SYMPOSIUM

Molecular Pathogenesis of Staphylococcal Osteomyelitis

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ABSTRACT Staphylococcus aureus is the most prominent musculoskeletal pathogen of man and animals. The persistent emergence of antibiotic-resistant strains has prompted renewed efforts to develop alternative protocols for the treatment and prevention of staphylococcal disease. These efforts have included attempts to develop an effective staphylococcal vaccine. Among the potential vaccine candidates are a group of surface proteins that act as adhesins by virtue of their ability to bind host proteins present in plasma and in the extracellular matrix. Because of our interest in the treatment and prevention of musculoskeletal infection, we have focused on adhesins that contribute to the colonization of bone and cartilage. Based on reports suggesting that colonization is a conserved characteristic of S. aureus strains that cause osteomyelitis and septic arthritis, we have paid particular attention to the factors that contribute to the ability to bind collagen. To date, only one collagen-binding adhesin (Cna) has been identified, and the gene encoding this adhesin (cna) is not present in most S. aureus strains.

(Key words: osteomyelitis, Staphylococcus, vaccine, collagen binding, adhesin)

INTRODUCTION

Staphylococcus aureus is one of the most prominent bacterial pathogens of man and animals. It has a particular propensity to infect tissues of the musculoskeletal system, as evidenced by the fact that it is the single leading cause of osteomyelitis (Waldvogel, 1988). The treatment of these infections is complicated by two factors. The first and most pervasive is the persistent emergence of antibiotic-resistant strains. Indeed, the increasing prevalence of methicillin-resistant strains, which are almost always resistant to other commonly used antibiotics (Waldvogel, 1995), together with the ominous appearance of strains with reduced susceptibility to the glycopeptide antibiotics (Smith et al., 1999), means that S. aureus represents a more serious threat now than at any time since the pre-antibiotic era. The second complicating factor is the inability to deliver effective concentrations of antibiotics to the site of infection. Indeed, the difficulties associated with the effective resolution of bone infections are largely independent of the resistance status of the S. aureus strain responsible for the infection. This is due, at least in part, to the ability of the bacterium to attach to surfaces (e.g., bone and implanted biomaterials) and to grow as an almost impenetrable biofilm (Mayberry-Carson et al., 1984; Evans et al., 1998). In most cases, the presence of this

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Abbreviation Key: agr = accessory gene regulator; Bsp = bone sialoprotein; Cna = collagen-binding adhesin; cna = collagen-binding adhesin gene; ica = intercellular adhesin; IVET = in vivo expression technology; MSCRAMM = microbial surface components recognizing adhesive matrix molecules; PIA = polysaccharide adhesin; PNSG = poly-N-succinyl β-1-6 glucosamine; RAP = RNAIII-activating peptide; RIP = RNAIII-inhibiting peptide; RNAIII = regulatory RNA.
biofilm necessitates surgical intervention to debride the infected area (Mader and Calhoun, 1995).

The continued emergence of antibiotic-resistant strains has led to a renewed effort to develop alternative protocols for the treatment and prevention of staphylococcal disease. This effort has taken several forms, including the obvious approach of developing new antimicrobial agents. However, these agents are, almost invariably, derivatives of existing antibiotics rather than truly novel drugs, and it is virtually certain that they will also be defeated by the remarkable capacity of the bacterium to develop resistance. That realization, together with advances in our understanding of S. aureus virulence factors, has prompted a number of investigators to take the more direct approach of developing pathogen-specific therapeutic agents. These efforts are based on the presumption that such agents would lessen the selective pressure for resistance and would avoid some of the other adverse consequences of broad-spectrum drugs, not the least of which is the development of secondary infections (Casedevall, 1996).

