



Key role of poly- γ -DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*

Stanislava Kocianova,¹ Cuong Vuong,¹ Yufeng Yao,¹ Jovanka M. Voyich,¹ Elizabeth R. Fischer,² Frank R. DeLeo,¹ and Michael Otto¹

¹Laboratory of Human Bacterial Pathogenesis and ²Microscopy Core Facility, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Hamilton, Montana, USA.

Coagulase-negative staphylococci, with the leading species *Staphylococcus epidermidis*, are the predominant cause of hospital-acquired infections. Treatment is especially difficult owing to biofilm formation and frequent antibiotic resistance. However, virulence mechanisms of these important opportunistic pathogens have remained poorly characterized. Here we demonstrate that *S. epidermidis* secretes poly- γ -DL-glutamic acid (PGA) to facilitate growth and survival in the human host. Importantly, PGA efficiently sheltered *S. epidermidis* from key components of innate host defense, namely antimicrobial peptides and neutrophil phagocytosis, and was indispensable for persistence during device-related infection. Furthermore, PGA protected *S. epidermidis* from high salt concentration, a key feature of its natural environment, the human skin. Notably, PGA was synthesized by all tested strains of *S. epidermidis* and a series of closely related coagulase-negative staphylococci, most of which are opportunistic pathogens. Our study presents important novel biological functions for PGA and indicates that PGA represents an excellent target for therapeutic maneuvers aimed at treating disease caused by *S. epidermidis* and related staphylococci.

Introduction

Over the past decade, *Staphylococcus epidermidis* has become the most prevalent pathogen involved in nosocomial infections (1). Usually an innocuous commensal microorganism on human skin, this member of the coagulase-negative group of staphylococci can cause severe infection after penetration of the epidermal protective barriers of the human body. In the US alone, *S. epidermidis* infections on in-dwelling medical devices, which represent the main type of infection with *S. epidermidis*, cost the public health system approximately \$1 billion per year (1). Importantly, *S. epidermidis* is frequently resistant to common antibiotics (1). It is, therefore, essential to find new molecular targets for drug development against *S. epidermidis* infections. The formation of surface-attached cellular agglomerations, so-called biofilms, is a major determinant of *S. epidermidis* pathogenicity (2). Biofilm formation is believed to contribute significantly to antibiotic resistance and protection from innate host defense (3). Resistance of *S. epidermidis* biofilms to some antibiotics might, in part, be due to a status of generally reduced metabolism (4). Yet, by and large, the specific mediators of biofilm resistance and immune evasion in *S. epidermidis* are unknown.

Poly- γ -DL-glutamic acid (PGA) is an anionic, extracellular polymer, in which the α -amino and γ -carboxy groups of D- or L-glutamic acid are linked by isopeptide bonds (5). PGA is produced primarily by *Bacillus* strains but also infrequently by other strains of bacteria, archaeobacteria, and some eukaryotes (5). On the whole, the biological role of PGA has remained unclear. Presumably, it serves as an external C and N reserve or protects from environmen-

tal factors such as high osmolarity (5). In *Bacillus anthracis*, a PGA capsule has a hitherto unique role in sheltering the bacteria from phagocytosis (6). Homologs of the *cap* locus for PGA biosynthesis are found in a restricted number of human pathogens, including the spirochaete *Leptospira interrogans*, which causes the waterborne disease leptospirosis (7), and *Fusobacterium nucleatum*, which causes periodontal disease (8). However, it is unknown whether PGA is produced and plays a role in virulence in these organisms.

Here we demonstrate PGA production in *S. epidermidis* and related coagulase-negative staphylococci. We constructed an isogenic *cap* mutant strain of *S. epidermidis* to analyze the role of PGA in protection from environmental factors and innate host defense and as a virulence determinant in an animal infection model. Our study indicates an important function of PGA during both the commensal and infectious lifestyles of *S. epidermidis* and presents PGA production as the first widespread mechanism of immune evasion in this principal opportunistic pathogen.

Results

The S. epidermidis genome contains the genes that code for PGA synthesis. To survive on the human skin and during infection of in-dwelling medical devices, *S. epidermidis* must have mechanisms to circumvent human innate host defense. Only recently, we discovered that the biofilm exopolysaccharide polysaccharide intercellular adhesin (PIA) protects *S. epidermidis* from major mechanisms of innate host defense (9). However, PIA is restricted to a subpopulation of *S. epidermidis*, and therefore, a ubiquitous principle protecting *S. epidermidis* from innate host defense has remained elusive. We searched the recently published *S. epidermidis* genome (10) for gene loci potentially involved in such protection. We found that, in contrast to its relative *S. aureus*, *S. epidermidis* has the *cap* locus that codes for production of the anionic exopolymer PGA. The *S. epidermidis cap* genes show high similarity to those of *B. anthracis* and *B. subtilis* (Figure

Nonstandard abbreviations used: PGA, poly- γ -DL-glutamic acid; PIA, polysaccharide intercellular adhesin; PMN, polymorphonuclear leukocyte; TSB, tryptic soy broth.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 115:688–694 (2005). doi:10.1172/JCI200523523.

