁷ CFU of wild-type *S. aureus* Newman or its isogenic *adsA* mutant and (C) *S. aureus* USA300 wild-type or its isogenic *adsA* mutant was enumerated as log₁₀ CFU per gram of tissue 5 d after infection. Data are representative of two independent analyses. Microscopic images of hematoxylin-eosin-stained kidney tissue were obtained after necropsy of mice infected with (B) wild-type *S. aureus* Newman and *adsA* mutants or (D) wild-type *S. aureus* USA300 and corresponding *adsA* mutants. Black arrows denote the abscess lesions. Data are representative samples of cohorts of five animals per bacterial strain and two independent analyses. Bars: (top) 1 mm; (bottom) 100 μ m. (E) Staphylococcal burden—wild-type (WT), *adsA*, or *adsA:padsA* *S. aureus* Newman—in blood of BALB/c mice ($n = 10$) was measured as log₁₀ CFU per 0.5 ml of blood retrieved by cardiac puncture at 30 and 90 min after intravenous challenge with 10⁷ CFU. Horizontal red bars represent mean CFUs in each cohort. Data are representative of three independent analyses using cohorts of 10 mice per bacterial strain per time point. Unpaired Student's *t* tests were used for statistical analysis.

MnCl₂, similar to the metal requirements of other adenosine synthases (Fig. 3 E; Zimmermann, 1992). On the other hand, incubation of AdsA with 5 mM ZnCl₂ or 5 mM CuSO₄ before the addition of [¹⁴C]AMP inhibited adenosine synthase activity (Fig. 3 E, lanes 6 and 7). A similar inhibitory effect was observed when EDTA, a divalent metal ion chelator, was added to the enzyme (Fig. 3 F). AdsA activity appears specific for adenosine monophosphate, as other nucleotide monophosphates were either not cleaved or were cleaved at a much reduced rate (Fig. 3 F).

Staphylococcal AdsA synthesizes adenosine during infection

Under physiological conditions, the concentration of AMP in the extracellular milieu is estimated to be in the nanomolar range. Immunological insult or tissue injury, however, causes release of AMP with concentrations up to 100 μ M. To us, it seemed plausible that these AMP stores may be converted to adenosine during staphylococcal infection. To assess the relative abundance of adenosine during staphylococcal infection, 1 ml of mouse blood was infected in vitro with 10⁵ CFU *S. aureus* for 60 min. Plasma was recovered by centrifugation, protein was removed, and samples were subjected to reversed-phase HPLC (RP-HPLC). For calibration, we separated chemically pure adenosine and determined its molecular mass in the eluate (Fig. 4 A). Chromatography of uninfected blood revealed the adenosine absorption peak, whose identity was confirmed by mass spectrometry (Fig. 4 B). The adenosine peak in blood was increased 10-fold after infection with *S. aureus* Newman (Fig. 4 C), whereas infection with the isogenic *adsA* mutant produced a <2-fold increase in adenosine (Fig. 4 D). To examine adenosine production in vivo, mice were infected by intravenous inoculation of 10⁷ CFU *S. aureus* and plasma adenosine abundance was quantified 60 min after infection. Animals infected with wild-type *S. aureus* Newman (Fig. 4 G) displayed increased amounts of adenosine in blood when compared with mice that had been mock infected (Fig. 4 F) or challenged with the *adsA* variant (Fig. 4 H). Thus, in agreement with the conjecture that staphylococci synthesize adenosine during infection, both the in vitro and the in vivo inoculation of mouse blood with *S. aureus* Newman cause an *adsA*-dependent increase in adenosine.

Adenosine reduces killing of staphylococci by neutrophils

We hypothesized that staphylococci escape phagocytic clearance in blood by synthesizing adenosine. If so, the survival defect of *adsA* mutant staphylococci in blood should be rescued by exogenous supplies of adenosine. This was tested, revealing a specific increase in the survival of *adsA* mutant staphylococci in the presence of 5 and 15 μ M adenosine (Fig. S3) but not with guanosine (Fig. 5 A). We wondered whether enhanced growth rates of *S. aureus* were not observed when adenosine was added to staphylococci incubated in laboratory media (Fig. 5 B), suggesting that the ability of adenosine to increase the bacterial load in blood may be exerted by its effect on phagocytic cells that otherwise kill the invading pathogen. In agreement with this

hypothesis, no difference in bacterial survival or growth was observed when wild-type and *adsA* mutant staphylococci were incubated in human serum or plasma (Fig. 5 C).

