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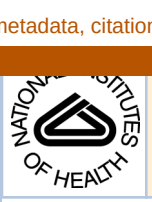

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# ACME encoded *speG* abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines

**Gauri S. Joshi**[†], **Jeffrey S. Spontak**[†], **David G. Klapper**, and **Anthony R. Richardson**[*]

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA

## Abstract

Polyamines, including spermine (Spm) and spermidine (Spd), are aliphatic cations that are reportedly synthesized by all living organisms. They exert pleiotropic effects on cells and are required for efficient nucleic acid and protein synthesis. Here, we report that the human pathogen *Staphylococcus aureus* lacks identifiable polyamine biosynthetic genes, and consequently produces no Spm/Spd or their precursor compounds putrescine and agmatine. Moreover, while supplementing defined medium with polyamines generally enhances bacterial growth, Spm and Spd exert bactericidal effects on *S. aureus* at physiologic concentrations. Small colony variants specifically lacking menaquinone biosynthesis arose after prolonged Spm exposure and exhibited reduced polyamine-sensitivity. However, other respiratory-defective mutants were no less susceptible to Spm implying menaquinone itself rather than general respiration is required for full Spm-toxicity. Polyamine hypersensitivity distinguishes *S. aureus* from other bacteria and is exhibited by all tested strains save those belonging to the USA-300 group of Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA). We identified one gene within the USA-300-specific Arginine Catabolic Mobile Element (ACME) encoding a Spm/Spd *N*-acetyltransferase that is necessary and sufficient for polyamine resistance. *S. aureus* encounters significant polyamine levels during infection, however the acquisition of ACME encoded *speG* allows USA-300 clones to circumvent polyamine-hypersensitivity, a peculiar trait of *S. aureus*.

## Introduction

Infections caused by the Gram-positive bacterium *Staphylococcus aureus* impose an enormous healthcare burden worldwide (Diekema *et al.*, 2001, Klevens *et al.*, 2007). *S. aureus* infections are notoriously difficult to treat given the highly invasive nature of this organism combined with its multiple antimicrobial resistance determinants (Chambers & Deleo, 2009). Since its first identification in the 1960s, Methicillin-Resistant *S. aureus* (MRSA) has become endemic in most hospital settings around the developed world (Barrett *et al.*, 1968, Klevens *et al.*, 2007). It has been estimated that MRSA infections result in additional annual healthcare costs exceeding £ 3 billion in the UK alone (Cosgrove *et al.*, 2005, Gould, 2006). However, since the turn of the last century, MRSA has spread beyond healthcare settings and into the community (*i.e.* Community Associated MRSA or CA-MRSA) (DeLeo *et al.*, 2010). CA-MRSA clones are phylogenetically distinct from traditional hospital associated MRSA (HA-MRSA) strains. Pulsed-field gel electrophoresis (PFGE) typing has revealed that HA-MRSA clones often belong to USA-100 and USA-200 PFGE types whereas CA-MRSA isolates generally fall into the USA-300, USA-400 or USA-1000 groupings (McDougal *et al.*, 2003). CA-MRSA first emerged simultaneously in

[*]**Corresponding author:** Department of Microbiology & Immunology, CB7290, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA., anthony_richardson@med.unc.edu.

[†]Authors contributed equally to this work.

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western Australia and in the midwestern United States in the 1990s (Centers for Disease Control and Prevention, 1999, Udo *et al.*, 1993). This original CA-MRSA clone belonged to the USA-400 PFGE type and spread globally for a decade. Subsequently, a new CA-MRSA clone has emerged belonging to the USA-300 group. USA-300 clones have spread quickly around the world replacing USA-400 in most areas and are currently responsible for the vast majority of CA-MRSA disease (DeLeo *et al.*, 2010, Stemper *et al.*, 2006). In addition to their hypertransmissibility, USA-300 clones have been demonstrated to be more virulent than other CA-MRSA and HA-MRSA isolates in modeled infections for reasons that are not yet clear (Li *et al.*, 2009, Montgomery *et al.*, 2008).

Given the rapid global spread of CA-MRSA, significant research effort has been undertaken to explain the hypervirulence and hypertransmissibility associated with USA-300 clones. Complete genome sequences for archetypical USA-400 (MW2) and USA-300 (FPR3757) clones have been published and have revealed numerous differences that might help explain the emergence of this new pandemic (Baba *et al.*, 2002, Diep *et al.*, 2006). For instance, most CA-MRSA clones harbor a phage-encoded Panton-Valentine Leukotoxin (PVL) that confers hypervirulence in a rabbit lung infection model (Diep *et al.*, 2010). In contrast, HA-MRSA clones are rarely PVL-positive (Fey *et al.*, 2003). Moreover, CA-MRSA clones consistently produce higher levels of α-type Phenol-Soluble Modulins (PSMs), small secreted peptides with chemoattractant and leukocidal activities, than do HA-MRSA isolates (Wang *et al.*, 2007). However, both PVL- and elevated αPSM-production are conserved among CA-MRSA isolates belonging to both USA-400 and USA-300 groupings. Therefore, these factors are unlikely to explain the dominance of USA-300 clones in most regions of the developed world. A distinguishing feature of USA-300 genomes compared with other CA-MRSA isolates is the presence of an Arginine Catabolic Mobile Element (ACME) (Diep *et al.*, 2006). This 33-gene island is juxtaposed to the SCC*mec*IVa cassette in virtually all USA-300 clones (Diep *et al.*, 2008, Goering *et al.*, 2007). The island encodes an arginine deiminase system, an oligopeptide transporter, various transposases and integrases, as well as an uncharacterized alcohol dehydrogenase and acetyl-transferase. ACME has been shown to directly enhance virulence in a rabbit model of bacteremia and is strongly associated with USA-300 clones but rarely found in any other CA-MRSA or HA-MRSA isolates (Diep *et al.*, 2008). However, neither the molecular mechanism(s) nor the extent to which ACME confers an advantage to USA-300 has been clearly established.

Polyamines are aliphatic compounds consisting of at least two primary amino groups. Generally, the term “polyamine” refers to a group of compounds including putrescine, agmatine, spermidine and spermine that are all synthesized from L-arginine. Additionally, diamine compounds such as cadaverine and histamine, which are decarboxylated lysine and histidine, respectively, are also referred to as polyamines. The synthesis of L-arginine-dependent polyamines differs among organisms, but is regarded as being present in one form or another in all living cells (Hamana & Matsuzaki, 1992, Tabor & Tabor, 1985, Cohen, 2000). For instance, mammals express arginases that convert L-arginine to L-ornithine, which is subsequently decarboxylated to produce putrescine (Figure 1). Putrescine is converted to spermidine and then spermine by sequential addition of aminopropyl groups derived from decarboxylated *S*-adenosylmethionine. In bacteria, arginase is not common and spermine is rarely synthesized. Rather, most species first decarboxylate L-arginine to produce agmatine (Figure 1). Agmatine can then be ureohydrolyzed directly to putrescine or alternatively, agmatine can be deaminated/decarboxylated through *N*-carbamoylputrescine to produce putrescine. The variety of pathways dedicated to the synthesis of these important biological compounds implies convergent evolution with some enzymatic steps independently evolving three distinct times in animals, fungi and plants (Hamana & Matsuzaki, 1992, Minguet *et al.*, 2008).