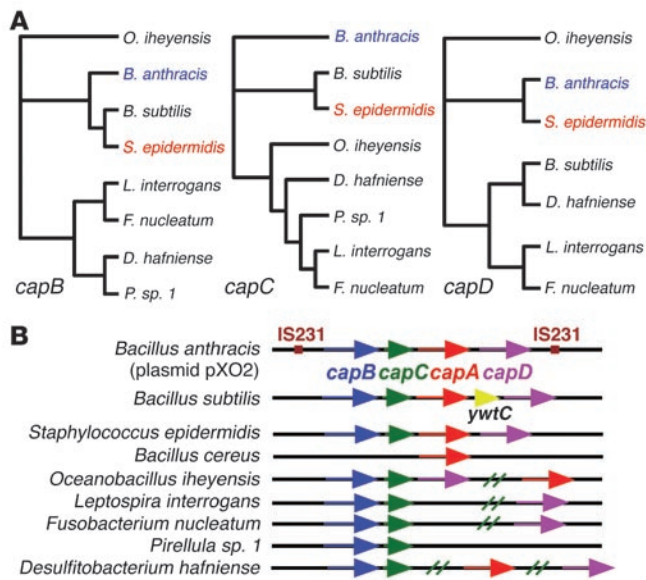


Figure 1

Molecular genetic comparison of bacteria with genes encoding a putative PGA synthesis machinery. **(A)** Phylogenetic trees based on sequence comparisons of the *capB* (amide ligase), *capC* (unknown function), and *capD* (depolymerase) genes. CapA is a putative PGA exporter. We have excluded a comparison of *capA* genes, because *capA* homologs were not found in all the organisms and comparison of transporters is normally less indicative of phylogenetic relations. Of the microorganisms shown, production of PGA has been demonstrated previously only in *B. anthracis* and *B. subtilis*, and in this study, in *S. epidermidis*. **(B)** *cap* genes and homologs in bacteria for which genetic information is available. The *B. anthracis cap* gene cluster is located on a plasmid and flanked by IS231 insertion sequences. All other genes are located in the bacterial chromosomes.

1A). Of note, in contrast to *B. subtilis* and several human pathogens that have parts of the *cap* genes, the genetic organization of the *cap* locus of *B. anthracis* is well conserved in *S. epidermidis* (Figure 1B).

The *S. epidermidis cap* gene locus drives production of surface-attached PGA. To analyze the role of the *cap* genes in *S. epidermidis*, we constructed a deletion mutant (*S. epidermidis Δcap*), in which the entire *cap* locus was replaced by a spectinomycin resistance cassette. To complement the mutant, we cloned the *cap* operon with its natural promoter in a plasmid and transformed the mutant with that plasmid (pRB*capBCAD*). First, we determined PGA production in the mutant, wild-type, and complemented strains. Real-time PCR data indicated that the *cap* genes in *S. epidermidis* are expressed (data not shown). Accordingly, immunodetection of PGA in cell surface extracts of

the wild-type and complemented strains demonstrated that PGA is synthesized by *S. epidermidis* and attached to the bacterial cell surface (Figure 2A). The PGA production level in the complemented strain was 147% of that of the wild-type strain. There was no detectable signal in the *cap* deletion strain or in any samples obtained from culture filtrates (Figure 2A and data not shown). Scanning electron microscopy with immunogold labeling confirmed that PGA is located on the *S. epidermidis* cell surface (Figure 2B). Next, we purified the immunoreactive material and performed quantitative and qualitative analyses to verify that it constitutes PGA. In contrast to *B. anthracis* PGA, which is entirely composed of D-glutamic acid (11), *S. epidermidis* PGA consisted of about equal amounts of D- and L-glutamic acid (Figure 2C). Notably, PGA production in *S. epidermidis* was far less than that reported for *B. anthracis* (1.2×10^{-6} g/l for *S. epidermidis* versus 5–10 g/l for *B. anthracis*; ref. 12). Taken together, our data demonstrate that the *cap* locus in *S. epidermidis* drives production of a surface-attached form of DL-PGA.

The *cap* gene locus and PGA production are ubiquitous among *S. epidermidis* strains. Some virulence factors in staphylococci, such as PIA, are mostly restricted to invasive strains (13, 14). In contrast,