Although many different antimicrobial factors have been revealed in blood, PMNs, particularly phagocytic neutrophils, play a dominant role in innate immune defenses against staphylococci (Verdrengh and Tarkowski, 1997; Mölne et al., 2000; Voyich et al., 2005). To ascertain the fate of staphylococci in blood, we incubated *S. aureus* Newman expressing GFP in anticoagulated blood and measured bacterial phagocytosis and clearance by immune cells (Fig. 5 D). To distinguish extracellular from intracellular bacteria, blood and cell samples were treated with lysostaphin, a glycyl-glycine endopeptidase that quickly lyses extracellular staphylococci and abrogates GFP fluorescence (Fig. S4). Blood samples were stained with anti-GR1 and infected neutrophils were analyzed by flow cytometry (Fig. S5). 15 min after staphylococcal inoculation into blood, neutrophils had internalized similar numbers of lysostaphin-resistant wild-type and *adsA* mutant staphylococci, measured as GFP fluorescence of GR1-positive cells (Fig. 5 D and Fig. S5). Staphylococcal uptake involves neutrophil opsonophagocytosis, which is not affected by adenosine synthesis, as similar amounts of C3b were found deposited on the surface of wild-type and *adsA* mutant staphylococci (Fig. S6). Upon 30 min of incubation,

adsA mutant staphylococci were killed by neutrophils, as the GFP fluorescence of GR1-positive cells declined over time (Fig. 5 D and Fig. S5). In contrast, GFP fluorescence of wild-type staphylococci within neutrophils was not diminished (Fig. 5 D). GFP fluorescence could not be identified in isolated monocytes and T or B lymphocytes of infected mouse blood, suggesting that C3b deposition primarily triggers staphylococcal phagocytosis by neutrophils (Fig. S4). Phagocytosis is thought to play a dominant role in the clearance of *S. aureus* from blood. Cytochalasin D, a molecule that inhibits actin polymerization, is known to block phagocytosis (Casella et al., 1981). In some cell types, e.g., macrophages, cytochalasin D can also inhibit the binding of yeast particles to complement-independent phagocytic receptors (Bos and de Souza, 2000). In our assay, where GFP-expressing staphylococci are taken up by neutrophils, 10 μ M cytochalasin D inhibited phagocytosis and increased the survival of both wild-type and *adsA* mutant staphylococci (Fig. S7).

B. anthracis survives in blood and synthesizes adenosine

To investigate whether other pathogenic bacteria express adenosine synthases, we first examined bacterial genome sequences with BLAST searches for predicted translation products harboring the adenosine synthase domain of AdsA; homologues were identified in several different species

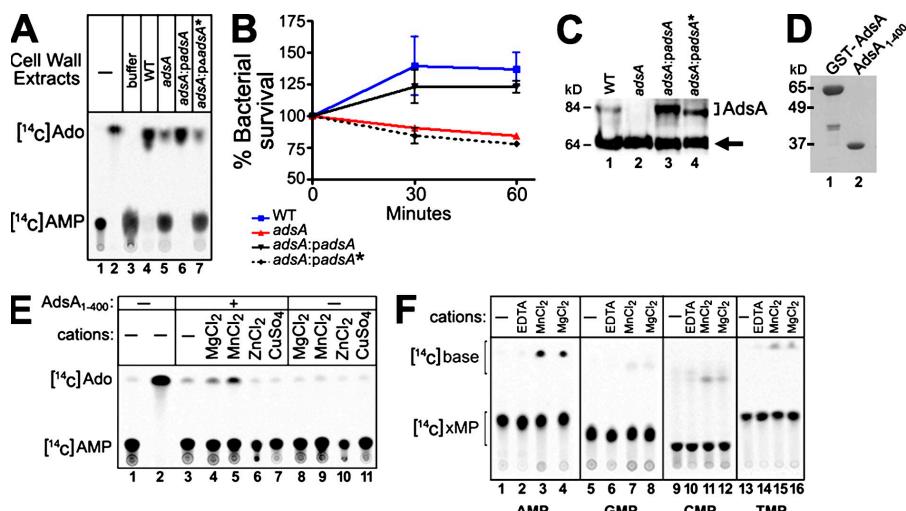


Figure 3. 5'-Nucleotidase activity of AdsA is essential for virulence. (A) Lysostaphin-generated cell-wall extracts of staphylococcal strains were incubated with radiolabeled [¹⁴C]AMP. The generation of [¹⁴C]adenosine ([¹⁴C]Ado) was measured by TLC using a PhosphorImager (Storm; GE Healthcare). Data are representative of two independent analyses. (B) *S. aureus* strains in A were analyzed for their ability to survive in lepirudin-anticoagulated fresh blood obtained from BALB/c mice. Data are representative of two independent analyses conducted in triplicate; error bars represent the SEM. (C) The abundance of AdsA in cell-wall lysates was measured by immunoblotting analyses with anti-AdsA rabbit polyclonal sera. The following strains were tested: *S. aureus* Newman wild-type (WT), *adsA*, *adsA:padsA* (harboring the *padsA* plasmid for the expression of wild-type *adsA*), and *adsA:padsA** (harboring the *padsA** plasmid for the expression of an *adsA* variant lacking the 5'-nucleotidase signature sequence). The arrow denotes protein A immunoreactive signals. Data are representative of four independent analyses (D) Recombinant GST-AdsA was purified from *E. coli* and cleaved with thrombin to generate AdsA₁₋₄₀₀, and purified proteins were analyzed by Coomassie-stained SDS-PAGE. Data are representative of five independent analyses. (E) Radiolabeled [¹⁴C]AMP was incubated in the presence or absence of 2 μ M of purified AdsA₁₋₄₀₀ and in the presence or absence of 5 mM of various metal ions. Radioactive signals for [¹⁴C]AMP and [¹⁴C]Ado after TLC were captured by a PhosphorImager. Data are representative of six independent analyses. (F) Radiolabeled [¹⁴C]AMP (lanes 1-4), [¹⁴C]GMP (lanes 5-8), [¹⁴C]CMP (lanes 9-12), and [¹⁴C]TMP (lanes 13-16) were incubated in the presence or absence of 2 μ M of purified AdsA₁₋₄₀₀ in the presence or absence of 5 mM of various metal ions, as indicated. Radioactive signals for [¹⁴C]xMP (nucleotide mono-phosphate) and [¹⁴C]nucleotide free base after TLC were captured by a PhosphorImager. Data are representative of three independent analyses.