**PATHOGEN-SPECIFIC THERAPY IN S. AUREUS**

Because S. aureus is an opportunistic pathogen whose ability to cause disease is as much a function of the host as of the bacterium, efforts to develop antistaphylococcal therapeutic agents have followed two basic tracks. The first targets the bacterium directly and revolves around the identification of genes whose products are required for survival within the host. The identification of these genes has been greatly facilitated by the development of genetic systems that allow for the detection of bacterial genes that are specifically expressed in vivo during the course of infection (Mei et al., 1997; Coulter et al., 1998; Lowe et al., 1998). The logic of these in vivo expression technology (IVET) systems (Mahan et al., 1993) is that inhibiting the function of the corresponding gene products will impair the ability of the bacterium to grow within the host and cause disease. For example, Coulter et al. (1998) used an IVET system to identify a gene (putP) that encodes a putative proline permease. Schwan et al. (1998) subsequently demonstrated that mutation of putP impairs the ability of S. aureus to cause disease. Based on that finding, it was suggested that proline scavenging is required in vivo and that proline analogs could potentially be used as therapeutic agents against staphylococcal infection.

The second approach is aimed at enhancing the host capacity to clear the infection with the primary example being attempts to develop an effective antistaphylococcal vaccine (Lee, 1998; McKenney et al., 1999). With respect to musculoskeletal disease, this approach is particularly attractive because it has the potential to stop the infection before it becomes established and thereby avoid the problems associated with the delivery of therapeutic agents. This approach is somewhat controversial because the overall utility of a vaccine is unclear in a situation in which one of the primary factors contributing to the onset of disease is the immune status of the host. However, certain groups of patients who are at extreme risk of infection are not immunocompromised (e.g., surgical patients awaiting orthopedic implants), and even immunocompromised patients could possibly benefit from passive immunotherapy. Additionally, S. aureus is an equally prominent animal pathogen, and the utility of a staphylococcal vaccine capable of preventing disease in food animals cannot be overestimated. This is particularly true because the widespread use of antibiotics in agriculture undoubtedly contributes to the emergence of antibiotic-resistant strains.

**VACCINE CANDIDATES**

The development of an effective vaccine depends on the identification of appropriate immunogens. Among the more interesting candidates are the signaling molecules required for toxin production. The primary focus of this approach is the regulatory system known as the accessory gene regulator or agr. Importantly, mutation of agr has been shown to result in reduced virulence in animal models of staphylococcal disease, including an osteomyelitis model (Gillaspy et al., 1995). The agr locus encodes a two-component signal transduction system that controls expression of a regulatory RNA (RNAIII). RNAIII serves a dual role in that it represses synthesis of many surface proteins and enhances production of virtually all S. aureus toxins (Projan and Novick, 1997). The agr locus mediates RNAIII production via a quorum-sensing system that responds to the accumulation of extracellular signaling molecules produced by the bacteria (Balaban and Novick, 1995). Although there is some controversy regarding the nature of these signaling molecules, it is clear that they occur in two forms (Balaban and Novick, 1995; Balaban et al., 1998). One of these activates agr and is known as RAP (RNAIII-activating peptide). The other inhibits activation and is known as RIP (RNAIII-inhibiting peptide). Whether the signaling molecule acts as an activator or an inhibitor is a strain-dependent characteristic (i.e., the cognate form is an activator that is also capable of inhibiting agr induction in unrelated strains) (Ji et al., 1997). Of most relevance to this discussion, however, is the recent demonstration that immunization with RAP or treatment with RIP can protect mice against S. aureus infection (Balaban et al., 1998).

*S. aureus* exopolysaccharides are a second vaccine candidate. Because of the potent antiphagocytic properties of the *S. aureus* capsule (Thakker et al., 1998), several studies have focused on the possibility of using purified capsular polysaccharides to induce opsonizing antibody and thereby enhance phagocytosis. Indeed, immunization with type-5 capsular polysaccharides has been shown to provide protection in animal models of bacteremia (Fattom et al., 1996) and endocarditis (Lee et al., 1997). In addition, McKenney et al. (1999) recently demonstrated that vaccination with a noncapsular polysaccharide (poly-N-succinyl β-1-6 glucosamine, or PNSG)
also provides protection against *S. aureus* infection. Importantly, PNSG production is a function of the ica (intercellular adhesin) locus, which is found in both *S. aureus* and *Staphylococcus epidermidis* (Heilmann et al., 1996; Cramton et al., 1999). Moreover, PNSG production is required for biofilm formation in both species (Heilmann et al., 1996; Cramton et al., 1999). This is particularly relevant because formation of a biofilm is a characteristic and complicating feature of all staphylococcal musculoskeletal infections.