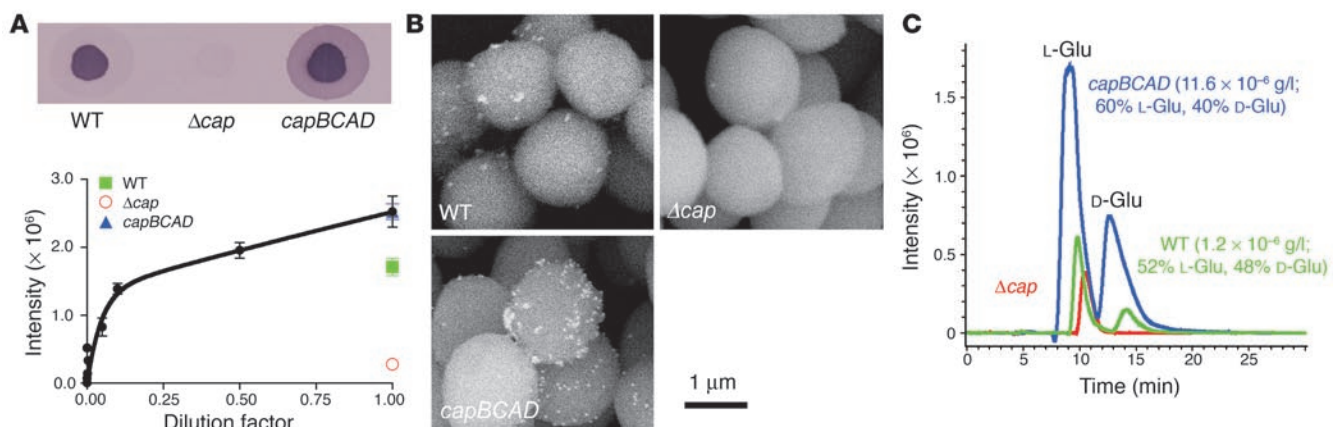


Figure 2

PGA production in *S. epidermidis*. **(A)** Relative expression of PGA determined by immuno-dot blot analysis. PGA was extracted from bacterial cell surfaces as described in Methods. A calibration curve was obtained by dilution of the most intensive sample obtained from the PGA-overexpressing complemented strain *S. epidermidis Δcap* (pRB*capBCAD*). Results are the mean \pm SEM of 4 experiments for the samples and the mean \pm SEM of 4 different serial dilutions for the calibration curve. A representative blot is shown at the top. **(B)** Detection of *S. epidermidis* PGA with immunoscanning electron microscopy. PGA was detected with anti-PGA antiserum. **(C)** Analysis of D-glutamic (D-Glu) and L-glutamic (L-Glu) acid in *S. epidermidis* PGA by stereoselective chromatography and liquid chromatographic–mass spectrometric detection of glutamic acid. To determine D- and L-glutamic acid amounts, the L-glutamic acid background detected in the *cap* mutant strain (Δcap) was subtracted from PGA expression strains. **(A–C)** Δcap , isogenic *cap* deletion strain; *capBCAD*, complemented strain *S. epidermidis Δcap* (pRB*capBCAD*).

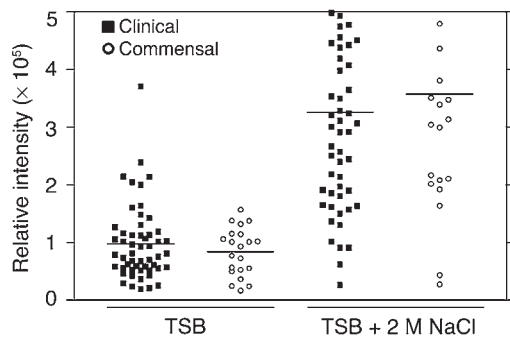


Figure 3
PGA production in *S. epidermidis* strains. PGA expression in *S. epidermidis* strains of clinical and commensal origin, and under low and high salt conditions, determined by immuno-dot blot analysis. Cells were grown for 24 hours at 37°C with shaking at 200 rpm. PGA was then purified as described in Methods. Horizontal bars show the group mean. The membrane background was subtracted from each sample. The experiment, including purification and detection, was repeated twice with very similar results.

all 74 *S. epidermidis* strains of clinical and commensal origin that we investigated had the *cap* gene locus as shown by analytical PCR (data not shown) and were positive for PGA by immuno-dot blot analysis (Figure 3), indicating that PGA production is ubiquitous in *S. epidermidis*. Remarkably, there was no significant difference in PGA production between commensal and clinical strains (Figure 3). We therefore hypothesized that PGA is important for the survival of *S. epidermidis* as a skin commensal organism and during infection.

PGA contributes to resistance of S. epidermidis to high salt concentration. We first determined whether PGA impacts survival of *S. epidermidis* under conditions found in its natural habitat, i.e., the human skin. The environment on human skin is characterized by high and varying salt concentration. *S. epidermidis* can withstand very high salt concentrations of up to approximately 2 M NaCl, but the reasons for this extraordinary resistance are not completely clear (13). Growth and viability of the *cap* mutant strain were significantly impaired at high concentrations of NaCl (shown for 2 M

NaCl in Figure 4, A and B), whereas growth at physiological salt concentration was similar for the wild-type and mutant strains (data not shown). Furthermore, quantitative real-time PCR analysis using a *capB* probe (Figure 4C) and immuno-dot blots (data not shown) demonstrated that *S. epidermidis* upregulates *cap* expression in response to high NaCl concentration. Additionally, in most strains of our collection, PGA production was greater at high NaCl concentration (Figure 3). These data suggest that PGA contributes to survival of *S. epidermidis* on human skin.

S. epidermidis PGA protects from key components of innate host defense. Next, we investigated whether PGA contributes to *S. epidermidis* virulence. Unlike its more aggressive relative *S. aureus*, *S. epidermidis* does not have a large arsenal of virulence factors (1). Rather, it causes relatively silent, chronic infections during which resistance against attacks by the innate immune system is of special importance to bacterial survival (1). Therefore, we analyzed whether *cap* expression in *S. epidermidis* affects resistance to key components of innate host defense against bacterial infections, namely antibacterial peptides and neutrophil phagocytosis. The *cap* mutant strain had significantly reduced resistance to 2 representative antibacterial peptides from human skin and neutrophil specific granules, LL-37 and human β -defensin 3 (Figure 5, A and B) and significantly increased susceptibility to phagocytosis by human neutrophils (Figure 5C). The phagocytosis rate was 42% higher with the mutant strain compared to the wild-type strain, which is a relatively large difference compared with what we observe with other single pathogen factors. These findings indicate that PGA plays a critical role in protecting *S. epidermidis* from the microcidal effects of innate host defense components.

PGA is indispensable for S. epidermidis persistence on in-dwelling medical devices in an animal infection model. The role of PGA in protection from innate host defense components suggested that PGA facilitates pathogen survival during *S. epidermidis* infection. To test this hypothesis, we compared persistence of wild-type and isogenic *cap* mutant strains in a mouse model of subcutaneous catheter infection. Importantly, biofilm formation on plastic material and intercellular aggregation in vitro did not differ between wild-type and *cap* mutant strains (data not shown), indicating that colonization and persistence in the animal model were not due to differences in the physicochemical interaction with the catheter