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In general polyamines exert pleiotropic effects on the basic cellular physiology of all organisms, from archaea to animals and plants. In contrast to metal cations, polyamines distribute their positive charges along a flexible hydrocarbon backbone. Thus, they are ideal for dissipating repulsive forces exerted between negatively charged nucleic acids and protein surfaces. Indeed, intracellular polyamines are abundantly bound to ribosomes, chromatin and mRNA and this binding facilitates macromolecular synthesis (Cohen, 2000). Moreover, increased polyamine synthesis is well documented in rapidly dividing tissues undergoing high rates of nucleic acid synthesis such as during embryogenesis, tumorigenesis, wound healing and inflammation (Moinard *et al.*, 2005, Seiler & Atanassov, 1994, Thomas & Thomas, 2001). Interestingly, polyamine production is also associated with modulating inflammation by a variety of transcriptional and post-transcriptional mechanisms (Seiler & Atanassov, 1994, Zhang *et al.*, 2000). In bacteria, exogenous polyamine supplementation promotes replication for many of the same reasons outlined above. Additionally, polyamines have been shown to affect specific gene expression in bacteria by binding to mRNA and tRNA structural elements (Igarashi & Kashiwagi, 2000, Shah & Swiatlo, 2008). They have also been implicated in modulating outer membrane permeability through interactions with porin proteins and can act as non-specific antioxidants in aerobically cultured bacteria (Iyer *et al.*, 2000, Ha *et al.*, 1998, Khan *et al.*, 1992). Given the plethora of functions attributed to polyamines, their synthesis represents a conserved feature across all three kingdoms of life.

Here we report the absence of *de novo* polyamine production by the human pathogen *S. aureus*. More importantly, we show that exogenous polyamines inhibit rather than enhance *S. aureus* growth and that they can be bactericidal at concentrations known to exist within the human host (Zhang *et al.*, 2000). During growth within inflamed tissue, *S. aureus* likely encounters significant polyamine levels, which may act to specifically limit *S. aureus* proliferation to some extent. This unique polyamine hypersensitivity is inherent to nearly all tested strains of *S. aureus* and distinguishes this species from nearly all life on earth. The only notable exceptions are clones belonging to the newly emerged USA-300 CA-MRSA group. USA-300 strains exhibit complete resistance to high levels of exogenous polyamines, and we show here that this polyamine-resistant phenotype depends on the presence of a spermine/spermidine acetyl-transferase encoded within the ACME island. Thus, the acquisition of ACME by USA-300 isolates resulted in complete resistance to physiologic polyamine concentrations and may represent a selective advantage that explains the dominance of these clones in CA-MRSA disease today.

## Results

### *S. aureus* lacks *de novo* polyamine synthesis

Bacteria can synthesize polyamines from L-arginine through agmatine and/or L-ornithine by a variety of pathways (Figure 1). Inspection of thirteen staphylococcal genomes revealed no homologues to SAM-decarboxylase or spermidine synthase suggesting that *S. aureus* cannot produce spermidine from putrescine. The only putative polyamine biosynthetic enzyme encoded by *S. aureus* (SACOL0523) belongs to the family of aspartate amino-transferases, which include ornithine/arginine/lysine decarboxylases that catalyze the formation of putrescine/agmatine/cadaverine depending on substrate specificity. Thus, the polyamine biosynthetic capabilities of *S. aureus* are limited to potentially one species from this ubiquitous family of compounds. Larger phylogenetic analyses revealed that many gram-positive bacteria and a few gram-negative species also lack *de novo* polyamine synthesis (Figure S1). Indeed, *Streptococcus pyogenes* and *S. agalactiae* lack identifiable biosynthetic homologues, while *S. pneumonia*, an organism known to make polyamines, has a complete biosynthetic pathway (Shah *et al.*, 2011). Similarly, Enterococci, Lactococci, *Haemophilus* ssp., *Borrellia* spp., certain species of *Francisella* and Mollicutes such as *Mycoplasma* and *Ureaplasma* spp. lack genes encoding *de novo* polyamine synthesis as well (Figure S1). We

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sought to determine whether these compounds were indeed synthesized *de novo* by some of these organisms implying new pathways exist for the production of polyamines.

Accordingly, we assayed for *de novo* polyamine production by a number of bacterial species cultivated in chemically defined medium devoid of exogenous polyamines (Table 1). In contrast to *E. coli*, *B. subtilis*, and *P. aeruginosa*, organisms known to produce polyamines *de novo*, whole-cell extracts of *S. aureus* lacked detectible levels of any tested polyamine species (Figure S2 and Table 1). *E. coli* and *B. subtilis* preferentially produce the L-arginine-derived polyamines spermidine and putrescine, while *P. aeruginosa* additionally produced large amounts of cadaverine, a lysine-derived polyamine (Table 1). The lack of any detectible polyamine species in *S. aureus* confirms predictions from genomic inspection. Moreover, *S. agalactiae* strain A909 also produced no detectible polyamines when cultivated in broth culture, again underscoring the lack of predicted biosynthetic pathways in many streptococcal genomes (data not shown). Curiously, *F. novicida* seems to have lost a genetic island retained by *F. tularensis* subspecies *holarctica* and *tularensis* that is required for the synthesis of L-arginine-derived polyamines. In line with this prediction, *F. tularensis holarctica* strain LVS produced high levels of spermidine, putrescine and agmatine, while none of these were detected in *F. novicida* (data not shown). Overall, these data are in direct conflict with the dogma that all living organisms synthesize polyamines, and *S. aureus* is not alone as an organism devoid of *de novo* polyamine biosynthesis.

### *S. aureus* exhibits hypersensitivity towards exogenous spermine/spermidine

Addition of 1mM spermine or spermidine to synthetic medium devoid of polyamines generally enhanced the growth of *E. coli* and *B. subtilis* but severely limited the growth of *S. aureus* (data not shown). Polyamine sensitivity was assessed here by measuring the zone of inhibition surrounding a sterile filter disk impregnated with 250 mM of a particular polyamine species (Figure 2A). In contrast to other Gram-positive and Gram-negative species, *S. aureus* is highly sensitive to spermine (Figure 2A) and spermidine (Figure S3), but not putrescine, agmatine or cadaverine (data not shown). While spermine concentrations at this level can significantly raise the media pH, no inhibition zone was observed in *S. aureus* around discs supplemented with 250 mM NaOH suggesting that spermine toxicity is not mediated through alkaline stress (data not shown). Spermine MIC values were consistently ~2 mM among sensitive *S. aureus* strains, but were > 10 mM for other species (*e.g. B. subtilis*, *L. monocytogenes*, *E. coli*, *S.* Typhimurium, or *P. aeruginosa*). Spermine was bactericidal at levels ≥ 3 mM and exposure to 5 mM spermine exerted 2-logs of killing within 3 h of exposure and > 3-logs killing after 24 h (Figure 2B). Spermidine also exhibited bactericidal activity albeit at higher concentrations (MIC/MBC levels ≥ 10-fold above spermine, above levels likely encountered in host tissue). The toxicity of polyamines towards *S. aureus* was more pronounced with increasing pH as previously observed (Rozansky *et al.*, 1954). At neutral pH and above, clear zones of inhibition could be detected around spermine discs, however acidifying the polyamine solution below neutrality abrogated toxicity towards *S. aureus* (Figure S4A). Thus, polyamine toxicity towards *S. aureus* is inversely proportional to the net cationic charge of polyamines since they become sequentially deprotonated at elevated pH.

Considering that *S. aureus* is not the only bacterial species unable to synthesize polyamines, we tested whether the lack of *de novo* synthesis correlated with polyamine sensitivity. Gram-negative *F. novicida*, *H. influenzae* and *H. ducreyi* do not encode polyamine synthetic pathways and the lack of polyamine production in *F. novicida* was experimentally verified, yet none of these species exhibited heightened sensitivity to spermine (data not shown). In contrast, both group A and B streptococci were sensitive to exogenous spermine and spermidine to levels similarly observed in staphylococci (Figure S3). Thus, lacking *de novo*

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polyamine synthesis and hypersensitivity to spermine/spermidine are unrelated traits with hypersensitivity being associated with specific Gram-positive pathogens including *S. aureus*.