The third category of vaccine candidates is a group of surface proteins that function as adhesins by virtue of their ability to bind host proteins present in plasma and in the extracellular matrix. These adhesins have been referred to as MSCRAMM to denote their role as microbial surface components recognizing adhesive matrix molecules (Patti et al., 1994a). Included among the host protein targets are fibronectin, fibrinogen, elastin, von Willebrand factor, thrombospondin, vitronectin, bone sialoprotein, laminin, and collagen (Foster and Hook, 1998). Because these adhesins are exposed on the surface of *S. aureus* cells and bind directly to their host protein targets, antibodies directed against the adhesins would not only act as opsonins but could also directly inhibit the binding of *S. aureus* to host tissues. Because of our interest in the treatment and prevention of musculoskeletal infection, we are particularly interested in those MSCRAMM adhesins that promote bone infection.

In most cases, the ability to bind host proteins is highly conserved among different strains of *S. aureus*. In fact, the ability to bind several host proteins is redundant. For instance, *S. aureus* produces two MSCRAMM adhesins (FnbpA and FnbpB) that bind fibronectin and at least two (ClfA and ClfB) that bind fibrinogen (Foster and Hook, 1998). In contrast, only one collagen-binding adhesin (Cna) has been identified, and the gene encoding this adhesin (*cna*) is not present in all strains (Ryding et al., 1997; Smeltzer et al., 1997a), which might suggest that the collagen adhesin is not the best choice for a vaccine. However, evidence suggests that collagen binding is a conserved characteristic of strains that cause osteomyelitis (Holderbaum et al., 1987; Buxton et al., 1990). This finding implies that the ability to bind collagen is an important contributing factor, at least with respect to musculoskeletal infection, and that prophylactic strategies that inhibit collagen binding could be effective for the prevention of osteomyelitis. However, the conclusion that collagen binding is a more highly conserved characteristic of musculoskeletal isolates is not universally accepted. Indeed, two recent studies concluded that collagen binding is no more prevalent among musculoskeletal isolates that it is among isolates from other forms of staphylococcal disease or, for that matter, isolates obtained from the nasopharynx in the absence of disease (Ryding et al., 1997; Thomas et al., 1999).

In an effort to resolve the dispute regarding the correlation between collagen binding and the ability to cause osteomyelitis, we carried out a series of experiments aimed at addressing three important issues. First, because collagen binding might not be as rare a phenotype as the limited prevalence of *cna* would suggest, we compared a diverse group of *cna*-negative and *cna*-positive strains to determine whether *S. aureus* produces a collagen-binding adhesin other than Cna. Second, because many bacterial virulence factors are clustered within pathogenicity islands that include additional virulence determinants, we characterized the genetic element that encodes *cna* to determine whether the presence of *cna* is associated with additional virulence factors that are relevant with respect to the pathogenesis of musculoskeletal disease. Finally, to directly assess the role of Cna in musculoskeletal infection, we used a rabbit model of acute, posttraumatic osteomyelitis to determine whether mutation of *cna* results in a reduced capacity to cause disease. We also used a modification of our model to determine whether vaccination directed at inhibiting the ability to bind collagen might be a viable approach for the prevention of staphylococcal musculoskeletal infection.

### COLLAGEN BINDING IN *S. AUREUS*

Switalski et al. (1989) was the first to describe a specific *S. aureus* adhesin capable of binding collagen. Patti et al. (1992) subsequently cloned the gene encoding this adhesin (*cna*) and demonstrated that Cna is both necessary and sufficient for the adherence of *S. aureus* to collagen fibrils in cartilage. This result strongly suggests that Cna is the only collagen-binding adhesin produced by *S. aureus*. However, Ryding et al. (1997) examined over 200 strains of *S. aureus* and found eight that bound collagen despite the absence of *cna*. Although most of these strains exhibited relatively low-level binding in comparison with *cna*-positive strains, these results clearly suggest the existence of a second collagen-binding adhesin. Additionally, the possibility that the level of collagen binding observed in the *cna*-negative strains is sufficient to facilitate the colonization of bone cannot be ruled out.