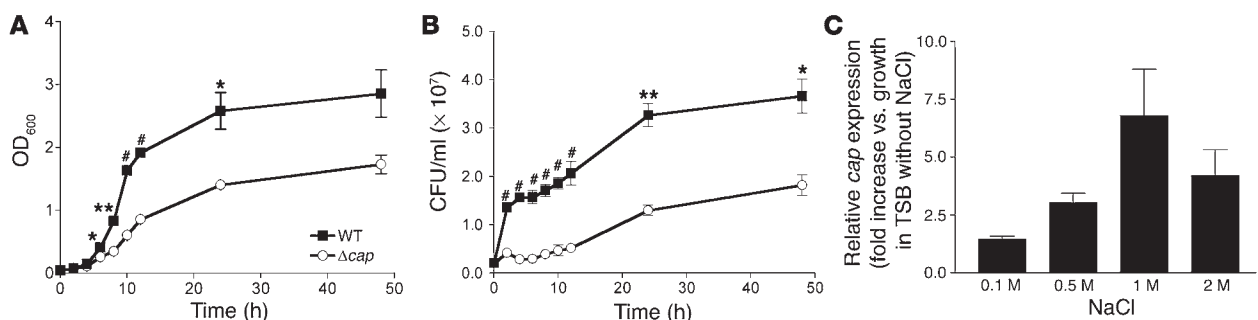


Figure 4
Role of PGA in osmoprotection and inducibility of *cap* expression by NaCl. (A) Growth (OD₆₀₀) and (B) viability (CFU) of wild-type and *cap* mutant strains in Luria-Bertani medium supplemented with 2 M NaCl. Bacteria were inoculated from an overnight preculture (1:1,000) and grown in flasks at 37°C with shaking at 200 rpm. Values are the mean \pm SEM of 3 experiments. **P* < 0.05; ***P* < 0.01; #*P* < 0.001 (wild-type versus mutant strain). (C) Quantitative real-time PCR analysis of NaCl inducibility of *cap* expression. Bacteria were grown as in (A) with the indicated concentrations of NaCl. Cells were harvested after 6 hours of growth, RNA was isolated, and real-time PCR was performed using a *capB* probe. Values are the mean \pm SEM of 3 experiments.

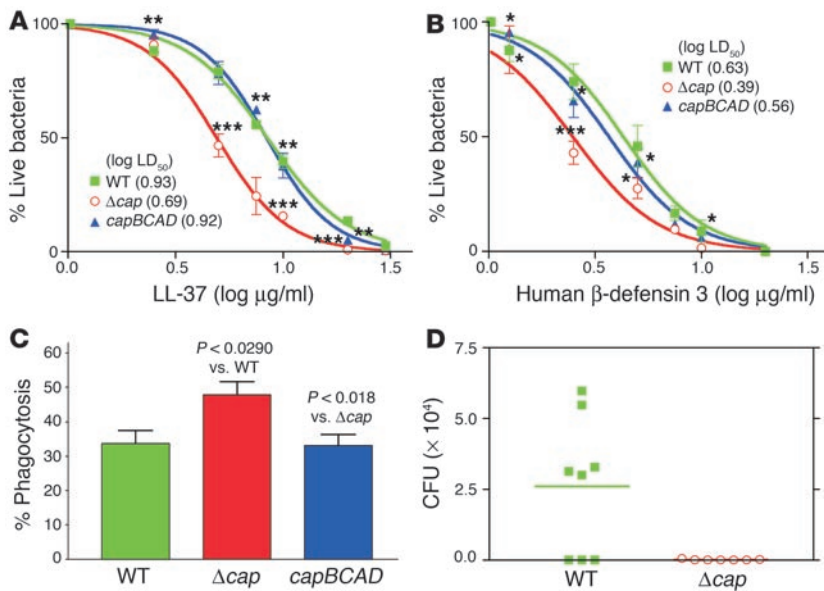


Figure 5 Role of PGA in immune evasion and virulence of *S. epidermidis*. (A and B) Resistance to cationic antimicrobial peptides. Washed *S. epidermidis* cells (approximately 10⁵) were incubated with LL-37 (A) or human β-defensin 3 (B) in various concentrations for 2 hours at 37°C. Thereafter, *S. epidermidis* survivor cells were counted by plating. Results are shown as dose-response curves. The log LD₅₀ values for all strain/peptide combinations are given in the key. Statistical analyses are for each peptide concentration. Values of significance were calculated against the wild-type (for Δcap) and Δcap (for capBCAD) strains. (C) Resistance to neutrophil phagocytosis. Phagocytosis by human neutrophils was determined after 30 minutes of incubation with *S. epidermidis* at a ratio of 20 bacteria per PMN. (D) Mouse model of subcutaneous device-related infection. Catheter pieces with equal amounts of adhered *S. epidermidis* cells (2 × 10⁵) were placed under the dorsum of the animals. CFU on implanted devices 1 week after infection were counted. The horizontal bar shows the group mean. *P < 0.05; ***P < 0.01; ****P < 0.001.

material. Most mice (5 of 8) infected with the wild-type strain had significant numbers of bacteria (approximately 3 × 10⁴–6 × 10⁴ CFU) on implanted catheters after 1 week of infection, whereas all 7 mice infected with the cap mutant strain had completely cleared the infection (Figure 5D). This pronounced difference indicates that PGA is a key factor for the success of *S. epidermidis* in device-associated infection, which represents the predominant type of disease caused by this organism.

A group of coagulase-negative staphylococci related to *S. epidermidis* also produces PGA. We know from publicly available genome sequencing data that the cap locus is absent from *S. aureus*. To investigate whether other staphylococcal species have the genetic information for PGA production, we tested a series of staphylococcal strains for the presence of the cap genes by DNA-DNA hybridization using capB and capD probes and a cocktail of probes from all 4 cap genes. Of 22 strains, representing 16 different species and subspecies, genomic DNA from 11 strains hybridized with the probes (Table 1). Except for *S. saprophyticus* strains, all strains with a signal in the Southern blot also produced surface-attached PGA, which was demonstrated using anti-PGA antisera (Table 1). Although there are some intraspecies differences, it is remarkable that all phylogenetically related members of the *S. epidermidis* group (*S. epidermidis*, *S. capitis*, *S. warneri*, *S. saccharolyticus*, *S. caprae*, *S. hominis*, and *S. haemolyticus*) (15) have the ability to produce PGA, whereas it is far less distributed among other species (Table 1).

