**Staphylococcus aureus** synthesizes adenosine to escape host immune responses

Vilasack Thammavongsa, Justin W. Kern, Dominique M. Missiakas, and Olaf Schneewind

Department of Microbiology, University of Chicago, Chicago, IL 60637

**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.

Staphylococcus aureus is the leading cause of bloodstream, 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). *Staphylococcal* 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, A2A, A2B, and A3 (Haskó and Pacher, 2008). T lymphocytes express the high affinity A2A receptor as well as the low affinity A2B receptor (Thiel et al., 2003). Depending on their activation state, macrophages and neutrophils express all four adenosine receptors, whereas B cells harbor only A2A (Thiel et al., 2003). Engagement of A2A 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

Supplemental material can be found at: http://doi.org/10.1084/jem.20090097

© 2009 Thammavongsa et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.jem.org/misc/terms.shtml). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike–No Mirror Sites license, as described at http://creativecommons.org/licenses/by-nc-nd/3.0/).
inflammation, hypoxia, organ injury, and traumatic shock is mediated by two sequential enzymes. Ecto-ATP diphosphohydrolase (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, 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 bacula 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 clfA and sasH (Staphylococcus aureus surface protein), hereafter named adsA, displayed consistent survival defects. The phenotype of clfA 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 ILHHTnDHGrL (residues 124–134) and YdamaVGNNHEFD (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 staphylococcal virulence and abscess formation
To investigate the contribution of adsA to invasive staphylococcal disease, BALB/c mice were infected by intravenous inoculation with 10⁷ 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 × 10⁷ 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 10⁷ 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 lysozyme (Schindler and Schuhardt, 1964), and cell-wall extracts were incubated with radiolabeled [14C]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*. Immuno blotting 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 ± 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 µm.
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 MgCl_2 or 5 mM MnCl_2, similar to the metal requirements of other adenosine synthases (Fig. 3 E; Zimmermann, 1992). On the other hand, incubation of AdsA with 5 mM ZnCl_2 or 5 mM CuSO_4 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).

Staphyloccocal 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 µM. 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 10^9 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 10^9 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 µM 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 \( \textit{adsA} \) 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 \( \textit{S. aureus} \) Newman expressing \( \textit{GFP} \) 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 \( \textit{GFP} \) 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 \( \textit{adsA} \) mutant staphylococci, measured as \( \textit{GFP} \) 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 \( \textit{adsA} \) mutant staphylococci (Fig. S6). Upon 30 min of incubation, \( \textit{adsA} \) mutant staphylococci were killed by neutrophils, as the \( \textit{GFP} \) fluorescence of GR1-positive cells declined over time (Fig. 5 D and Fig. S5). In contrast, \( \textit{GFP} \) fluorescence of wild-type staphylococci within neutrophils was not diminished (Fig. 5 D). \( \textit{GFP} \) 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 \( \textit{S. aureus} \) 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 \( \textit{GFP} \)-expressing staphylococci are taken up by neutrophils, 10 \( \mu \text{M} \) cytochalasin D inhibited phagocytosis and increased the survival of both wild-type and \( \textit{adsA} \) mutant staphylococci (Fig. S7).

\( \textit{B. anthracis} \) 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.
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 (Table S1).

Figure 4. S. aureus AdsA synthesizes adenosine in blood. (A) RP-HPLC to quantify 100 µM 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 µM (B, no staphylococci), 13.2 µM (C, WT S. aureus Newman), and 2.1 µM (D, adsA mutant staphylococci). Data are representative of three independent analyses. (E–H) RP-HPLC analyses of 50 µM 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 conducted in triplicate; error bars indicate SEM.