(Table S1). For example, the genome of *B. anthracis* encodes an AdsA homologue (*B. anthracis* surface protein; available from GenBank/EMBL/DDBJ under accession no. BAS4031) with a 5'-nucleotidase signature sequence (YdvisLGN-HEFN; residues 131–142), an N-terminal signal peptide, and a C-terminal LPXTG sorting signal, indicating that this surface protein is also deposited by sortase A in the cell-wall envelope (Gaspar et al., 2005). To determine whether *B. anthracis* AdsA functions as an adenosine synthase and contributes to escape from innate immune responses, we constructed a deletion mutant of *B. anthracis* *adsA* by allelic replacement (Fig. 6). Mutanolysin, a muralytic enzyme that cleaves *N*-acetylmuramyl-(β 1 \rightarrow 4)-*N*-acetylglucosamine within

peptidoglycan (Yokogawa et al., 1974), was used to generate cell-wall lysates. Cell-wall extracts of wild-type bacilli harbored adenosine synthase activity, whereas extracts derived from *adsA* mutant bacilli displayed reduced activity (Fig. 6 A). Deletion of the structural gene *adsA* abolished the expression of adenosine synthase (Fig. 6 B), abrogated the surface display of AdsA by *B. anthracis* (Fig. 6 C), and reduced the ability of bacilli to synthesize adenosine (Fig. 6 A). Residual amounts of AMP hydrolysis are attributed to other phosphatases, including alkaline phosphatase. We expressed and affinity tagged *B. anthracis* AdsA in *E. coli* and purified the enzyme. Similar to *S. aureus* AdsA, adenosine synthase activity of *B. anthracis* AdsA was observed in the presence of 5 mM

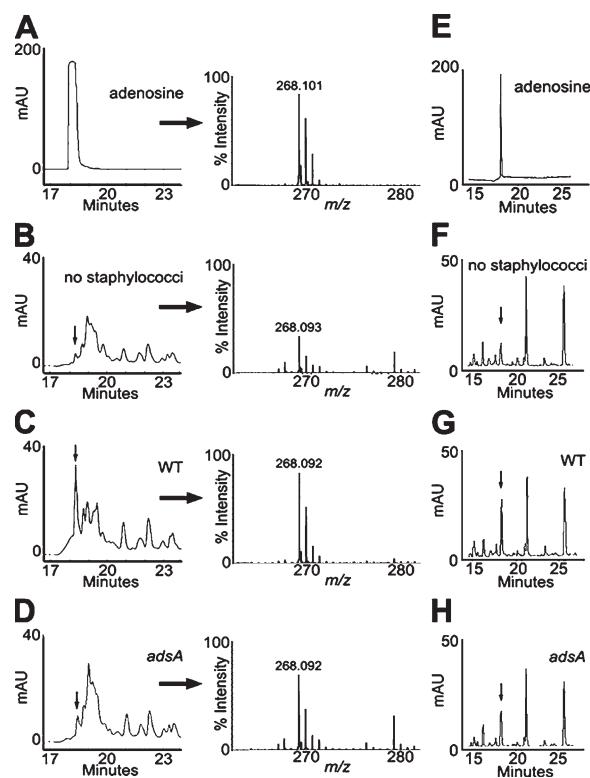


Figure 4. *S. aureus* AdsA synthesizes adenosine in blood. (A) RP-HPLC to quantify 100 μ M adenosine (left) and identify its monoisotopic ions by MALDI-MS (right). 1 ml of lepirudin-anticoagulated mouse blood was incubated without (B) or with 10^5 CFU of wild-type *S. aureus* Newman (WT; C) or its isogenic *adsA* variants (D) for 1 h. Plasma was deproteinized, filtered, and subjected to RP-HPLC to quantify adenosine (left) and identify its monoisotopic ions by MALDI-MS (right). Arrows denote corresponding adenosine peaks. Calculated abundance of adenosine in plasma extrapolated from the purified adenosine control was 1.1 μ M (B, no staphylococci), 13.2 μ M (C, WT *S. aureus* Newman), and 2.1 μ M (D, *adsA* mutant staphylococci). Data are representative of three independent analyses. (E–H) RP-HPLC analyses of 50 μ M adenosine (E) or plasma collected from mice that had been mock infected (F), or from animals that were challenged with 10^7 CFU wild-type (WT; G) or (H) *adsA* mutant bacteria. Data are representative of two independent analyses. The calculated abundance of plasma adenosine in mice extrapolated from the purified adenosine control was 2.8 ± 0.6 μ M (F, no staphylococci), 16.4 ± 2.1 μ M (G, WT *S. aureus* Newman), and 7.4 ± 3.2 μ M (H, *adsA* mutant staphylococci). mAU, milliabsorbance units of HPLC eluate.