Discussion

We are only beginning to understand strategies used by pathogenic bacteria to evade the efficient bactericidal mechanisms of innate host defense (16). To survive in its natural habitat, i.e., on human skin, *S. epidermidis* needs to protect itself from antibacterial peptides of human epithelial cells. Furthermore, to persist during human infection, it must have efficient protection from ingestion by neutrophils and associated bactericidal mechanisms. Here we present PGA as the first widespread mechanism of immune evasion in *S. epidermidis*. Notably, we demonstrate a hitherto unknown role for PGA in resistance to cationic antimicrobial peptides. Further, PGA was critical for the establishment of chronic infection by *S. epidermidis*. The difference in persistence of the cap mutant and wild-type strains was more pronounced than with any other virulence determinant of *S. epidermidis* that has been investigated by an animal infection model. Inasmuch as PGA production in *S. epidermidis* is approximately 1 million times lower than in *B. anthracis*, one can conclude that the efficiency of PGA-mediated protection from innate host defense in the case of *S. epidermidis* is extraordinary. The mechanism of protection is unclear. Presumably, it is based on the strong anionic properties of PGA. For example, PGA may bind antibacterial peptides, which are usually cationic (17), and thus prevent them from reaching their target, the cytoplasmic membrane. However, protection by PGA may also be mediated, for example, by binding of PGA to other surface polymers, which might enhance the protective effect.

Except for the role that PGA has in protecting *B. anthracis* from phagocytosis, the biological function of PGA, particularly in noninfectious organisms, has remained speculative. It is known that several halophilic bacteria synthesize PGA (5). Therefore, it has been assumed that PGA serves to protect from high osmolarity. In our study, we show for the first time that PGA contributes to resistance against high salt concentration. It has been speculated that binding of water molecules by negatively charged PGA protects bacteria from dehydration (18). However, the low level of PGA production suggests that mechanisms other than direct binding of water by PGA might be involved in the protective effect in *S. epidermidis*.

Importantly, PGA production did not affect the aggregation behavior or biofilm formation of *S. epidermidis*, demonstrating that our observations are not due to secondary effects of cell aggregation. However, recent results that we obtained by microarray analysis of gene expression in *S. epidermidis* biofilms indicated that the cap locus is upregulated in biofilms (4). PGA may thus contribute to the overall resistance of *S. epidermidis* biofilms to innate immunity, although it does not appear to directly influence biofilm formation.

The *S. epidermidis* cap locus shows high similarity to that of *B. anthracis*. The genetic information for PGA production in *B. anthracis* is located on a plasmid (pXO2) (19, 20) and shows high genetic mobility (21). It might have been transferred between *B. anthracis* and staphylococci and from one strain of *Staphylococcus* to another. Coinfection of humans or human ancestors may represent a



Table 1
PGA production in staphylococci

Strain	ATCC no.	PGA production ^A	Presence of <i>capB</i> and <i>capD</i> genes ^B
<i>S. epidermidis</i> group			
<i>S. capitis</i> subsp. <i>capitis</i>	ATCC 27840	+	+
<i>S. capitis</i> subsp. <i>ureolyticus</i>	ATCC 49324	+	+
<i>S. caprae</i>	ATCC 51548	+	+
<i>S. haemolyticus</i>	ATCC 29970	+	+
<i>S. warneri</i>	ATCC 17917	+	+
<i>S. warneri</i>	ATCC 49518	–	–
<i>S. saccharolyticus</i>	ATCC 14953	+	+
<i>S. hominis</i> subsp. <i>hominis</i>	ATCC 25615	+	+
<i>S. hominis</i> subsp. <i>hominis</i>	ATCC 27844	–	–
Other staphylococci			
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	ATCC 43808	–	–
<i>S. pulveri</i>	ATCC 51698	–	–
<i>S. simulans</i>	ATCC 31432	–	–
<i>S. simulans</i>	ATCC 700576	+	+
<i>S. simulans</i>	ATCC 27848	–	–
<i>S. xyloso</i>	ATCC 49148	–	–
<i>S. xyloso</i>	ATCC 29966	–	–
<i>S. saprophyticus</i>	ATCC 35552	–	+
<i>S. saprophyticus</i>	ATCC 15305	–	+
<i>S. cohnii</i> subsp. <i>cohnii</i>	ATCC 29972	–	–
<i>S. cohnii</i> subsp. <i>urealyticum</i>	ATCC 49328	–	–
<i>S. lugdunensis</i>	ATCC 43809	+	+
<i>S. intermedius</i>	ATCC 49052	–	–
<i>S. aureus</i>	All strains with known genome sequence	ND	–

^ABy immuno-dot blot analysis. ^BBy Southern blot analysis. ND, not determined.

likely scenario for these events. Of note, most of the staphylococcal strains that produce PGA are opportunistic human pathogens. Consistent with its role in *S. epidermidis*, PGA might thus contribute to survival of those strains in the human host, although this notion remains to be demonstrated.

In conclusion, PGA appears to play a key role for the survival of *S. epidermidis* in the human host during colonization and infection. Remarkably, though produced in very small amounts, PGA contributes significantly to the pathogenicity of *S. epidermidis*, which underscores the efficiency of PGA-mediated protection. PGA may represent an excellent target for drug or vaccine development against infection with *S. epidermidis* and possibly other staphylococcal pathogens. Anti-PGA antibodies have been suggested for the treatment and prophylaxis of *B. anthracis* infections. Although PGA has a generally low immunogenicity, recent studies have shown that anti-PGA antibodies efficiently protect from anthrax infection in animal models (22–25). Our data indicate that they might also be valuable for the treatment of chronic infections by *S. epidermidis* and certain other pathogenic staphylococci.