In an effort to resolve this discrepancy, we compared 32 unrelated strains of *S. aureus* with respect to the presence or absence of *cna* and the ability to bind collagen. Although we also observed what could be interpreted as low-level binding in *cna*-negative strains, the level of binding was relatively consistent and, more importantly, was not inhibited in the presence of excess amounts of unlabeled collagen. Based on that finding, we concluded that it represented the background associated with our binding assay, rather than the low-level activity of a second collagen-binding adhesin (Gillaspy et al., 1998). Indeed, we have found an almost direct correlation between the presence of *cna* and the ability to bind collagen (Gillaspy et al., 1998). The only exceptions were one strain that encoded but did not express *cna* and two heavily-encapsulated strains (Smith diffuse and M) that encoded and expressed *cna* but bound only minimal amounts of collagen. Comparison of the heavily-encapsulated strains with their corresponding capsule mutants clearly indicated that the failure to bind collagen was due to masking of the adhesin by capsular polysaccharides (see below).
Most importantly, the fact that the only exceptions involved cna-positive strains that did not bind collagen (rather than cna-negative strains that did bind collagen) supports the conclusion that cna encodes the primary collagen-binding adhesin in S. aureus.

Interestingly, we examined the heavily-encapsulated, cna-positive strains in a rabbit model of acute, posttraumatic osteomyelitis and found that they are relatively avirulent in comparison with our prototype osteomyelitis isolate (UAMS-1) (Smeltzer et al., 1997b). This finding is particularly interesting because these same strains are exceptionally virulent in other models of staphylococcal disease. More specifically, we compared UAMS-1 and one of the heavily-encapsulated strains (Smith diffuse) with a murine peritonitis model and found that UAMS-1 had an LD$_{50}$ in excess of $10^5$ cfu, whereas Smith diffuse had an LD$_{50}$ of only $10^3$ cfu (Smeltzer et al., 1997b). In contrast, UAMS-1 had an ID$_{50}$ in our rabbit model of $10^5$ cfu, whereas Smith diffuse had an ID$_{50}$ in excess of $10^6$ cfu. Although it is premature to conclude that this difference is related to the inability to bind collagen, these results clearly emphasize the existence of strain-specific virulence differences that are directly relevant to the pathogenesis of musculoskeletal disease.

The possibility that capsular polysaccharides can mask adhesins on the surface of S. aureus cells warrants comment, because capsular polysaccharides and adhesins are currently under consideration as vaccine candidates. However, heavily-encapsulated strains like Smith diffuse and M are not representative of the microencapsulated serotype 5 and 8 strains that are most often responsible for disease in both man and animals (including poultry) (Daum et al., 1990; Foster, 1991; Foutrel et al., 1998). Indeed, we did not observe a significant degree of inhibition when we compared collagen binding in the microencapsulated serotype 8 strains UAMS-1 and Becker and their corresponding capsule mutants (Gillaspy et al., 1998; Snodgrass et al., 1999). No inhibition occurred even when we assayed cells grown under conditions thought to maximize capsule production (e.g., growth on Columbia agar) (Lee et al., 1993; Snodgrass et al., 1999). These results suggest that most strains that cause infection do not produce enough capsule to mask Cna to an extent that limits its ability to bind collagen or its utility as an immunogen, which is consistent with our demonstration that Cna is exposed on the surface of S. aureus cells growing in bone (Gillaspy et al., 1997b).

**THE CNA GENETIC ELEMENT**

We examined 25 unrelated strains of S. aureus and found that cna was present in only 10 strains (Smeltzer et al., 1997a). The subsequent analysis of cna-positive strains by pulsed-field gel electrophoresis confirmed that cna is encoded with the chromosome. Its presence in the chromosome is relevant because it suggests that cna may be encoded within a pathogenicity island, which can be defined as a large chromosomal segment that encodes multiple virulence factors and is present only in pathogenic strains (Hacker et al., 1997). If the above is true, then cna could be directly linked to additional virulence factors that are more relevant culprits with respect to the pathogenesis of musculoskeletal disease. In other words, the ability to bind collagen could be nothing more than a phenotypic marker of strains capable of causing musculoskeletal disease.