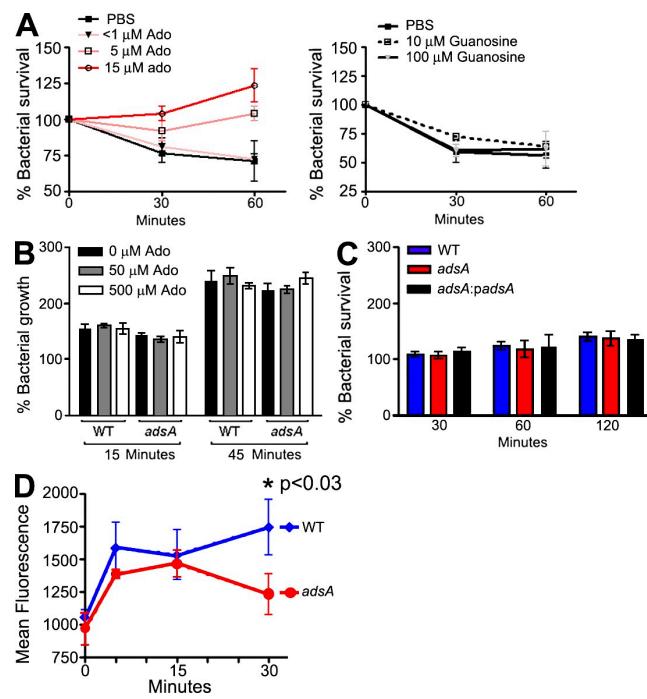


Figure 5. Immunosuppressive effects of adenosine contribute to *S. aureus* survival in blood. (A) Survival of *adsA* *S. aureus* Newman in mouse blood in the presence of increasing concentrations of adenosine (left) or guanosine (right) as indicated. Extracellular adenosine concentration was quantified by RP-HPLC immediately before enumerating bacterial CFUs, as described in Fig. S3. Data are representative of two independent analyses conducted in duplicate (left) and are representative of five independent analyses (right); error bars represent the SEM. (B) Growth of wild-type and *adsA* *S. aureus* Newman in laboratory growth media with 0–500 μ M adenosine (Ado) for 15 and 45 min. Data are means of two independent analyses conducted in triplicate. Error bars represent the SEM. (C) Survival of wild-type, *adsA*, or *adsA*:*padsA* *S. aureus* Newman in human plasma. Data are averaged of two independent analyses conducted in triplicate; error bars indicate SEM. (D) The *adsA* gene is required for staphylococcal escape from neutrophil killing. Mouse blood was inoculated with *S. aureus* Newman expressing GFP, comparing the wild type (WT) and its isogenic *adsA* variant. At the indicated time points, samples were treated with 10 μ g/ml lysostaphin, followed by isolation of neutrophils by FACS using anti-GR1 staining and phagocytosis and survival of staphylococci measured as mean GFP fluorescence. Data are representative of two independent analyses conducted in triplicate; error bars indicate SEM. *p < 0.03.

$MnCl_2$ ($K_M = 2.01$ nM), whereas 5 mM $MgCl_2$ reduced its activity (Fig. 6 D). When inoculated into mouse blood, we observed increased phagocytic clearance of the *adsA* mutant, as compared with the wild-type parent *B. anthracis* Sterne (Fig. 6 E). Collectively, these experiments suggest that *B. anthracis* also utilizes AdsA to synthesize adenosine and escape host innate immune responses.

Several other Gram-positive bacteria harbor putative 5'-nucleotidase genes (Table S1), and we analyzed their ability to synthesize adenosine. Similar to *S. aureus* and *B. anthracis*,

cell-wall extracts derived from *Enterococcus faecalis* and *S. epidermidis* both synthesized adenosine from AMP, but *B. subtilis* did not (Fig. 7). The *B. subtilis* chromosome encodes a gene with a 5'-nucleotidase signature motif (Kunst et al., 1997); however, it is currently not known whether or not this gene is expressed and its predicted product displays activity. Nevertheless, we surmise that the ability of bacterial pathogens to synthesize adenosine and release this immunosuppressive compound may represent an important virulence strategy in many different hosts.

DISCUSSION

This study provides evidence that *S. aureus* and other bacterial pathogens generate adenosine to promote their survival during host infection. We identified and characterized AdsA, a cell wall-anchored protein of *S. aureus* with 5'-nucleotidase signature sequences, and its functional homologue in *B. anthracis*. We show that *adsA* is required for synthesis of adenosine during infection and that the structural gene contributes to the survival of *S. aureus* and *B. anthracis* in animal or human blood. Further, in a renal abscess model of *S. aureus* infection in mice, *adsA* was required for pathogen replication in organ tissues and for the formation of abscess lesions.