Methods

Bacterial strains, growth conditions, and basic molecular biology methods. The clinical isolate *S. epidermidis* 1457 (26) was used in this study. Bacteria were grown in tryptic soy broth (TSB) unless otherwise noted. Antibiotics were used at the following concentrations: chloramphenicol, 10 µg/ml; spectinomycin, 100 µg/ml; and ampicillin, 100 µg/ml. Cultures were incubated at 37°C with shaking at 200 rpm. DNA manipulation, isolation of plasmid DNA, and transformation of *Escherichia coli* were performed using standard

procedures. Staphylococcal plasmid DNA was prepared with the Qiagen Plasmid Midi Kit as described (27). *S. epidermidis* was transformed by electroporation as described (28). PCRs were performed with Ready-To-Go PCR Beads (Amersham Biosciences) as recommended by the manufacturer. DNA was sequenced using Big Dye Terminator cycle sequencing (version 3.0) on an ABI3700 sequencer (Applied Biosystems). Nucleotide sequences were analyzed using the program Vector NTI Suite (InforMax). Primers for DNA amplifications by PCR (Table 2) were purchased from Sigma Genosys. The 74 strains used for epidemiological studies were obtained from N. El Solh (Institut Pasteur, Paris, France) and are predominantly nonclonal (29).

Real-time PCR. RNA isolation was performed using a Fast-Prep BLUE Kit (Q-BioGene Inc.). Oligonucleotide primers and probes were designed using Primer Express 2.0 software (Applied Biosystems). The probe used for analysis of *cap* expression was located within the *capB* gene. TaqMan analysis was performed in a 384-well MicroAmp Optical using a 7900 Sequence Detector (Applied Biosystems). Standard curves were determined with purified chromosomal template DNA at concentrations ranging from 0.001 ng/ml to 10 ng/ml. Assays were performed in triplicate using cDNA samples, and 16S rRNA as control, with a standard cycle protocol.

Southern blot analysis. Equal amounts of EcoRV-digested genomic DNA were separated by gel electrophoresis on a 0.7% agarose gel and visualized by ethidium bromide staining. DNA was transferred onto a nylon membrane (GE Osmonics Lab-store) and probed with digoxigenin-labeled PCR product amplified from the *capB* or *capD* genes of *S. epidermidis* 1457 or with a cocktail of probes amplified from the *capA*, *capB*, *capC*, and *capD* genes. Prehybridization, hybridization, posthybridization, and immunologic detection were performed as described in the

technical update accompanying the nonradioactive DNA Labeling and Detection Kit (Roche Applied Science). For immunologic detection, the membrane was incubated with a 1:5,000 dilution of sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase. Probe-target hybrids were detected using the chromogenic substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Immuno-dot blot assay. Surface-attached PGA was released from the cell surface by boiling bacteria for 30 minutes at 100°C or by autoclaving. PGA was then purified by acid precipitation as described (12). Aliquots (3 µl) of the samples were spotted on a nitrocellulose membrane, air-dried, and PGA was detected with anti-PGA antiserum using a scanner and Total Lab Version 2003 software (Nonlinear USA). The assay was calibrated by serial dilution of the most intensive sample. The value detected in the wild-type strain was set to 100% and production values in the other strains were expressed relative to that value. Anti-PGA antiserum (kindly provided by R. Schneerson, National Institute of Child Health & Human Development, NIH, Bethesda, Maryland, USA) was developed against PGA from *Bacillus pumilus*. For *Staphylococcus* strains other than *S. epidermidis*, a strain with a signal higher than that of the background of the *S. epidermidis* Δ*cap* mutant was considered a PGA producer.

Construction of an isogenic cap deletion mutant and cap-complementing vector. To delete *capBCAD* in *S. epidermidis* 1457, PCR-amplified regions flanking the *cap* locus and a spectinomycin resistance cassette were cloned into plasmid pBT2 (30), yielding plasmid pBTΔ*cap*, which was used for allelic replacement as described (31). The proper integration of the resistance gene marker *spc* was verified by direct sequencing of the genomic DNA at the borders of the PCR-derived regions. Lack of *cap* transcript in the *cap* mutant strain was verified by real-time PCR (data not shown). *S. epidermidis* 1457 in which



Table 2
Oligonucleotides used in this study

For TaqMan analysis of *cap* expression

capF CATGAAGCTGAGAATGCACCTTGATT
CapProbe AACATCCACGGCCCGG
capR CTATCCCTTCTATGAATCCGCTATT

For allelic replacement of the *cap* locus

CapEco CCACCAATATCCGTTGCTTGAGGTGCAGCAGAATTCATCC
CapBam GGGTATTGGTACACCAGGTGGGAACAAGGATCCAACAATTC
CapSal GCGCAACACACGCTATAATGAGTAACAGTGCACCTTTACCTCTC
CapHind CATGTCTTTACCATTTAAGCTTCCAATAAGTATAAATGCGAGG

For construction of complementation vectors

capBam2 CAATCATCATACTACTTCTTTCATTATTGGATCCGCTTACAC
capXba GACTTCTCCATACCTCTCCTCCTCTAGACGTAATATC

To confirm lack of *cap* expression in the *cap* mutant strain by real-time PCR

CAP1 CCCTTCTATGAATCCGCTATTCTACCACCCGGGCGGTGG
CAP2 GACGTCATACCAGAATCATATTTACGGAAGTTCCG
C2P1 GATATTGATCACAACCTCACC
C2P5 GCCATTATCTGTGTTTTCC

For amplification of the *capB* probe (Southern blot)