## *S. aureus* polyamine-sensitivity requires menadione biosynthesis but is independent of respiration

To gain insights into the mechanism(s) underlying *S. aureus* polyamine sensitivity, we isolated spontaneous mutants exhibiting increased spermine resistance compared with parental WT strain Newman. Plating ~ $1 \times 10^9$ cfu of *S. aureus* strain Newman onto BHI agar supplemented with 20 mM spermine resulted in the appearance of small, non-pigmented colonies arising within 48 to 72 h at a frequency of ~$1 \times 10^{-6}$·plated $cfu^{-1}$. Forty-two out of the 47 (89%) isolated colonies were stable, slow-growing, non-pigmented small colony variants (SCVs) that exhibited reduced spermine sensitivity when assayed via disc diffusion (Figure 3). These spermine-resistant SCVs were exposed to exogenous menadione, hemin, thiamine or thymidine to determine the nature of the mutation that generated the SCV phenotype. All but 7 exhibited restored growth and pigmentation when supplemented with exogenous menadione, while the remaining strains were not rescued by any of the four supplements (Figure 3A). Moreover, supplementation of these SCVs with exogenous menadione also restored full spermine toxicity (data not shown). Consistently, insertional inactivation of *menD*, which exhibits typical SCV phenotypes, resulted in an elevation of spermine MIC and MBC that could be reversed by the addition of exogenous menadione (Table 2 and Figures 3B and 3C). As menaquinone is the sole quinone electron carrier species in *S. aureus*, loss of *de novo* biosynthesis would result in cells unable to respire. Accordingly, spermine disc diffusion zones and MIC levels were determined for *S. aureus* cultured anaerobically demonstrating that without oxygen, spermine exerts reduced toxicity towards *S. aureus* strains COL and Newman (Table 2).

However, several pieces of data argue against respiration in general as being required for full spermine toxicity but rather menaquione itself is necessary for the cytotoxic effects of spermine on *S. aureus*. First, supplementing anaerobic cultures with 20 mM nitrate to promote anaerobic respiration did not restore any spermine-toxicity (Table 2). Moreover, *menD*::$Er^R$ strains exhibit increased spermine resistance anaerobically in the absence of respiration (Table 2). Finally, a Δ*hemB* mutant, an SCV that also lacks respiratory activity, was still susceptible to spermine (Figure 3B). The fact that spermine toxicity in *S. aureus* does not require respiration *per se* but is dependent on the presence of menaquinone is consistent with the fact that of all the know genetic etiologies responsible for *S. aureus* SCV phenotypes, menaquinone auxotrophy was most commonly isolated variant with heightened spermine resistance. We also addressed whether slow growing cells in general, which are often difficult to kill with bactericidal agents, exhibit reduced susceptibility to spermine. Decreasing incubation temperatures slows the growth of *S. aureus* yet spermine becomes increasingly toxic at these lower temperatures (Figure S4). Thus, the presence of menaquinone itself is necessary for full spermine sensitivity, but not necessarily due to its role in respiration or in achieving maximal growth rates.

## USA-300 clones of *S. aureus* resist polyamine cytotoxicity by expressing a predicted spermine/spermidine acetyltransferase harbored on the ACME island

In contrast to other *S. aureus* strains, USA-300 clones, including SF8300 and LAC, exhibit innate resistance to spermine and spermidine (Figures 2A and S3). This phenotype differs significantly with the spontaneously occurring menadione-auxotrophic SCVs in that USA-300 clones are fully pigmented and fast growing. Furthermore, supplementation with exogenous menadione did not confer polyamine-sensitivity to USA-300 clones (data not shown). However, an SF8300 derivative with a deletion encompassing the entire ACME island exhibited typical *S. aureus* polyamine sensitivity (Figures 2A and 4). The ACME

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island is a 33-gene composite element with regions of genetic synteny conserved in other coagulase negative staphylococci (Figure 4). One gene within ACME, SAUSA300_0053, encodes a predicted GNAT family acetyltransferase with homology to SpeG from *E. coli*, an *N*-1 spermidine acetyltransferase (51%/71% identical/similar). Δ*speG* USA-300 exhibited full spermine/spermidine sensitivity as determined in both SF8300 and LAC (Figures 4 and S3). Moreover, providing *speG* in *trans* complements the polyamine sensitivity of both the ΔACME and Δ*speG* mutants (Figures 4 and S3). Finally, expressing *speG* in non-USA-300 clones such as *S. aureus* COL or Newman provides complete polyamine resistance to these otherwise sensitive strains (Figures 4 and S3). Thus, ACME encoded *speG* is necessary and sufficient for polyamine resistance in USA300 *S. aureus*.

To assess whether the acetyltransferase activity of SpeG was responsible for conferring polyamine resistance, we employed the antitrypanosomal agent Berenil, which is known to inhibit human spermine/spermidine acetyltransferase (SSAT) activity (Libby & Porter, 1992). Mammals acetylate spermine and spermidine to sequentially convert them back to putrescine in order to maintain polyamine homeostasis. Human SSAT is therefore the mammalian equivalent of bacterial SpeG either of which should be inhibited by an acetyltransferase inhibitor such as Berenil. Administering Berenil inhibited the growth of USA300 in the presence of 5 mM spermine in a dose dependent fashion, consistent with the compound's role as a spermine/spermidine acetyltransferase inhibitor (Figure 5). In contrast, Berenil was not detrimental to the growth of USA300 *S. aureus* in the absence of exogenous polyamines (Figure 5). Thus, SpeG SSAT-activity is required for USA300 polyamine resistance. To test whether the product of SpeG, mono-acetylated spermine, is sufficient to eliminate its toxicity towards *S. aureus*, polyamine sensitive *S. aureus* Newman was exposed to exogenous *N*-1-acetylspermine. Acetylated spermine had no effect on the growth of *S. aureus* (Figure S5) supporting the notion that toxicity is not correlated with net polyamine charge since both nontoxic *N*-1-acetylspermine and toxic spermidine have the same net +3 charge.

Q-RT PCR analysis revealed that the *speG* transcript is abundant in USA-300 clones and constitutively expressed throughout all stages of growth. Additionally, high-level expression is independent of the presence or absence of exogenous spermine (Figure S6).

## Discussion

The assertion that polyamines constitute a class of essential compounds synthesized by all living organisms has been directly challenged here. The fact that entire genera of unrelated bacteria seem to lack biosynthetic genes implies that the requirement for polyamine biosynthesis has been lost several times over the course of bacterial evolution. Indeed, species with "streamlined" genomes including *Mycoplasma*, *Ureaplasma* and *Haemophilus* universally lack any homologues to polyamine biosynthetic genes. Moreover, organisms with "larger" genomes can be identified that also lack *de novo* polyamine synthesis including *F. novicida*, *Borrelia* ssp., and various species of the Lactobacilliales (*e.g. S. pyogenes*, *S. agalacticiae*, *E. faecalis* and certain strains of *L. lactis*). Some of these organisms may still require polyamine import from their mammalian hosts making them true "polyamine auxotrophs". This is inline with our data that while *H. influenzae*, *H. ducreyi* and *F. novicida* lack polyamine biosynthesis, they exhibit no growth inhibition in the presence of exogenous spermine or spermidine. In fact, Chamberlain's defined media for *Francisella* cultivation calls for supplementation with spermine (Chamberlain, 1965). Thus, it would appear that "polyamine auxotrophy" is a naturally occurring phenomenon though it is limited to select bacterial species. In contrast to true "polyamine auxtrophs", *S. aureus* lacks *de novo* polyamine synthesis but grows quite well in their absence. In fact, exogenous spermidine and spermine are highly toxic to *S. aureus*. Taken together, these data imply that

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*S. aureus* is not a true "polyamine-auxotroph", but rather has evolved "polyamine-independence", a remarkable evolutionary feat given the wide range of biological roles assigned to these molecules.