To address the possibility that cna is linked to other factors, we carried out a series of Southern blots with genomic DNA isolated from cna-positive and cna-negative strains and with DNA probes corresponding to cna and to the chromosomal regions immediately upstream and downstream of cna (Gillaspy et al., 1997a). Surprisingly, the probes corresponding to the regions adjacent to cna hybridized to chromosomal DNA from all strains, including those that did not encode cna. We subsequently cloned the DNA fragments containing the junctions between conserved and unique DNA and confirmed by sequencing analysis that cna is encoded within a discrete chromosomal element that extends only 202 bp upstream of the cna start codon and 100 bp downstream of the cna stop codon (Figure 1). This element does not include any genes other than cna, and its presence does not disrupt a gene that is present in cna-negative strains (Gillaspy et al., 1997a). These results confirm that cna is not encoded within a pathogenicity island and strongly suggest that the only consistent difference between cna-positive and cna-negative strains is the ability to bind collagen.

**THE CNA ADHESIN AND DISEASE**

The results discussed above support the following conclusions: 1) S. aureus does not produce a collagen-binding adhesin other than Cna and 2) the cna-encoded adhesin is not directly linked to additional S. aureus virulence factors. However, both of these conclusions must be interpreted with caution. For instance, it remains possible that there is a second collagen-binding adhesin that is not expressed in vitro but is expressed at higher, biologically-
relevant levels in vivo. Additionally, the possibility that cna-positive strains encode additional virulence factors at some other chromosomal location cannot be excluded. Based on that possibility, direct address of the correlation between Cna and the pathogenesis of musculoskeletal infection remains important. Although several authors have attempted to resolve this issue with survey studies correlating collagen-binding with disease presentation, the results of these studies are contradictory. More directly, some studies have concluded that isolates that cause osteomyelitis and septic arthritis almost invariably bind collagen (Buxton et al., 1990; Holderbaum et al., 1987; Switalski et al., 1993), whereas others have found that the ability to bind collagen is no more prevalent among musculoskeletal isolates than it is among isolates from other forms of staphylococcal disease (Ryding et al., 1997; Thomas et al., 1999). Clearly, the isolation of strains that do not bind collagen from patients suffering from bone and joint infection suggests that collagen binding is not a necessary prerequisite for musculoskeletal infection. However, survey studies attempting to correlate a phenotype with a disease are complicated by a number of factors, not the least of which is the basis used to define the phenotype. For example, Ryding et al. (1995) found that serum of patients infected with S. aureus strains that do not bind collagen contains anti-Cna antibody. This finding is consistent with the hypothesis that some strains that do not bind collagen when assayed in vitro may bind collagen when growing in vivo. Additionally, because S. aureus is an opportunistic pathogen that can be found in a significant proportion of the population in the absence of disease, the isolation of S. aureus from an infected patient does not necessarily mean that the isolate is responsible for the infection. For example, we have two isolates obtained from patients suffering from osteomyelitis, neither of which encodes cna or binds collagen. However, both were obtained from draining sinus tracts rather than by bone biopsy. Because the isolation of S. aureus from a draining sinus tract is not a reliable indicator of the etiology of bone infection (Mackowiak et al., 1978), the inclusion of these two strains in a survey study could potentially obscure important correlations between phenotype and disease. It is also possible that some strains may encode adhesins that can compensate for the absence of Cna. For example, the ability to bind bone sialoprotein (Bsp) is also a strain-dependent characteristic of S. aureus (Patti et al., 1994b).

We believe the permutations involved in interpreting survey studies make it impossible to draw definitive conclusions about the correlation between Cna and the ability to cause infection. Unfortunately, the number of experimental studies that address the issue on a direct, experimental basis is limited. It has been demonstrated that S. aureus binds directly to collagen fibrils in cartilage (Voytek et al., 1988) and that Cna is responsible for this binding (Switalski et al., 1993). We have also demonstrated that Cna is present on the surface of S. aureus cells growing in bone (Gillaspy et al., 1997b), and Ryding et al. (1995) has confirmed that infection with a cna-positive strain elicits an anti-Cna antibody response. Although not definitive, these observations confirm that Cna is expressed in vivo during the course of S. aureus infection.