5'-Nucleotidases are present in many different bacterial species, but their potential as virulence factors has hitherto not been appreciated. We provide evidence that AdsA may be a clinically relevant virulence factor of CA-MRSA strain USA300, the causative agent of the majority of community-acquired skin and soft-tissue infections in the United States (Carleton et al., 2004). Roche et al. (2003) previously reported that *sasH* (*adsA*) was significantly associated with invasive disease isolates as compared with nasal carriage isolates, further distinguishing a pivotal role for *adsA* in staphylococcal disease. Although the extraordinary immunosuppressive attributes of adenosine are generally known, to the best of our knowledge, this study provides the first mechanistic link

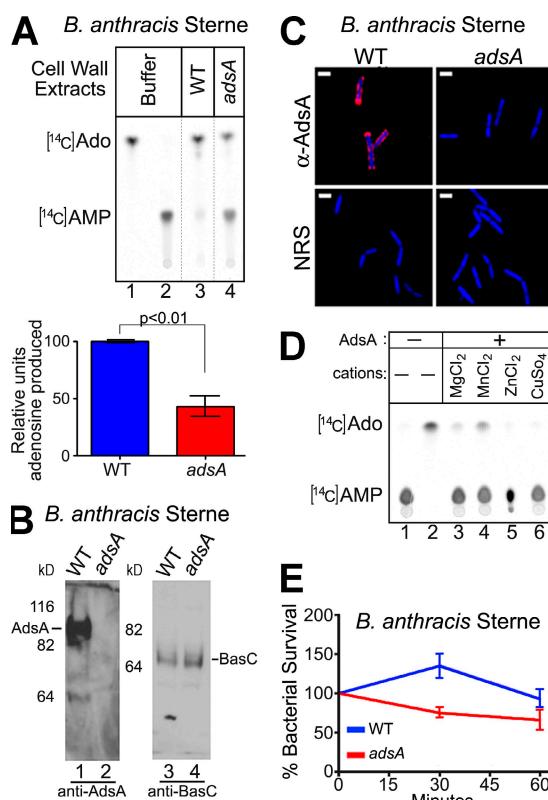


Figure 6. 5'-Nucleotidase activity of AdsA enhances *B. anthracis* survival in blood. (A, top) Mutanolysin extracts from *B. anthracis* strain Sterne (WT) or its isogenic *adsA* variant were incubated with radiolabeled [¹⁴C]AMP. The generation of adenosine was measured by TLC. (bottom) Quantification of relative abundance of adenosine. Data are the mean of three independent analyses; error bars indicate SEM. (B) Mutanolysin extracts were analyzed by immunoblotting with antibodies directed against *B. anthracis* AdsA or BasC (anti-BasC), a control protein not involved in adenosine production. Bars, 5 μ m. (C) Fluorescence microscopy images of wild-type *B. anthracis* Sterne and its isogenic *adsA* mutant stained with antiserum against *B. anthracis* AdsA (top) or nonreactive serum (NRS) and Cy3-labeled secondary antibodies (red), as well as Hoechst staining of nucleic acids (blue). Data are representative of two independent analyses. (D) Radiolabeled [¹⁴C]AMP was incubated with 2 μ M of purified *B. anthracis* AdsA in the presence of 5 mM of the indicated metal cations, and generation of [¹⁴C]adenosine ([¹⁴C]Ado) was measured by TLC and a PhosphorImager. Data are representative of three independent analyses. (E) Survival of wild-type and *adsA* *B. anthracis* Sterne in rat blood over time, measured as CFUs on agar plates. Data are the mean of two independent analyses; error bars indicate SEM.

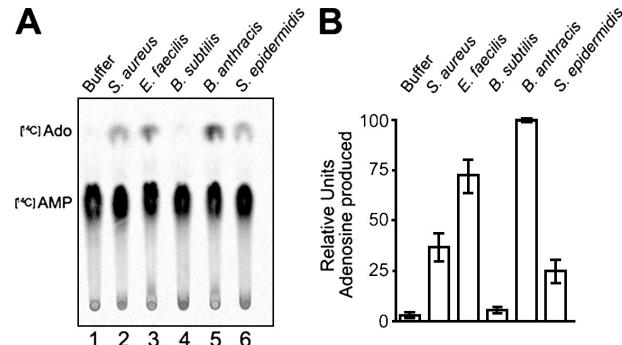


Figure 7. Hydrolysis of AMP is observable in various Gram-positive pathogens. (A) Cell-wall proteins from the indicated bacterial strains were released with mutanolysin digestion, and mutanolysin extracts were incubated with radiolabeled [¹⁴C]AMP. Hydrolysis of AMP into adenosine was assessed by separating the compounds by TLC. (B) Quantification of the relative abundance of adenosine produced in cell-wall extracts from A. Data represent the mean of three independent analyses; error bars indicate SEM.

between bacterial pathogenesis and adenosine synthesis. *S. aureus* is primarily an extracellular pathogen, and the main thrust of host defenses against this pathogen relies on PMNs and phagocytic killing (Lowy, 1998). Adenosine is known to inhibit neutrophil degranulation (Bouma et al., 1997), adhesion to vascular surfaces (Firestein et al., 1995), and superoxide burst (Cronstein et al., 1990; Gunther and Herring, 1991; Kaufmann et al., 2007). Our experiments reveal the effect of adenosine on staphylococcal survival in blood at an early stage of infection (30–90 min), when tissue damage and AMP release is limited. At a later stage of infection, when *S. aureus* seeds abscesses and causes liquefaction necrosis, AMP levels are expected to increase as a consequence of staphylococcal toxins, e.g., α -hemolysin, leukocidins, or phenol-soluble modulins, that form pores in membranes and precipitate cellular lysis (Diep and Otto, 2008).