The complete absence of detectible polyamine production by *S. aureus* cultured in chemically defined medium was unexpected given the presence of a putative ornithine/arginine/lysine decarboxylase (SACOL0523) encoded in the *S. aureus* genome. We assessed polyamine levels in multiple *S. aureus* strains, thus it is unlikely that this enzyme happened to be inactive in our chosen isolate (data not shown). Rather, SACOL0523 may not be expressed under our laboratory conditions, though similar enzymes from other organisms are highly expressed in media lacking exogenous polyamines resulting in abundant *de novo* synthesis (Table 1 and Figure S2). Alternatively, it is possible that the product of this enzyme (putrescine, agmatine, or cadaverine) is an intermediate consumed by a downstream reaction such that steady-state levels are below our limit of detection. Another explanation is that SACOL0523 is misannotated and is merely an aspartate aminotransferase family member other than an Orn/Arg/Lys decarboxylase. In any case, under conditions where most bacterial species synthesize copious amounts of polyamines, *S. aureus* produces none to detectible levels.

Here we also show that, in addition to the lack of robust polyamine production in *S. aureus*, this species is hypersensitive to their presence in the environment (Figure 2). This observation was first reported over fifty years ago in a study that found staphylococcal ssp. to be significantly more sensitive to spermine than other tested bacteria (Rozansky *et al.*, 1954). In a follow-up study, this same group showed that *S. aureus* spermine hyper-sensitivity was apparent under both aerobic and anaerobic conditions as long as the bacteria were metabolically active (Grossowicz *et al.*, 1955). However, these studies used bacteria plated on solid media, then incubated in anaerobic jars. We also observe significant inhibition zones around spermine discs in anaerobic jars (data not shown), but when cultured under true anaerobiosis no zones of inhibition are apparent along with significant increases in MIC values in liquid culture (Table 2). Given the kinetics of spermine-induced killing (Figure 2), it is likely that significant killing occurs in anaerobe jars prior to achieving true anoxia. We therefore maintain that the absence of oxygen significantly curtails the bactericidal activity of spermine towards *S. aureus*.

The metabolic state of *S. aureus*, whether fermenting or respiring, does not directly correlate with spermine toxicity. First, *S. aureus* respiring anaerobically on nitrate are no more sensitive to spermine than are fermenting cells (Table 2). Second, different mutants unable to respire aerobically exhibit differential spermine susceptibility. Namely, *menD* mutants exhibit decreased spermine-sensitivity while Δ*hemB* mutants do not. This explains why of all SCV etiologies, menaquinone auxotrophy was most commonly isolated in mutants with heightened spermine-resistance. This cannot be attributed to the specific absence of menaquinone in BHI medium compared with other known SCV auxotrophies including heme, thiamine or thymidine. For example, Δ*hemB* mutants still require heme supplementation to BHI for normal growth and pigmentation, thus heme levels in BHI are not high enough to completely rescue full heme-auxotrophy. Therefore, if heme-auxotrophy led to increased spermine-resistance, these mutants should have been isolated even on BHI medium. It seems as though spermine toxicity is mediated by menaquinone rather than by menaquinone-dependent respiration *per se*, both aerobically and anaerobically (Figure 3 and Table 2). However, it is clear that oxygen potentiates spermine toxicity towards *S. aureus*.

Previous studies on the antibacterial effects of spermine on *S. aureus* were all carried out prior to the emergence of USA-300 or MRSA in general, thus highly resistant *S. aureus* were never observed. The recent acquisition of ACME by CA-MRSA represents one of the

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most distinguishing features of USA-300. Virtually all tested USA-300 strains harboring the SCC*mec* IVa cassette also carried ACME (Diep *et al.*, 2008, Goering *et al.*, 2007). Only USA-300 clones carrying other SCC*mec* cassettes (~10% of USA-300 isolates) lack detectible ACME sequences (Goering *et al.*, 2007). Thus, the predominant CA-MRSA lineage causing disease today is almost universally ACME positive, and consequently, likely resistant to exogenous spermine/spermidine. Whether this polyamine-resistance plays a role in *S. aureus* virulence, and therefore contributes to the success of the USA-300 lineage has yet to be demonstrated. The presence of ACME has been associated with virulence in a rabbit sepsis model, but not in murine sepsis or wound-healing models (Diep *et al.*, 2008, Montgomery *et al.*, 2009). However, the role for ACME, and particularly *speG*, in CA-MRSA virulence may be subtle and therefore difficult to assess in acute infection models. ACME entered into a clinically relevant clonal complex known as CC8 (Li *et al.*, 2009, Tenover & Goering, 2009). Therefore, it's quite possible that ACME merely contributes to as opposed to constituting virulence in an already established invasive clone.

Interestingly, ACME was acquired by USA-300 from *S. epidermidis* where it can be detected in half of all isolates (Miragaia *et al.*, 2009). Whereas, significant diversity exists among ACME islands from *S. epidermidis*, the most common form (ACME-1.02) is identical to the island found in USA-300 *S. aureus* and encodes a *speG* (Miragaia *et al.*, 2009). Consequently, only two out of six tested *S. epidermidis* isolates proved sensitive to spermine/spermidine implying that *speG* is more common among *S. epidermidis* (Figure S3). In addition, we found a *S. saprophyticus* clone that was also completely resistant to spermine, underscoring the extent to which this phenotype has spread among staphylococci. In fact, recent studies have identified a plasmid-borne ornithine decarboxylase catalyzing putrescine production in rare strains of *S. epidermidis* (Coton *et al.*, 2010). Thus, "polyamine independence" may be eventually lost to this genus of human commensals/pathogens.

Here we have demonstrated that the spermine/spermidine-acetyltransferase activity of ACME encoded SpeG affords USA-300 clones with complete resistance to antistaphylococcal polyamines. It is unclear how acetylation of polyamines abolishes their toxicity towards *S. aureus*. In contrast to the membrane associated BltD spermidine-acetyltransferase of *B. subtilis*, SpeG from *E. coli* is a cytosolic protein as is predicted of ACME encoded SpeG (Fukuchi *et al.*, 1994, Woolridge *et al.*, 1999). This is consistent with an intracellular spermine target that is unreactive with *N*-acetylspermine. While polyamine acetylation does decrease the overall positive charge associated with these molecules, this alone cannot account for the protective effect of acetylation. First, non-toxic *N*-1 acetylspermine has the same net +3 charge as unmodified spermidine, but the latter is still bactericidal to *S. aureus*. Second, polyamines exert greater toxicity at high pH (Figure S4) at which their net positive charge is diminished through deprotonization (Rozansky *et al.*, 1954). Rather, our data fit within a hypothetical model whereby rapid import of spermine/spermidine leads to their menaquinone-dependent enzymatic modification into toxic intermediates, a process that is inhibited by SpeG-mediated acetylation and less pronounced in the absence of oxygen. Interestingly, a *menD* mutant was more resistant than WT to spermine even under anaerobiosis (Table 2). This may indicate that in complex medium (Brain Heart Infusion) there are metabolites that allow *S. aureus* to oxidize low levels of menaquinone that can be used to toxify spermine. However, the nature of these electron acceptor(s) is currently undefined. Alternatively, menaquinone may act directly with spermine at membrane interfaces and cells still synthesize reduced levels of menaquinone anaerobically. More work is required to fully understand the mechanism of *S. aureus* spermine toxicity.

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Growing within inflamed tissue, *S. aureus* must contend with increased polyamine production from the reported stimulation of host biosynthetic enzymes (Seiler & Atanassov, 1994, Zhang *et al.*, 2000). Acquisition of ACME and SpeG by CA-MRSA represents an adaptation to this environment and may help to explain the extreme success of USA-300 clones.