More definitive evidence supporting the hypothesis that Cna makes an important contribution to the pathogenesis of musculoskeletal infection comes from the direct comparison of a cna-positive clinical isolate (Phillips) and an isogenic mutant (PH100) in which cna was inactivated by allele replacement (Patti et al., 1994b). This comparison confirmed that the mutant was less virulent than its cna-positive parent strain in animal models of septic arthritis (Patti et al., 1994b) and endocarditis (Hienz et al., 1996). Additionally, the introduction of cna into a cna-negative strain enhanced virulence in the septic arthritis model (Patti et al., 1994b). These results clearly indicate that Cna is, in fact, an important virulence factor contributing to the pathogenesis of S. aureus musculoskeletal infections.

We also compared the virulence of Phillips and PH100 by using our rabbit model of posttraumatic osteomyelitis. In the first set of experiments, we evaluated the capacity of each strain to cause osteomyelitis by directly introducing bacteria into the intramedullary space of an excised bone segment (Smeltzer et al., 1997b). In a second set of experiments, we evaluated the possibility of using Cna as a vaccine by comparing unimmunized rabbits with rabbits that had been immunized with a 55-kDa recombinant fragment corresponding to the Cna ligand-binding A domain (kindly provided by Joseph M. Patti, Inhibitex, Inc., Alpharetta, GA 30004). In the second case, the excised segment was either inoculated directly with bacteria or was placed back into the defect in its original orientation without inoculation. In those cases in which the segment was replaced without inoculation, bacteria were subsequently introduced into the bloodstream via ear vein injection. These experiments were done using the wild-type Phillips strain.

The results of the direct comparison of Phillips and PH100 were inconclusive. Specifically, we observed only a slightly reduced infection rate (from 45 to 35%) with the PH100 cna mutant. Additionally, no difference was observed between the immunized and unimmunized rabbits when the infection was introduced directly into the bone (Table 1). However, when the inoculation was done by the intravenous route, 67% (four of six) of the unimmunized rabbits died compared with only 17% (one of six) of the immunized rabbits. Also, S. aureus was isolated from the bone of 50% (three of six) of the unimmunized rabbits and only 17% (one of six) of the immunized rabbits (Table 1). These results clearly suggest that immunization with Cna can provide protection against septic death and

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limit the ability to colonize bone. Further evidence supporting the utility of Cna as an immunogen comes from the results of Nilsson et al. (1998), who demonstrated that active immunization with recombinant Cna and passive immunization with Cna-specific antibody protected mice against intravenous challenge with *S. aureus*.

**DISCUSSION**

Osteomyelitis is a devastating disease that can be clinically subdivided into two general forms. One of these arises from the invasion of bone from a contiguous focus of infection. This form is most common in adults and typically results from the direct introduction of bacteria into the bone (posttraumatic osteomyelitis) or by invasion of the bone from an adjacent, soft tissue infection (Mader and Calhoun, 1995; Lew and Waldvogel, 1997). These infections are rarely resolved without surgical debridement. The second form is hematogenous osteomyelitis, which arises from blood-borne seeding of the bone and is typically observed in children. Importantly, this form appears to be very similar to the disease observed in poultry (Emslie and Nade, 1983). Hematogenous osteomyelitis usually appears as an acute infection and, in comparison to the disease in adults, is relatively easy to treat (Mader and Calhoun, 1995). However, even in this case, treatment is increasingly compromised by the continued emergence of antibiotic-resistant strains. Because of the difficulties associated with the treatment of osteomyelitis, an attractive alternative is the development of effective protocols that can stop an infection before it becomes established. However, the development of such protocols will require a clear understanding of the initiating events that lead to the colonization of musculoskeletal tissues.