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 µM 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 µg/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.
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 μm. (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 μM 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 (Eppel 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 can be reversed by adenosine.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. aureus* strains were grown in TSB at 37°C. *S. aureus* strain USA300 was obtained through the Network onAntimicrobial Resistance in *Staphylococcus aureus* (National Institute of Allergy and Infectious Diseases [NIAID]). All mutants used in this study were obtained from the Phoenix (BNÉ) 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 *lursa aurea* isolations were transduced into wild-type *S. aureus* Newman or USA300 using bacteriophage q8S 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 µg/ml−1 kanamycin). Cultures were diluted 1:100 and plated on LB agar (20 µg/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 pTetpro-GFP, a pCL55 derivative with a tetracycline-inducible promoter, was electroporated into *S. aureus* T90 by generating T90 (tetom/gfp). T90 is a variant of *S. aureus* Newman generated by homologous recombination to excise bacteriophage NIM4.3 from the *gfb* like gene (Bae et al., 2006). *pTetpro*-GFP integration into the *gfb* 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′-TTTCCTGGAGGATCCACTCCGCTATCTAATGGTCTG-3′), P56 (5′-TTGAGCTCTAAGAATCCAATGGGAGAATATATAAGA-3′), P57 (5′-TTTGGAGCTACGTGCTCCGACGCAGCATT-3′), mP58 (5′-TTTGG- GAATCTCAACCGATTCATCCAGCAGCC-3′), F10 (5′-TACGAATTCCAGTCTTGGGCAACTGGAATGAAA-3′), R10 (5′-TGTGAATCTTCTCTAGCTAGCTCTCTCTAGTGCG-3′), F12 (5′-TCTGGATCGCTGACGACGCA-3′), R12 (5′-TGTTGGAATCTTATTTGATTAATGCTAT-3′), V1 (5′-TACGAATTCCAGTCTTGGGCAACTGGAATGAAA-3′), F15 (5′-TACGAATTCCAGTCTTGGGCAACTGGAATGAAA-3′), R15 (5′-TGTTGGAATCTTATTTGATTAATGCTAT-3′), V2 (5′-TACGAATTCCAGTCTTGGGCAACTGGAATGAAA-3′), F18 (5′-TACGAATTCCAGTCTTGGGCAACTGGAATGAAA-3′), R18 (5′-TGTTGGAATCTTATTTGATTAATGCTAT-3′). Ligation of P10/RP10 (adsA + 700 bp upstream from start site) PCR products into pOS1 (EcoRI) generated padsA. Triple ligation of P50/FP10 and VTFD/RP10 (deletion of amino acids 189–201) PCR products into pOS1 (EcoRI) generated pAdsA. Insertion of P55/P56 (5′-3′) flanking sequence) and P57/P58 (5′-3′) flanking sequence) PCR products into plM4 (EcoRI, ScaI, and Xmal sites) generated pJK34. This plasmid was used to delete the *hscA* coding sequence. Ligation products were transformed into *E. coli* DH5α, and purified (nonmethylated) plasmid DNA was transformed into *E. coli* K1077 (dam−, dnm−), 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 #1864/#1912 into pCLES (Lee et al., 1991) using BamHI/KpnI sites resulted in plasmid pTetpro-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 (10^6 CFU ml^-1). Viable staphylococci were enumerated by colony formation on tryptic soy agar plates to quantify the infectious dose. Mice were anesthetized by intraperitoneal injection of 80–120 mg ketamine and 3–6 mg xylazine per kilogram of body weight. 100 µl of bacterial suspension (10^6 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 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.). 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 10^7 CFU of staphylococci by retroorbital injection. At 30 or 90 min, 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.). 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.

**Chemicals.** Mutanolysin (Sigma-Aldrich) was suspended at a concentration of 5,000 U/ml 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. Lyso- staphin 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 (10^6 CFU/ml^-1). Whole 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. Experiments were performed with triplicate determinations of CFUs.

**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 30 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 that of standard adenosine. 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 lyso- staphin. After lysostaphin treatment, samples were diluted with ice cold PBS plus 10 µM cytochalasin D, and erythrocytes were lysed with erythrocyte lys- in bufer (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 FACSCount (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 dependence of complement (C3b deposition 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.

We thank members of our laboratory, F. Bagnoli, and G. Grandi for critical comments and discussion.


SUPPLEMENTAL MATERIAL

Thammavongsa et al., http://www.jem.org/cgi/content/full/jem.20090097/DC1

Figure S1. Histopathological examination of kidneys isolated from mice infected with USA300. Microscopic images of hematoxylin-eosin–stained kidney tissue obtained after necropsy of mice infected for 4 d with wild-type S. aureus USA300 (bottom) and corresponding adsA mutants (top). Arrows denote a central concentration of staphylococci and PMN infiltrates. Data are representative samples of cohorts of five animals per bacterial strain and two independent analyses. Bars, 1 mm.