We observed further that *adsA*-deficient *S. aureus* were cleared more rapidly from the bloodstream of BALB/c mice than wild-type staphylococci, correlating with the reduced ability to grow during infection and/or seed abscesses; presumably, lower numbers of *adsA* mutant staphylococci can disseminate through the vasculature and form abscess lesions.

In addition to neutrophils, tissue macrophages are essential for the clearance of bacterial infections. Adenosine directly impairs the ability of macrophages to combat infection by decreasing the phagocytic activity of these cells (Eppell et al., 1989). Further, adenosine attenuates macrophage antibacterial activity by suppressing the production of superoxide (Edwards et al., 1994) and nitric oxide (Haskó et al., 1996; Xaus et al., 1999), both of which are integral to the killing of phagocytosed bacteria. Macrophage deactivation by adenosine also suppresses antibacterial defense mechanisms by decreasing the production of proinflammatory cytokines that both orchestrate inflammatory/immune functions of other cell types and act as regulators of macrophage function (Haskó and Szabó, 1998). For example, TNF is a regulator of neutrophil, endothelial cell, and lymphocyte function, and the production of TNF has been shown to be under negative control of adenosine. Finally, an important consideration is that adenosine, through the activation of macrophages, may decrease T cell antibacterial effector mechanisms. In support of this conjecture, adenosine decreases the expression of MHC II on macrophages (Edwards et al., 1994) and dendritic cells (Panther et al., 2001), and it decreases macrophage production of IL-12, a pivotal stimulus for Th1-type immune responses (Haskó et al., 2000).

Examination of the genomes of Gram-positive bacteria identified genes with similarities to putative 5'-nucleotidases. Many of these proteins harbor sortase A-specific sorting signals (LPXTG), indicating that their mature products are localized in the bacterial cell-wall envelope. Such localization appears relevant, as surface-exposed adenosine synthases should release their product in close proximity to phagocytic cells. Other microorganisms secrete 5'-nucleotidases into the extracellular environment, as recent studies have described a panel of nucleotide-metabolizing enzymes secreted by

Trichinella spiralis parasites (Gounaris, 2002) and *Vibrio cholerae*, a Gram-negative pathogen (Punj et al., 2000). Collectively, our results suggest that *S. aureus* and other Gram-positive pathogens increase extracellular concentrations of the potent immunosuppressive molecule adenosine and, through this mechanism, are likely able to perturb immune defenses and promote their survival in host tissues.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains were grown in TSB at 37°C. *S. aureus* strain USA300 was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (National Institute of Allergy and Infectious Diseases [NIAID]). All mutants used in this study were obtained from the Phoenix (ΦNE) library (Bae et al., 2004). Each Phoenix isolate is a derivative of the clinical isolate Newman (Duthie and Lorenz, 1952) or USA300 (Carleton et al., 2004), as indicated in the figures. All *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman or USA300 using bacteriophage ϕ 85 and verified by PCR analysis. Chloramphenicol was used at 10 mg/liter⁻¹ for plasmid and allele selection with *padsA*. Erythromycin was used at 10 mg/liter⁻¹ for allele selection in *S. aureus* Newman and at 50 mg/liter⁻¹ for allele selection in USA300. Mutants of *B. anthracis* strain Sterne were generated with pLM4, containing a thermosensitive origin of replication. Plasmids with a 1-kb DNA sequence flanking each side of the mutation were transformed into *B. anthracis*, and transformants were grown at 30°C (permissive temperature) in Luria broth (LB; 20 μ g/ml⁻¹ kanamycin). Cultures were diluted 1:100 and plated on LB agar (20 μ g/ml⁻¹ kanamycin) at 43°C overnight (restrictive temperature). Single colonies were inoculated into LB broth without antibiotics and grown overnight at 30°C. To ensure loss of pLM4-based plasmid, these cultures were diluted four times into fresh LB broth without antibiotic pressure and propagated at 30°C. Cultures were diluted and plated on LB agar and colonies were examined for kanamycin resistance. DNA from kanamycin-sensitive colonies was analyzed by PCR for the presence or absence of mutant alleles.

S. aureus-expressing GFP. Plasmid pTet_{P_{ro}}-GFP, a pCL55 derivative with a tetracycline-inducible promoter, was electroporated into *S. aureus* TB9 to generate TB9 (*tet*_{P_{ro}}-*gfp*). TB9 is a variant of *S. aureus* Newman generated by homologous recombination to excise bacteriophage NMφ4 from the *geh* lipase gene (Bae et al., 2006). pTet_{P_{ro}}-GFP integration into the *geh* gene resulted in a lipase-negative phenotype, which was verified on egg-yolk plates.