By using chickens as a model system, Emslie and Nade (1983) demonstrated that hematogenous osteomyelitis arises from the deposition of bacteria at the metaphyseal growth plate. The circulatory architecture in this region includes very tight loops that turn away from the cartilaginous growth plate (Mader and Calhoun, 1995). Based on this finding, it was originally suggested that the deposition of bacteria in this region was due to mechanical factors associated with reduced blood flow in the metaphyseal capillary beds (Norden et al., 1994). However, it has become increasingly evident that additional factors are involved. Included among these factors are the relative lack of phagocytic cells in the metaphyseal blood vessels and an incomplete endothelial lining that allows bacteria to escape into the extravascular tissues (Emslie and Nade, 1983; Alderson et al., 1986; Mader and Calhoun, 1995).

It has also been suggested that a major contributing factor in bone and joint infection is the production of a bacterial glycocalyx that promotes the adherence of bacteria to host tissues (Mayberry-Carson et al., 1984; Alderson et al., 1986). The glycocalyx forms the foundation of the multilayered biofilm containing bacterial microcolonies (Mayberry-Carson et al., 1984; Evans et al., 1998). There is no doubt that the glycocalyx is a fundamental and clinically-relevant feature of staphylococcal osteomyelitis. However, it is not clear whether the glycocalyx promotes the initial adherence of bacteria or whether adherence is dependent on other factors, with glycocalyx production and biofilm formation being the consequence of attachment and the subsequent growth of sessile bacteria. This scenario implies a two-stage process in which bacteria first attach to a substrate (e.g., bone) and then attach to each other as the biofilm grows and matures. The two-stage process is consistent with the scenario described for *S. epidermidis*, which is a common cause of infections involving in-dwelling medical devices. In this case, the initial attachment appears to be dependent on the production of one or more protein adhesins, whereas the subsequent aggregation of bacteria into a biofilm is dependent on the production of exopolysaccharide adhesins (Heilmann et al., 1996). The latter have been referred to by various names, including the intercellular polysaccharide adhesin (PIA) (Heilmann et al., 1996) and PNSG (McKenney et al., 1999). The production of PIA, which is the nomenclature we prefer because it describes the relevant phenotype, is a function of the *ica* locus (Heilmann et al., 1996; McKenney et al., 1998; Ziebuhr et al., 1997).

*Staphylococcus aureus* was recently shown to encode the *ica* locus and to produce PIA when growing in vivo (Cramton et al., 1999; McKenney et al., 1999). However, the initial attachment of *S. aureus* to both biomaterials and host tissues also appears to be dependent on protein adhesins, most notably the MSCRAMM (Foster and Hook, 1998). This finding is consistent with the observation that *S. aureus* surface proteins are produced early during the course of infection, when the most important considerations for the bacterium are avoiding host defenses and colonizing an appropriate tissue. This scenario suggests that anti-MSCRAMM antibodies would be effective opsonins capable of enhancing phagocytosis during the early stages of infection, when the number of bacteria is relatively low. This result is consistent with our studies and those of Nilsson et al. (1998) demonstrating that immunization with Cna can protect against septic death. It also suggests that MSCRAMM-specific antibodies would inhibit the earliest stages of attachment and would thereby limit the ability to colonize the host. Evidence supporting this hypothesis comes from our studies demonstrating a reduced frequency of bone infection in rabbits immunized with Cna. Immunization based on specific staphylococcal MSCRAMM could therefore provide an important first line of defense capable of limiting the infection before it has a chance to form biofilm. We believe this consideration is particularly important in the treatment and prevention of musculoskeletal infection.

Finally, it is perhaps not surprising that cna-negative strains are capable of causing musculoskeletal infection, at least under some circumstances. One possible explanation revolves around the fact that such infections are often precipitated by trauma, in which case the recruitment of platelets and host proteins (e.g., fibrinogen, fibronectin) to the site of inflammation could provide a substrate for
S. aureus infection (Lowy et al., 1998). A second possibility is that some strains produce alternative MSCRAMM (e.g., the Bsp adhesin) that can compensate for the absence of Cna. However, even if Cna is not required for the colonization of musculoskeletal tissues, the cumulative data clearly supports the conclusion that Cna is sufficient for that purpose. Taken together, these results suggest that the inclusion of immunogens derived from conserved adhesins (e.g., FnbpA and ClfA) would be required to achieve maximum effectiveness. However, failure to include Cna would result in an immune response that would not necessarily limit the ability of a cna-positive strain to colonize musculoskeletal tissues.

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