CapSB2 ATGTGATGGAAGACCATATGGATGCTTAGGACCGGACACT
CapSB3 CGCTTTTGTAGACTGCGGTTTCATTAGCAGCGAATGCATTA
CapSB-D3 ATCCTTGAAGCATTAATGTGCTCCTAAAGGATTAGGTGC
CapSB-D4 CTCATTATCAGGACTAGGAGGTGGCGGTGCGACACTTAC
CapSB-A1 AATGACATCTGCACCAGCATTGCTAACGCATGTGCATAT
CapSB-A2 GAGAGACAAAGATAGCATTGCTATCACCTATTTAGGTAAC
CapSB-C1 GACAACACCTATACCTGAAACTTCAACATTTCAAATGGGGTC
CapSB-C2 GCTGAGAAATTTGGGATTAATCCAGCAGGGTTAGTCGTTCC

capBCAD was deleted was named *S. epidermidis* Δ *cap*. To complement for *capBCAD* in *S. epidermidis* Δ *cap*, *capBCAD* genes were cloned into plasmid pRB474 (30). The resulting plasmid was named pRB*capBCAD*.

Purification of PGA and detection of D- and L-glutamic acid. Cultures were grown in TSB medium supplemented with 1 M NaCl to induce PGA production. PGA was first purified as described above. Then, PGA samples were further purified by ion exchange chromatography using a RESOURCE Q 6 ml column (Amersham Biosciences) on an AKTA Purifier 10 (Amersham Biosciences) and a gradient from 0.2% acetic acid to 0.2% acetic acid/1 M NaCl in 20 column volumes at a flow rate of 5 ml/min. Fractions with positive reaction in an immuno-dot blot were combined, dialyzed against water, lyophilized, resuspended in 6 M HCl, and hydrolyzed at 110°C for 24 hours. Samples were lyophilized again and dissolved in 200 μ l of water. Ten microliters of the samples were then injected onto a Chirobiotic T column (Astec) using 0.1% triethylammonium acetate (pH 4.0) in 80% ethanol as elution buffer at a flow rate of 0.4 ml/min. Chromatography was performed using an Agilent 1100 series HPLC connected to a VL Trap mass spectrometer. The extracted ion chromatograms at 146 Da, the mass of glutamic acid, were used to determine the amounts of D- and L-glutamic acid by peak integration in comparison to pure D- and L-glutamic acid.

Isolation of human polymorphonuclear leukocytes and phagocytosis experiments. Human polymorphonuclear leukocytes (PMNs) were isolated from heparinized venous blood of healthy individuals with a standard method (32). All studies were performed in accordance with a protocol approved by the Institutional Review Board for Human Subjects of NIAID. Cell preparations contained approximately 99% PMNs and all reagents used contained <25.0 pg/ml endotoxin. Phagocytosis of *S. epidermidis* by human PMNs was analyzed by flow cytometry with a previously described method (32). Briefly, bacteria were cultured to stationary growth phase, washed in PBS, and labeled with fluorescein-5-isothiocyanate for 15 minutes. PMNs ($10^6/100 \mu$ l) and bacteria ($2 \times 10^7/100 \mu$ l) were combined in wells of serum-coated 96-well round-bot-

tom microtiter plates. Plates were incubated for 30 minutes at 37°C and the degree of phagocytosis was determined by flow cytometry.

Peptide bacterial killing assays. *S. epidermidis* cultures were harvested, washed with PBS buffer, and resuspended in 10 mM sodium phosphate buffer (pH 7.0). Bacterial killing assays were performed using a final concentration of approximately 10^5 *S. epidermidis* cells in each sample. Antimicrobial peptides were dissolved in the following solutions: human β -defensin 3, 10 mM acetic acid, and LL-37, 10% acetonitrile with 0.1% trifluoroacetic acid. The bacteria were exposed to a range of antimicrobial peptide concentrations (0, 5, 10, 20, 30, and 40 μ g/ml). An equal volume of the respective peptide dilution buffer was applied to control samples. Samples were incubated at 37°C for 2 hours and appropriate dilution series of the samples were plated on TSB agar. Survivor *S. epidermidis* cells were enumerated after 24 hours of incubation at 37°C. The percentage of killed *S. epidermidis* was calculated using the formula $(1 - [\text{CFU}_{\text{peptide}} / \text{CFU}_{\text{control}}]) \times 100$.

Scanning immunoelectron microscopy. Fifty-microliter aliquots of *S. epidermidis* cultures were washed with PBS buffer. Cells were resuspended in 200 μ l of anti-PGA antiserum and incubated at 37°C with agitation at 400 rpm for 12 hours. Samples were washed with PBS and pellets were subsequently incubated with goat anti-rabbit IgG conjugated with 20 nm of gold (BB International) at 37°C with agitation at 400 rpm for 2 hours. Following antibody labeling, the cell suspensions were attached to coverslips, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate. Samples were washed with distilled water, dehydrated in a graded ethanol series, critical-point dried under CO₂ with a Bal-Tec model cpd 030 drier (Balzers), mounted on aluminum studs, and sputter-coated with 100 angstroms of chromium in a model IBS/TM200S ion beam sputterer (South Bay Technologies) prior to viewing at 10 kV on a Hitachi S-4500 field emission scanning electron microscope (Hitachi) in backscatter imaging mode.

Murine model of device-related infection. Female Balb/c mice were used in a model of subcutaneous implanted device-related infection as described (33). Two catheter pieces of 1-cm length were placed under the skin of the dorsum of each animal. CFUs on catheters were counted before insertion and were in the range of 2×10^5 on all implanted catheters. CFU on excised catheters and surrounding tissues were counted after 1 week of infection. All studies were performed in accordance with a protocol approved by the Animal Care and Use Committee of Rocky Mountain Laboratories, NIAID.

Statistics and DNA sequence analysis. Statistical analysis was performed using GraphPad Prism version 4.0. DNA sequences were compared using Clustal W software.