Plasmids. The following primers were used for PCR amplification reactions: P55 (5'-TTTCCCGGGACGATCCAGCTCTAACGCTG-3'), P56 (5'-TTT-GAGCTCAAAGCAAATAGATAATCGAGAAATATAAAAAG-3'), P57 (5'-TTT-GAGCTCAGTTGCTCCAGCCAGCATT-3'), mP58 (5'-TTT-GAATCAAACGGATTCCATTCCAGCC-3'), FP10 (5'-TACGAATTCTG-ACCTGGCAGGCAATTGAAA-3'), RP10 (5'-TGTGAATTCTTAG-CTAGCTTTCTACGTCG-3'), FP3C (5'-TCGGGATCCGCTGAGCA-GCATACACCAATG-3'), RPB (5'-TGTGGATCCTTATTGATTAATT-TGTTCACTAATGC-3'), VTFD (5'-TACAGATCTAACCTACTGCATTCA-3'), VTRC (5'-TACAGATCTAACCTACTGCATTCA-3'), #184 (5'-CGGGATCCCCCTCGAGTTCATGAAAAAC-3'), and #192 (5'-GGGGTACCTTATTGTATAGTCATCCATGCCATGTG-3'). Ligation of FP10/RP10 (*adsA* + 700 bp upstream from start site) PCR products into pOS1 (EcoRI) generated *padsA*. Triple ligation of FP10/VTRD and VTFD/RP10 (deletion of amino acids 189–201) PCR products into pOS1 (EcoRI) generated p Δ adsA. Insertion of P55/P56 (*basA* 1-kb 5' flanking sequence) and P57/P58 (*basA* 1-kb 3' flanking sequence) PCR products into pLM4 (EcoRI, SacI, and XbaI sites) generated pJK34. This plasmid was used to delete the *basA* coding sequence. Ligation products were transformed into *E. coli* DH5 α , and plasmid DNA was transformed into *E. coli* K1077 (*dam*⁻, *dcm*⁻), and purified (nonmethylated) plasmid DNA was transformed into *B. anthracis* according to a previously developed protocol (Gaspar et al., 2005). Ligation of FP3C/RPB (1.2-kb truncation of *adsA* starting 5' after the signal peptide) PCR

products into pGEX-2T (GE Healthcare) generated the *adsA* expression vector pVT1, and this plasmid was transformed into *E. coli* BL21. Ligation of PCR amplification products generated with primers #184/#192 into pCL55 (Lee et al., 1991) using BamHI/KpnI sites resulted in plasmid pTet_{pro}-GFP (Gründling and Schneewind, 2007).

Animal experiments. All experimental protocols were reviewed, approved, and performed under regulatory supervision of the University of Chicago's Institutional Biosafety Committee and Institutional Animal Care and Use Committee. BALB/c mice were purchased from Charles River, and Sprague-Dawley rats were purchased from Harlan. Overnight cultures of *S. aureus* strains were diluted 1:100 into fresh TSB and grown for 3 h at 37°C. Staphylococci were centrifuged, washed twice, and diluted in PBS to yield an OD₆₀₀ of 0.5 (10⁸ CFU ml⁻¹). Viable staphylococci were enumerated by colony formation on tryptic soy agar plates to quantify the infectious dose. Mice were anaesthetized by intraperitoneal injection of 80–120 mg ketamine and 3–6 mg xylazine per kilogram of body weight. 100 µl of bacterial suspension (10⁷ CFU) was administered intravenously via retroorbital injection into 6-wk-old female BALB/c mice. On day 5, mice were killed by compressed CO₂ inhalation. Kidneys were removed and homogenized in PBS containing 1% Triton X-100. Aliquots of homogenates were diluted and plated on agar medium for triplicate determination of CFUs. The Student's *t* test was performed for statistical analysis using Prism software (GraphPad Software, Inc.). For histopathology, kidney tissue was incubated at room temperature in 10% formalin for 24 h. Tissues were embedded in paraffin, thin sectioned, stained with hematoxylin-eosin, and examined by microscopy.

To measure staphylococcal survival in blood, 6-wk-old female BALB/c mice were infected with 10⁷ CFU of staphylococci by retroorbital injection. At 30 or 90 min, mice were killed by compressed CO₂ inhalation and blood was collected by cardiac puncture using a 25-gauge needle. Aliquots were incubated on ice for 30 min in a final concentration of 0.5% saponin/PBS to lyse host eukaryotic cells. Dilutions were plated on TSA for enumeration of surviving CFUs at the two different time points.

Chemicals. Mutanolysin (Sigma-Aldrich) was suspended at a concentration of 5,000 U/ml⁻¹ in 100 mM sodium phosphate, pH 6, containing 1 mM PMSF and stored at –20°C. [¹⁴C]AMP, [¹⁴C]GMP, [¹⁴C]CMP, [¹⁴C]TMP, and [¹⁴C]adenosine were purchased from Moravek Biochemicals. Lysostaphin was purchased from AMBI and purified adenosine was purchased from Sigma-Aldrich.

Bacterial survival in blood. Overnight cultures of *S. aureus* strains were diluted 1:100 into fresh TSB and grown for 3 h at 37°C. Staphylococci were centrifuged, washed twice, and diluted in PBS to yield an OD₆₀₀ of 0.5 (10⁸ CFU/ml⁻¹). Whole blood was collected by cardiac puncture of Sprague-Dawley rats or BALB/c mice, and 5 µg/ml⁻¹ of lepirudin anticoagulant was immediately added. 100 µl of 10⁶ CFU/ml⁻¹ of bacteria were mixed with 900 µl of rat or mouse blood. For human blood studies, 100 µl of 10⁸ CFU/ml⁻¹ of bacteria was mixed with 900 µl of freshly drawn human blood. The tubes were incubated at 37°C with slow rotation for the time points indicated in the figures, at which time aliquots were incubated on ice for 30 min in a final concentration of 0.5% saponin/PBS to lyse eukaryotic cells. Dilutions of staphylococci were plated on TSA for enumeration of surviving CFUs. Experiments with blood from human volunteers involved protocols that were reviewed, approved, and performed under regulatory supervision of the University of Chicago's Institutional Review Board.