## Experimental Procedures

### Bacterial Strains and Culture Conditions

Strains used in this work are listed in Table S1. Bacteria were generally cultivated in Brain Heart Infusion Broth/Agar (Difco, Detroit, MI) or chemically defined PN media (Pattee and Neveln, 1975) in shaking cultures (250 rpm) at 37° C. The only exceptions were *Francisella* strains were cultivated in Chamberlain's chemically defined medium lacking spermine supplementation, *Haemophilus* species were grown on chocolate agar plates and streptococci were cultivated in standing BHI cultures. For cloning purposes, *E. coli* strain DH10B was routinely grown in LB medium. Media were supplemented with antibiotics when appropriate: ampicillin (*E. coli* 100 μg·ml$^{-1}$), chloramphenicol (*S. aureus* 20 μg·ml$^{-1}$), erythromycin (*E. coli* 300 μg·ml$^{-1}$, *S. aureus* 5 μg·ml$^{-1}$) or spectinomycin (*S. aureus* 500 μg·ml$^{-1}$, *E. coli* 100 μg·ml$^{-1}$). To achieve anaerobiosis, cells were either incubated at 37° C in anaerobe jars with GasPak™ EZ catalyst system (Becton Dickenson, Franklin Lakes, NJ) or in an anaerobe chamber under a $N_2/CO_2/H_2$ (90%/5%/5%) atmosphere (Coy Laboratory Products, Inc., Grass Lakes, MI).

Δ*speG* was constructed using allelic exchange to insert an unmarked, in-frame deletion allele into the SF8300 chromsome. ~1 kb 5' and 3' homology fragments were amplified using primers listed in Table S2, then linked using Splicing Overlapping Extension (SOEing) PCR. The resulting fragment was cloned into an *E. coli/S. aureus* shuttle vector (pBT2ts), and allelic exchange was performed as previously described (Richardson *et al.*, 2006, Bruckner, 1997). Δ*hemB*::Sp$^R$ mutants were constructed by cloning ~1kb 5' and 3' flanking homology regions on either side of a Sp$^R$ cassette cloned into an *E. coli/S. aureus* shuttle vector (pBT2ts). Allelic exchange was then performed as previously described (Richardson *et al*., 2006). *menD*::Er$^R$ mutants were generated by amplifying the *menD* locus with primers listed in Table S1 and cloning into pCR Blunt II Topo cloning vector (Invitrogen, Carlsbad, CA). A Er$^R$ cassette from Tn1545 was inserted into a blunted unique *Nde*I site within *menD* and the *menD*::Er$^R$ construct was moved into pBT2ts for allelic exchange.

### Determination of Polyamine Inhibition Zones

Bacterial strains were grown overnight from frozen stocks at 37° C in BHI broth. Cultures were washed and resuspended in 1 ml PBS to cell density of ~1 × 10$^7$ CFU·mL$^{-1}$. BHI agar plates (with/without 8 μg·ml$^{-1}$ menadione) were inoculated by spreading 200μl of re-suspended cells then adding 6mm sterile discs (Becton Dickinson, Sparks, MD) supplemented with 20μl of 250mM Spermidine, Spermine, Cadaverine, Agmatine, Putrescine, N$^1$ acetylspermine or NaOH (Sigma Aldrich, St Louis, MO). When appropriate, the pH of the polyamine free base solutions was adjusted with 1 N HCl. Plates were then incubated at 4° C, 25°C, 30 °C, 37°C or 42 °C and zone radii were measured after 24 hours of incubation.

### Determination of Spermine Minimum Inihibitory (MIC) and Minimum Bactericidal Concentration (MBC)

Spermine MIC was determined by inoculating brain heart infusion (BHI) broth containing various dilutions of spermine (free base) with 1×10$^5$ cfu of *S. aureus* under both aerobic and

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anaerobic conditions. Anaerobic cultures were additionally supplemented with 20 mM nitrate to allow for anaerobic respiration. The MIC was defined as the lowest concentration of spermine that prevented growth after incubation for 18h at 37° C. Spermine MBC was determined by plating wells lacking discernable overnight growth to identify the concentration of spermine that reduced viability by ≥ 3-logs over 24 h.

### HPLC Analysis of Polyamines

All chemicals, reagents and buffers were purchased through Sigma Aldrich (St. Louis, MO). Bacterial strains were grown overnight from frozen stocks at 37° C in chemically defined PN or Chamberlain's media. Cultures were then diluted 1:100 in 20 mL fresh PN/Chamberlain's media (Chamberlain, 1965) and allowed to grow to a cell density of ~$1.0 \times 10^9$ CFU·mL$^{-1}$. Cultures were centrifuged, washed and re-suspended in 1 ml PBS. Whole cell extracts were generated by TCA precipitation followed by centrifugation at 12,000 × g for 5 minutes. Whole cell extracts were derivatized with benzoyl chloride as previously described (Morgan, 1998). Derivatized polyamines were then chloroform extracted, evaporated to dryness, re-suspended in HPLC grade $H_2O$ to rehydrate any anhydrous methylbenzoate, then re-extracted with chloroform. The organic extract was washed 3 times with HPLC grade $H_2O$, evaporated to dryness and re-suspended in 200μL mobile phase (15% acetonitrile/0.05% triflouroacetic acid).

50μL samples were injected (Applied Biosystems 112A oven/injector) into an Applied Biosystems 140B solvent delivery system and monitored using an Applied Biosystems 785A programmable detection system at 229 nm. Polyamines were separated on a reverse phase Ambershchrom Resin, CG-300S column, 4.6 × 150 mm (Rohn & Haas, Philadelphia, PA) and eluted with a 20 minute linear gradient of mobile phase (15% to 75% acetonitrile) with a flow rate of 0.4 mL·minute$^{-1}$. Values were analyzed with a Peaksimple Chromatography Data System (SRI, Torrance, CA). Standard polyamine concentration curves were generated using derivatized putrescence dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, $N^1$ acetylspermine trihydrochloride, agmatine sulfate and cadaverine dihydrochloride (Sigma-Aldrich, St. Louis, MO) analyzed by the above stated method.

### RNA isolation and real time analysis

RNA was isolated from three independent cultures of USA 300 (SF8300) at various stages of growth with/without exogenous spermine (5mM) as described previously (Richardson et al 2006). Real-time PCR analysis was performed on the iCycler (Bio-Rad Laboratories) thermocycler. Standard curves with known amounts of genomic DNA were used to determine primer-pair efficiencies using SensiMix™ SYBR (Quantace). Then, using the Fluorescein One-Step kit (Quantace), quantitative transcript levels for both *rpoD* and *speG* could be determined in all RNA samples. *speG* transcript levels were then reported relative to *rpoD* levels.