Acknowledgments

The authors would like to thank Rachel Schneerson for providing anti-PGA antisera, Nevine El Solh for *S. epidermidis* strains, Aaron B. Carmody for technical assistance, and Donald Gardner, Michael Parnell, and Ralph Larson for help with animal studies.

Received for publication October 1, 2004, and accepted in revised form December 7, 2004.

Address correspondence to: Michael Otto, Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, 903 South 4th Street, Hamilton, Montana 59840, USA. Phone: (406) 363-9283; Fax: (406) 375-9677; E-mail: motto@niaid.nih.gov.



1. Vuong, C., and Otto, M. 2002. *Staphylococcus epidermidis* infections. *Microbes Infect.* **4**:481–489.
2. Götz, F. 2002. *Staphylococcus* and biofilms. *Mol. Microbiol.* **43**:1367–1378.
3. Costerton, J.W., Stewart, P.S., and Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science.* **284**:1318–1322.
4. Yao, Y., Sturdevant, D.E., and Otto, M. 2005. Genome-wide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into *S. epidermidis* biofilm pathophysiology and the role of phenol-soluble modulins in biofilm formation. *J. Infect. Dis.* **191**:289–298.
5. Oppermann-Sanio, F.B., and Steinbüchel, A. 2002. Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. *Naturwissenschaften.* **89**:11–22.
6. Little, S.F., and Ivins, B.E. 1999. Molecular pathogenesis of *Bacillus anthracis* infection. *Microbes Infect.* **1**:131–139.
7. Ren, S.X., et al. 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature.* **422**:888–893.
8. Kapatral, V., et al. 2002. Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J. Bacteriol.* **184**:2005–2018.
9. Vuong, C., et al. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell. Microbiol.* **6**:269–275.
10. Zhang, Y.Q., et al. 2003. Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol. Microbiol.* **49**:1577–1593.
11. Ashiuchi, M., and Misono, H. 2002. Biochemistry and molecular genetics of poly-gamma-glutamate synthesis. *Appl. Microbiol. Biotechnol.* **59**:9–14.
12. Hanby, W.E., and Rydon, H.N. 1946. The capsular substance of *Bacillus anthracis*. *Biochemistry.* **40**:297–307.
13. Otto, M. 2004. Virulence factors of the coagulase-negative staphylococci. *Front. Biosci.* **9**:841–863.
14. Galdbart, J.O., Allignet, J., Tung, H.S., Ryden, C., and El Solh, N. 2000. Screening for *Staphylococcus epidermidis* markers discriminating between skin flora strains and those responsible for infections of joint prostheses. *J. Infect. Dis.* **182**:351–355.
15. Saruta, K., et al. 1997. Rapid identification and typing of *Staphylococcus aureus* by nested PCR amplified ribosomal DNA spacer region. *FEMS Microbiol. Lett.* **146**:271–278.
16. Hornef, M.W., Wick, M.J., Rhen, M., and Normark, S. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* **3**:1033–1040.
17. Hancock, R.E., and Diamond, G. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.* **8**:402–410.
18. Hezayen, F.F., Rehm, B.H., Eberhardt, R., and Steinbüchel, A. 2000. Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. *Appl. Microbiol. Biotechnol.* **54**:319–325.
19. Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B., and Ivins, B.E. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun.* **49**:291–297.
20. Makino, S., Sasakawa, C., Uchida, I., Terakado, N., and Yoshikawa, M. 1988. Cloning and CO₂-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. *Mol. Microbiol.* **2**:371–376.
21. Green, B.D., Battisti, L., and Thorne, C.B. 1989. Involvement of Tn4430 in transfer of *Bacillus anthracis* plasmids mediated by *Bacillus thuringiensis* plasmid pXO12. *J. Bacteriol.* **171**:104–113.
22. Leppla, S.H., Robbins, J.B., Schneerson, R., and Shiloach, J. 2002. Development of an improved vaccine for anthrax. *J. Clin. Invest.* **110**:141–144. doi:10.1172/JCI200216204.
23. Schneerson, R., et al. 2003. Poly(gamma-D-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: a potential addition to the anthrax vaccine. *Proc. Natl. Acad. Sci. U. S. A.* **100**:8945–8950.
24. Kozel, T.R., et al. 2004. mAbs to *Bacillus anthracis* capsular antigen for immunoprotection in anthrax and detection of antigenemia. *Proc. Natl. Acad. Sci. U. S. A.* **101**:5042–5047.
25. Rhie, G.E., et al. 2003. A dually active anthrax vaccine that confers protection against both bacilli and toxins. *Proc. Natl. Acad. Sci. U. S. A.* **100**:10925–10930.
26. Mack, D., et al. 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. *Infect. Immun.* **62**:3244–3253.
27. Vuong, C., Gerke, C., Somerville, G.A., Fischer, E.R., and Otto, M. 2003. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J. Infect. Dis.* **188**:706–718.
28. Augustin, J., et al. 1992. Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. *Eur. J. Biochem.* **204**:1149–1154.
29. Galdbart, J.O., Morvan, A., Desplaces, N., and el Solh, N. 1999. Phenotypic and genomic variation among *Staphylococcus epidermidis* strains infecting joint prostheses. *J. Clin. Microbiol.* **37**:1306–1312.
30. Brückner, R. 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosum*. *FEMS Microbiol. Lett.* **151**:1–8.
31. Vuong, C., Götz, F., and Otto, M. 2000. Construction and characterization of an *agr* deletion mutant of *Staphylococcus epidermidis*. *Infect. Immun.* **68**:1048–1053.
32. Voyich, J.M., et al. 2003. Genome-wide protective response used by group A *Streptococcus* to evade destruction by human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* **100**:1996–2001.
33. Kadurugamuwa, J.L., et al. 2003. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect. Immun.* **71**:882–890.