Adenosine synthase activity. Overnight cultures of *S. aureus* strains were diluted 1:100 into fresh TSB and grown for 3 h at 37°C. Staphylococci were centrifuged and washed twice with PBS. 3 ml of cells was spun down and suspended in 100 µl TSM buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, and 0.5 M sucrose); lysostaphin (final concentration = 70 µg/ml⁻¹) was added and allowed to incubate for 30 min at 37°C. The solution was spun down for 5 min at 9,000 g, and supernatants containing released cell-surface proteins were collected. 15 µl of lysostaphin extracts were incubated with 3 µCi [¹⁴C]AMP for 30 min at 37°C. Samples were spotted on a silica

plate, followed by separation by TLC using a (75:25 isopropanol/double-distilled H₂O) 0.2-M ammonia bicarbonate solvent. For cell-wall extracts of *S. aureus*, *E. faecalis*, *B. anthracis*, and *S. epidermidis* digested with mutanolysin, mutanolysin was substituted for lysostaphin and used according to the manufacturer's recommended conditions. When assayed with purified proteins, 2 µM of purified AdsA or BasA was incubated in a final volume of 15 µl with 3 µCi [¹⁴C]AMP in the presence of the metal cations in TSM buffer indicated in the figures.

Adenosine concentration in blood. Whole-blood killing assay with staphylococci was performed as described in Bacterial survival in blood. Extraction of plasma was performed as previously described (Mo and Ballard, 2001). In brief, after conclusion of the whole-blood killing assay, blood samples were centrifuged at 16,000 g for 5 min and noncellular plasma was collected. 600 µl of plasma was extracted with 75 µl perchloric acid (1.5 M) and 1 mM EDTA. The 500-µl supernatant was withdrawn after centrifugation for 5 min at 16,000 g and neutralized with 29 µl of 4 M KOH. After ice cooling for 10 min, the sample was again centrifuged at 16,000 g for 5 min. The pH of the supernatant was finally adjusted to pH 6–7, diluted 1:4 with PBS, and filtered with a 0.22-µm syringe filter before RP-HPLC.

HPLC and mass spectrometry. The presence of adenosine production was determined by RP-HPLC. Samples were chromatographed on a 250 mm × 3 mm column (5-µm particle size; BDS Hypersil C18; Thermo Fisher Scientific). The mobile phase consisted of solution A (deionized H₂O/0.1% trifluoroacetic acid) and solution B (acetonitrile/0.1% trifluoroacetic acid). Adenosine was eluted with a solvent B gradient from 1 to 100%, run from 5 to 50 min. The solvent flow rate was 0.5 ml/min. Peaks were detected by their UV absorbance at 260 nm. The peak of adenosine in the HPLC chromatogram was identified by comparison of its retention time to the retention time of purified adenosine used as a standard sample. Fractions containing adenosine were cospotted with matrix (α -cyano-4-hydroxycinnamic acid) and subjected to matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) under reflector-positive conditions.

FACS analyses. Freshly prepared mouse blood was inoculated with *S. aureus* Newman expressing GFP and incubated at 37°C as described. Aliquots were removed and incubated for an additional 5 min with 10 µg/ml⁻¹ lysostaphin. After lysostaphin treatment, samples were diluted with ice cold PBS plus 10 µM cytochalasin D, and erythrocytes were lysed with erythrocyte lysis buffer (QIAGEN) for 30 min on ice. Blood leukocytes were washed three times by centrifuging at 400 g, and cells were suspended in PBS/1% FBS. Cells were stained with allophycocyanin-conjugated anti-GR1 or anti-F4/80 antibodies, or PE-conjugated anti-CD4, anti-CD8, or anti-B220 antibodies (eBioscience), and analyzed using a FACSCanto (BD).

Online supplemental material. Fig. S1 examines the contribution of *adsA* toward *S. aureus* USA300 abscess formation in mice. Fig. S2 documents that *adsA*-mediated adenosine production is responsible for enhanced survival of staphylococci in blood and that this phenotype can be inhibited with adenosine 5'-(α , β -methylene) diphosphate. The rapid clearance of exogenously added adenosine in blood is studied in Fig. S3. Fig. S4 examines the correlation between GFP fluorescence of staphylococci and bacterial viability in the presence of lysostaphin. Fig. S5 displays representative FACS dot plots and histograms demonstrating that wild-type and *adsA* mutant staphylococci are phagocytosed by neutrophils, but that only *adsA* mutants, not the wild type, are killed by immune cells. Fig. S6 analyzes the deposition of complement (C3b) on the staphylococcal surface in the presence or absence of AdsA. Fig. S7 uses cytochalasin D to study the contribution of phagocytosis toward staphylococcal killing by neutrophils. Table S1 lists Gram-positive bacteria encoding AdsA homologues, which are anchored to the cell-wall envelope. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090097/DC1>.

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