### Bioinformatics

To ascertain the presence/absence of polyamine synthetic genes across all published genomes, the polyamine subsystem of the SEED (http://theseed.uchicago.edu/FIG/index.cgi) was analyzed (Overbeek *et al.*, 2005).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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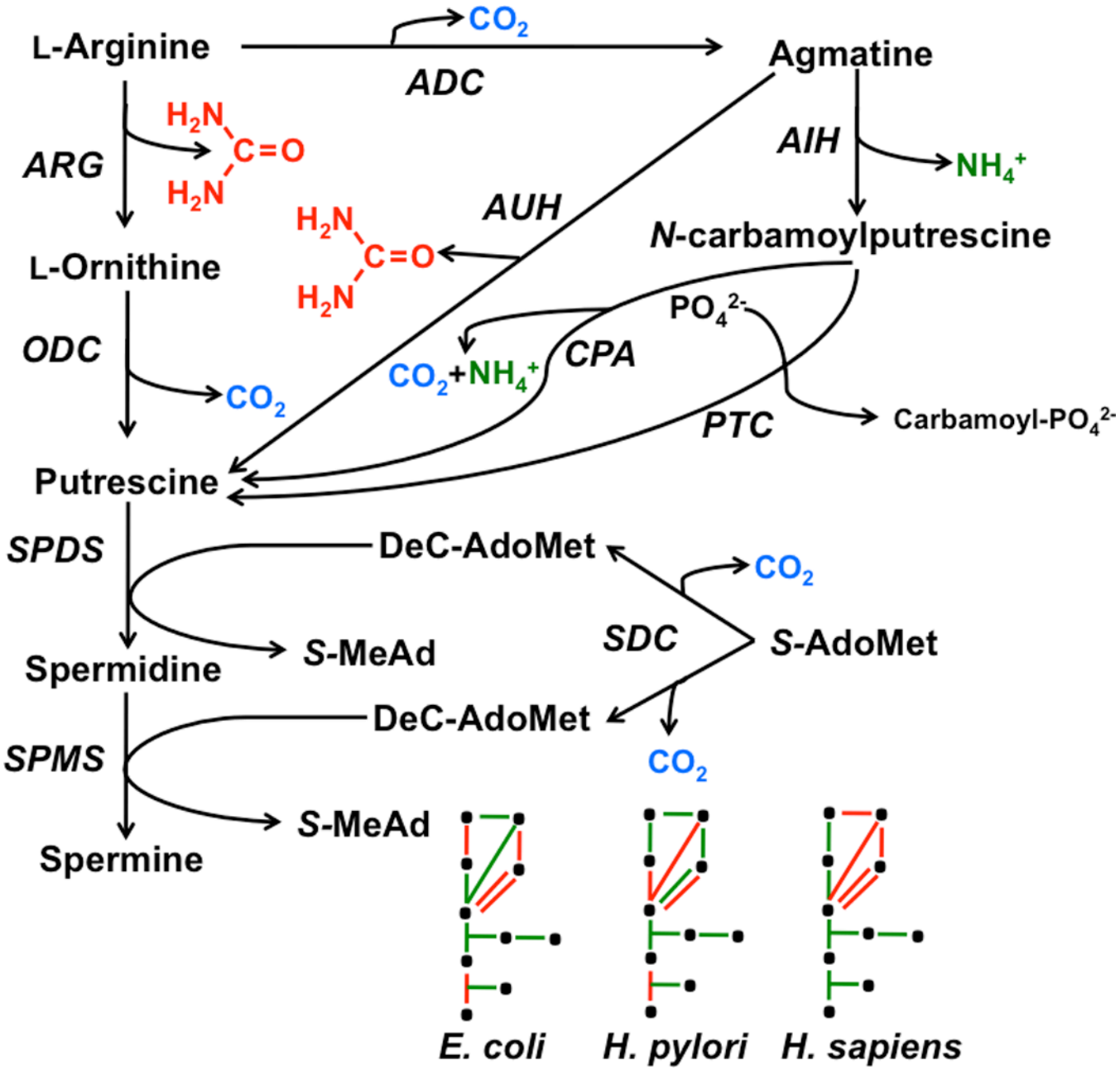


**Figure 1. Arginine-dependent polyamine biosynthetic pathways**

Four different biosynthetic pathways exist across all three kingdoms of life for the conversion of L-arginine to putrescine. Putrescine is then converted to spermidine through the addition of an aminopropyl group from decarboxylated *S*-adenosylmethionine (DeC-AdoMet). Glyphs in the lower right depict pathways present in *E. coli* as well as humans, green lines indicate enzymes in the above scheme that are present, red = absent. Abbreviations: ARG (Arginase), ODC (Ornithine decarboxylase), SPS (Spermidine synthase), *S*-MeAd (*S*-methyladenosine), SDC (*S*-adenosylmethionine decarboxylase), *S*-AdoMet (*S*-adenosylmethionine), ADC (Arginine decarboxylase), AUH (Agmatine ureohydrolase, Agmatinase), CPA (*N*-Carbamoylputrescine amidohydrolase) and PTC (Putrescine transcarbamoylase).

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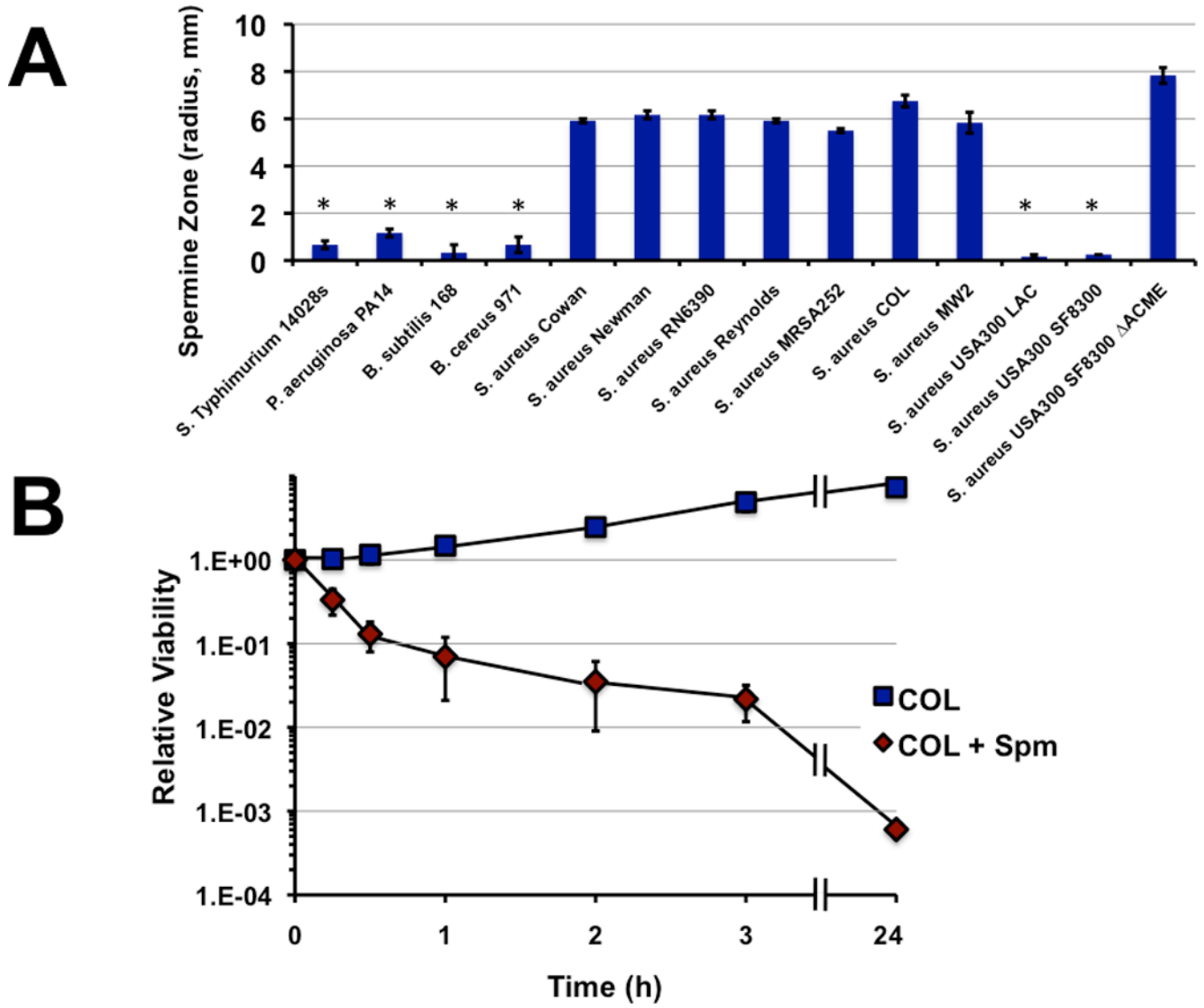


**Figure 2. Bactericidal effects of spermine on *S. aureus***

**A.** Zones of inhibition around discs supplemented with 20 µl of 250 mM spermine across various species and *S. aureus* strains. (*) denotes mean zone radii significantly ($p \leq 0.01$, $n \geq 3$) smaller than that of *S. aureus* COL (Students t-test). None of these strains exhibited zones around discs supplemented with 20 µl of 250 mM NaOH. **B.** Viable cfu of *S. aureus* strain COL over a 24-hour exposure to 5 mM Spermine.