Figure S2. Inhibition of adenosine synthase activity reduces S. aureus survival in blood. (A) Lysostaphin cell-wall extracts from the indicated bacterial strains were incubated with radiolabeled [14C]AMP alone or with radiolabeled [14C]AMP and increasing concentrations of adenosine 5'-(α,β-methylene) diphosphate. Generation of [14C]adenosine ([14C]Ado) was measured by TLC and a PhosphorImager. Data are representative of two independent analyses. (B) Survival of wild-type (WT) or adsA S. aureus Newman in mouse blood with increasing concentrations of 5'-(α,β-methylene) diphosphate. Data are representative of two independent analyses conducted in triplicate, and error bars represent the SEM. (C) RP-HPLC to quantify adenosine. 1 ml of lepirudin-anticoagulated mouse blood was incubated with 10^5 CFU of wild-type S. aureus Newman alone or with either 100 or 500 μM adenosine 5'-(α,β-methylene) diphosphate for 1 h. Plasma was deproteinized, filtered, and subjected to RP-HPLC to quantify adenosine (left). Arrows denote corresponding adenosine peaks. Calculated abundance of adenosine in plasma extrapolated from the purified adenosine control: no detectable adenosine in blood without staphylococci, 16 μM after infection with wild-type S. aureus Newman, 1.5 μM in the presence of 100 μM 5'-(α,β-methylene) diphosphate, and, finally, adenosine was not detectable in the presence of 500 μM of inhibitor. Data are representative of two independent analyses.
Figure S3. Abundance of exogenously added adenosine in blood. (A) RP-HPLC to quantify adenosine. 1 ml of lepirudin-anticoagulated mouse blood was incubated with $10^5$ CFU adsA variants for 1 h with 20 (B), 50 (C), 100 (D), or 200 μM (E) of added adenosine. Plasma was deproteinized, filtered, and subjected to RP-HPLC to quantify adenosine. Arrows denote corresponding adenosine peaks. The calculated abundance of adenosine in plasma extrapolated from the purified adenosine control was not detected (ND) for B and C, 5 μM for D, and 15 μM for E. Data are representative of two independent analyses. mAU, milliabsorbance units of HPLC eluate.
Figure S4. **Lysostaphin treatment results in loss of GFP fluorescence and bacterial viability.** $10^7$ CFU of wild-type or *adsA* mutant staphylococci was incubated with either 0, 5, or 10 μg ml$^{-1}$ lysostaphin for 5 min at 37°C. Bacteria were either immediately fixed in 10% paraformaldehyde and visualized by confocal microscopy (A), or serially diluted and CFUs were enumerated by plating on agar (B). Data are representative of two independent analyses conducted in quadruplicate. DIC, differential interference contrast microscopy. Bars, 10 μm.
Figure S5. Flow cytometry–based analyses for observing association of *S. aureus* with cells in blood. 1 ml of mouse blood was inoculated with $10^5$ CFU of GFP-expressing wild-type (WT) or *adsA* mutant *S. aureus*. (A) Cells were isolated 15 min after inoculation, treated with 10 μg/ml lyso- staphin, stained with anti-GR1, and analyzed by flow cytometry. (left) A bivariant dot plot of isolated cells isolated after 15 min. The horizontal axis shows forward scatter (FSC) and the vertical axis shows side scatter (SSC). (right) Bivariant dot plot of gated granulocytes from the left panel showing the percentage of granulocytes that were anti-GR1 positive. The horizontal axis shows forward scatter and the vertical axis shows anti-GR1–PE. (B) Histogram plots of gated cells from A (right) showing GFP fluorescence of GR1-positive cells inoculated with either wild-type or *adsA* staphylococci. The horizontal axis shows the intensity of GFP fluorescence and the vertical axis shows cell counts. The gate shows the percentage of GFP-positive cells. (C) *S. aureus* is primarily associated with neutrophils. (left) A bivariant dot plot of isolated cells isolated after 15 min. The horizontal axis shows forward scatter and the vertical axis shows side scatter. (right) Histograms of gated cells (R1, R2, and R3) from the left panel. The horizontal axis shows the intensity of GFP fluorescence and the horizontal axis shows cell counts. The gate shows the percentage of GFP-positive cells. Data are representative of two independent analyses conducted in triplicate.

Figure S6. *AdsA* does not effect complement deposition. Wild-type, *adsA*, and *adsA:padsA* *S. aureus* were grown to mid-log phase (absorbance$_{600nm}$ = 0.5), washed twice with PBS, and incubated with PBS/10% human sera at 37°C for the indicated times. Cells were immediately placed on ice, and surface-bound C3b was detected with FITC-conjugated goat F(ab′)$_2$ anti-human C3b. The binding of antibody to $10^5$ bacteria was measured by flow cytometry. Data are representative of two independent analyses conducted in quadruplicate. Error bars represent the SEM. MFI, mean fluorescence intensity.
Figure S7. **Inhibition of phagocytosis enhances bacterial survival.** Survival of wild type (WT) or adsA S. aureus Newman in mouse blood with 10 μM cytochalasin D. Data are representative of three independent analyses conducted in duplicate, and error bars represent the SEM.

Table S1. **Gram-positive bacteria harboring 5'-nucleotidase genes**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Function</th>
<th>Accession no.^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. anthracis</td>
<td>2',3'-cyclic-nucleotide 2'-phosphodiesterase</td>
<td>Q6HTQ7</td>
</tr>
<tr>
<td>B. cereus</td>
<td>5'-nucleotidase domain protein</td>
<td>A7GMX9</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>5'-nucleotidase family protein</td>
<td>Q0SRM9</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>5'-nucleotidase family protein</td>
<td>Q839U0</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Putative uncharacterized protein</td>
<td>B8DG18</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>Putative uncharacterized protein</td>
<td>Q8YAJ5</td>
</tr>
<tr>
<td>S. aureus strain MW2</td>
<td>Putative 5'-nucleotidase</td>
<td>Q8NYQ6</td>
</tr>
<tr>
<td>S. epidermis</td>
<td>5'-nucleotidase family protein</td>
<td>Q5HQ0E0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Putative surface-anchored 5'-nucleotidase</td>
<td>A2RF30</td>
</tr>
<tr>
<td>S. mutans</td>
<td>Putative 5'-nucleotidase</td>
<td>Q8CVSC5</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>5'-nucleotidase family protein</td>
<td>A8XAM1</td>
</tr>
<tr>
<td>S. suis</td>
<td>Putative 5'-nucleotidase</td>
<td>A4V27</td>
</tr>
</tbody>
</table>

^aAvailable from GenBank/EMBL/DDBJ.