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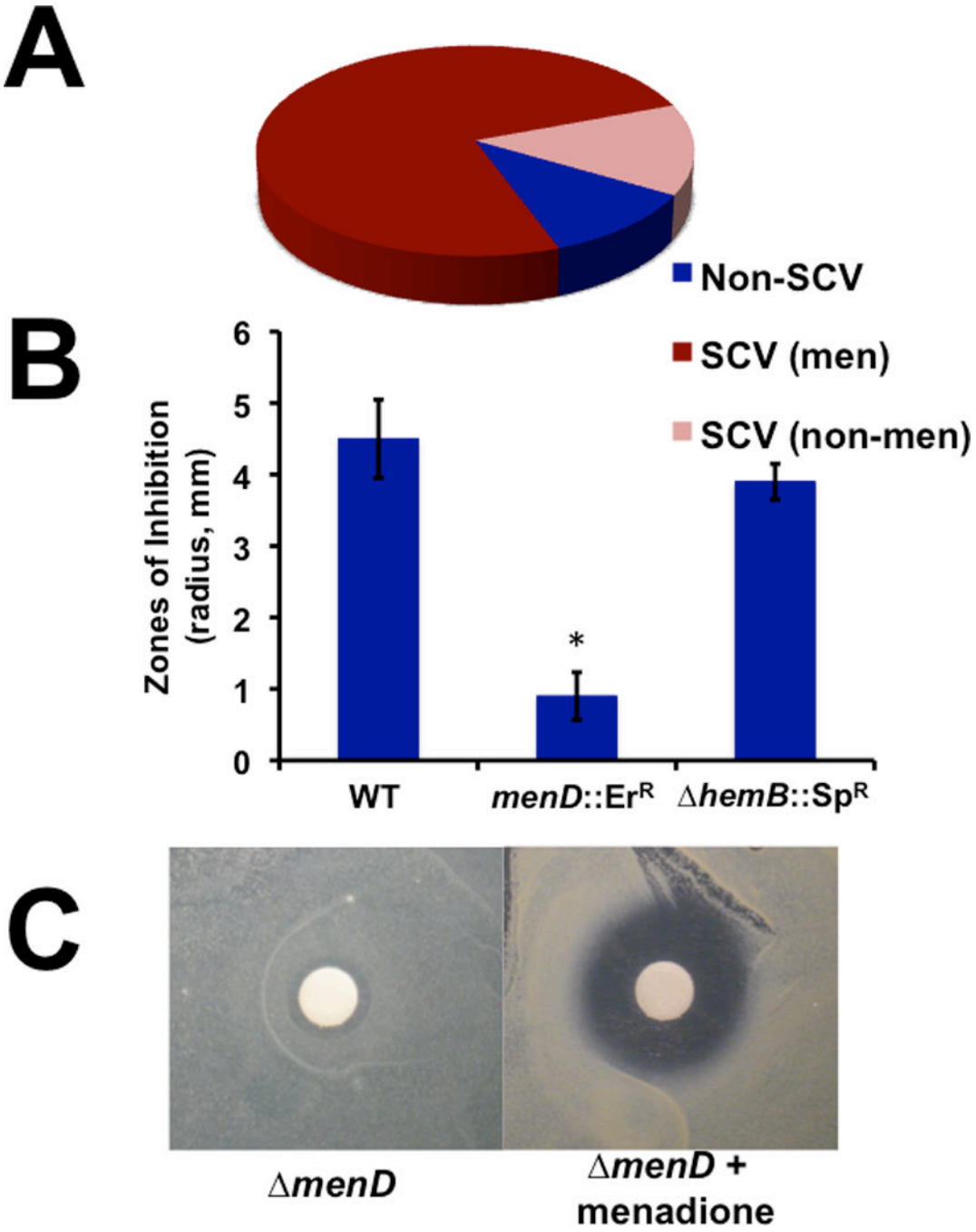


**Figure 3. Spontaneous spermine-resistance results primarily in menadione-requiring small colony variants (SCVs)**

**A.** Proportions of 47 characterized spontaneous spermine-resistant clones that were SCVs (89%) versus mutants exhibiting normal growth (11%). Normal growth could be restored to 83% of these SCVs by supplementing 8 μg·ml$^{-1}$ of menadione. The remaining strains were not rescued by supplementing menadione, heme, thiamine or thymidine. Spermine-resistant mutants were isolated by plating $10^9$ cfu of *S. aureus* strain Newman on BHI plates supplemented with 20 mM Spermine. **B.** Zones of inhibition around 250 mM spermine discs using *S. aureus* strain Newman and isogenic SCV mutants; *menD*::Er$^R$ and Δ*hemB*. (*) indicates that the only spermine zone of the *menD*::Er$^R$ mutant was significantly smaller

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than that observed in WT ($p \leq 0.05$, $n \geq 3$, Student's t-test) **C.** Supplementing BHI medium with 8 $\mu g \cdot ml^{-1}$ menadione restored WT growth, normal pigmentation and full spermine susceptibility to a *menD*::$Er^R$ mutant of *S. aureus* strain Newman.

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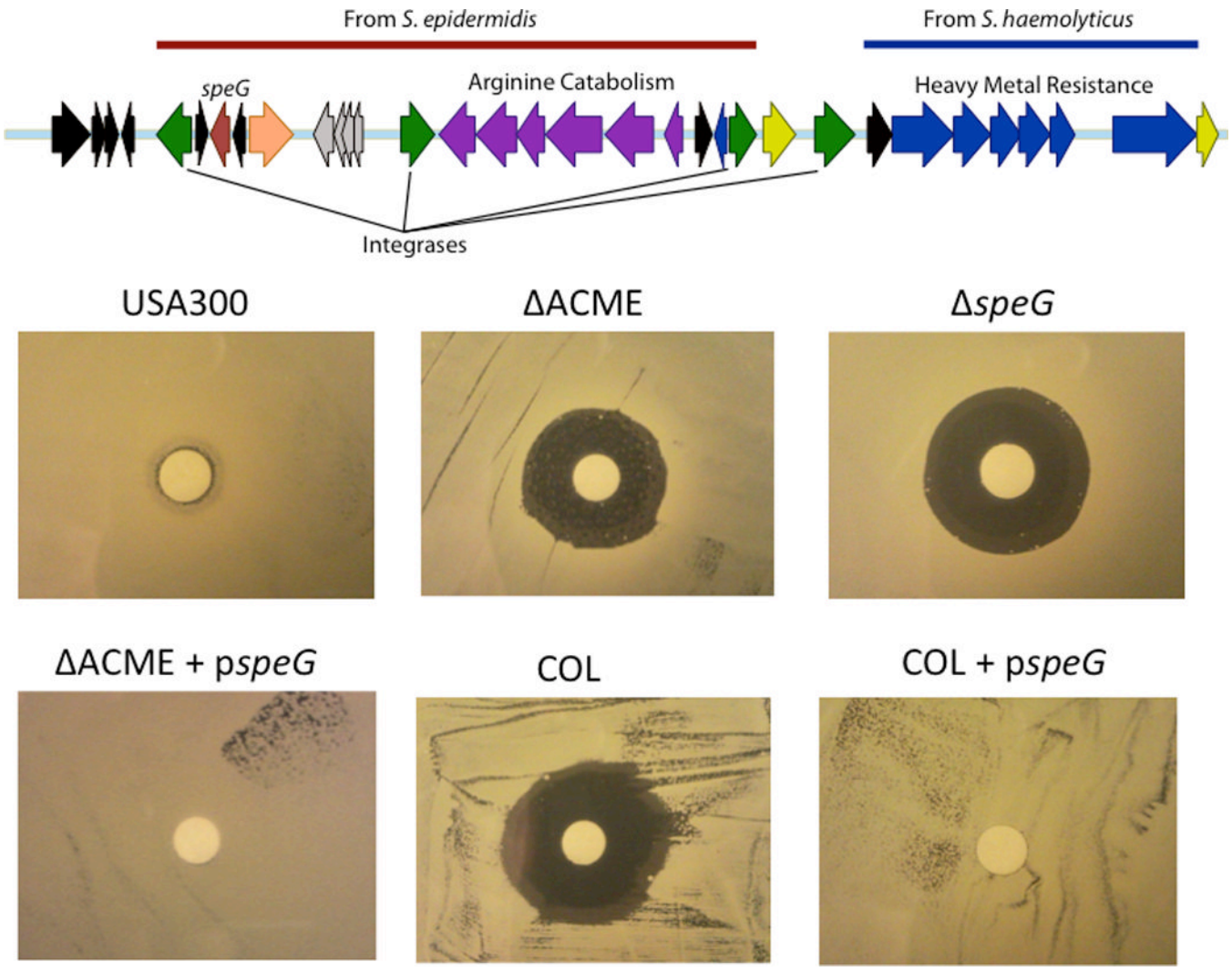


**Figure 4. CA-MRSA isolates belonging to the USA-300 PFGE-type exhibit full spermine resistance due to an ACME encoded spermine/spermidine acetyltransferase (*speG*)**

TOP: Schematic of the 33 gene ACME island found in nearly every USA-300 clone harboring the SCCMEC type IVa. BOTTOM: *speG* is necessary and sufficient for USA300 spermine-resistance. Spermine inhibition zones of USA-300 strain SF8300 and isogenic ΔACME and Δ*speG* strains as well as MRSA isolate COL and ΔACME harboring a plasmid-borne *speG* allele.

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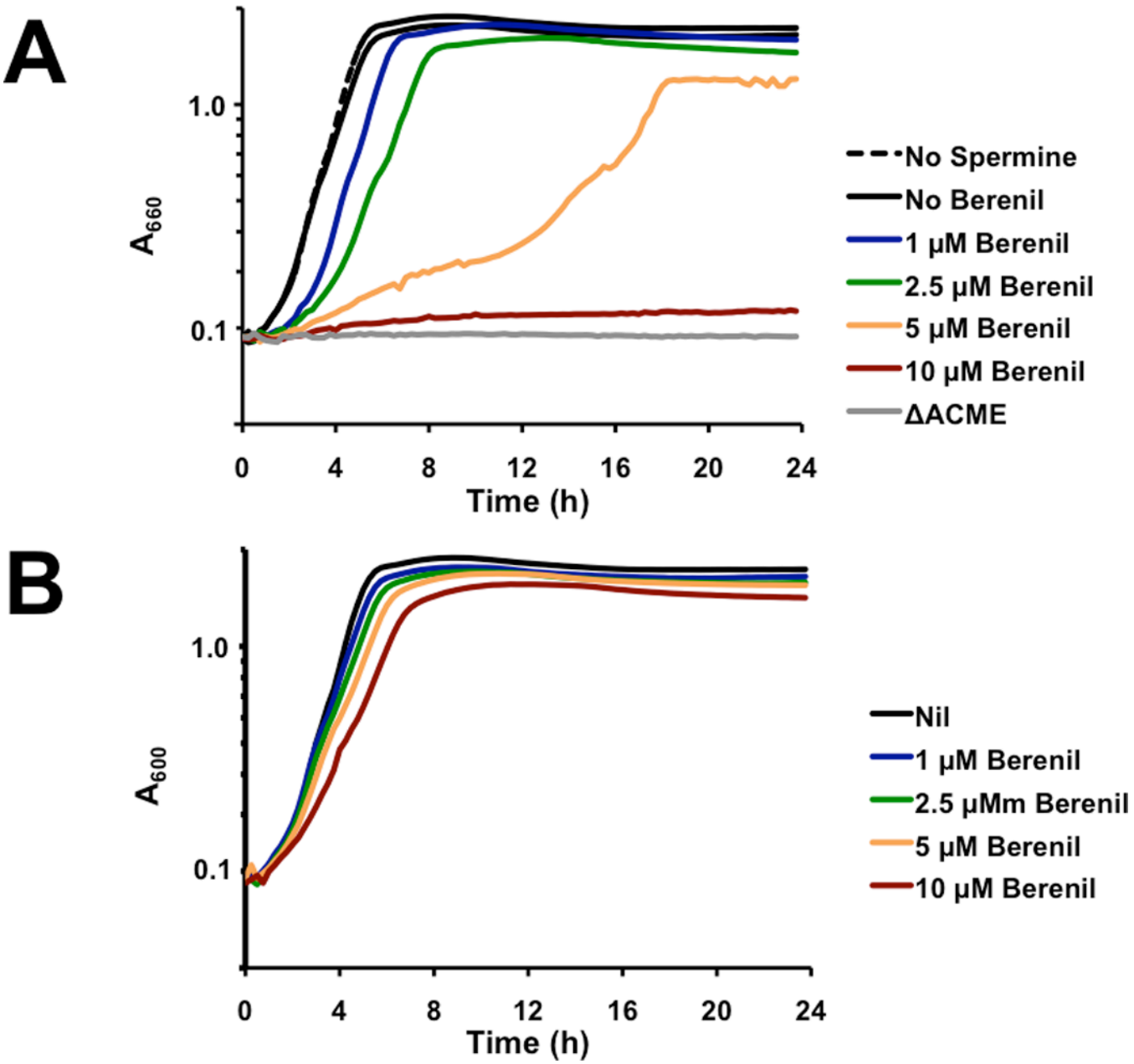


**Figure 5. SpeG spermine-acetyltransferase activity is required for USA300 spermine-resistance**

Growth of USA300 in the presence of 5 mM Spermine requires *speG* and can be inhibited by the addition of 10 μM Berenil, a spermine/spermidine acetyltransferase inhibitor. This concentration of Berenil had negligible effects on the growth of USA300 in the absence of spermine.

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## Table 1

Lack of *de novo* polyamine synthesis in *S. aureus*.

| Organism(Strain) | Agmatine | Putrescine | Cadaverine | Spermidine |
|---|---|---|---|---|
| *E. coli* (MG1655) | 50.0±(5.2) | 94.7±(5.3) | N.D. | 90.7±(11.2) |
| *P. aeruginosa* (PA14) | N.D. | 51.2±(4.7) | 20.8±(7.2) | 72.6±(8.0) |
| *B. subtilis* (168) | 15.2±(0.7) | 3.2±(0.9) | N.D. | 428.8±(8.1) |
| *S. aureus* (COL) | N.D. | N.D. | N.D. | N.D. |
| *S. aureus* (USA300) | N.D. | N.D. | N.D. | N.D. |

.Values represent mean polyamine levels (nmol) detected per $10^{10}$ cfu of indicated organism ± (S.D.), n ≥ 3.

.N.D. = Not Detected

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## Table 2

Minimum Inhibitory and Bactericidal Concentrations of Spermine [a]

| *S. aureus strain* | Aerobic | Anaerobic | Anaerobic + $NO_3^-$ |
|---|---|---|---|
| COL | 2 (3) | 5 (10) | 5 |
| COL *menD*::$Er^R$ | 4 (5) | 10 (15) | ND |
| Newman | 3 (3) | 7.5 (10) | 8 |
| Newman *menD*::$Er^R$ | 5 (6) | 13 (>15) | ND |

[a] MIC(MBC) mM, error was < 10% of listed values for all measurement

ND: Not Determined