

Osteomyelitis and the role of biofilms in chronic infection

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Abstract

Understanding the mechanisms implicated in the initial attachment, development, and maturation of a biofilm phenotype are of tremendous importance for their effect on the medical, industrial, and public health arenas. This review explores the current understanding of the nature of biofilms and the impact that molecular interactions between the bacteria themselves, as well as between bacteria and the host, may have on biofilm development and phenotype using the nonmotile Gram-positive coccus, *Staphylococcus aureus*, as an example.

Introduction

Osteomyelitis is defined as an infection of the bone (Mader *et al.*, 1997). The pathogenesis of osteomyelitis has been explored clinically and different types of osteomyelitis can be classified according to the source of the infection (i.e. hematogenous or contiguous focus) and the vascular capability of the host (i.e. with or without generalized vascular insufficiency) (Lew & Waldvogel, 2004).

Types and causes of osteomyelitis

Hematogenous osteomyelitis

Hematogenous osteomyelitis, which is caused by the seeding of bacteria from the bloodstream, accounts for 20% of osteomyelitis cases. Primary hematogenous osteomyelitis is caused by the direct seeding of bone from a bacterial species in the blood. Although found in the adult population, it is more predominant in infants and children (Lew & Waldvogel, 2004). Hematogenous osteomyelitis in adults is more commonly caused by secondary infection where the bacteria gain access to the bloodstream and seed distal bone and

marrow sites. Infections are also caused by the reactivation of a quiescent focus of hematogenous osteomyelitis that developed in infancy or childhood and ‘arrested.’ The most common site of involvement is the distal part of the tibia, and the lesion is typically single and located near the metaphysis. There are two common types of hematogenous osteomyelitis: Long bone and vertebral osteomyelitis. Both are most often caused by a single bacterial species (discussed later), though vertebral osteomyelitis secondary to trauma may be polymicrobial. Patients generally present with fever, lethargy, tenderness over the infection site, and decreased range of motion (Carek *et al.*, 2001).

In long bone osteomyelitis, the metaphyses of the long bones (tibia, femur) are most frequently involved (Shirtliff *et al.*, 1999), which is explained by the anatomy of the metaphyseal region. Here, the blood flow becomes sluggish and disordered. The slowing of blood flow allows bacteria to settle and initiate colonization and an inflammatory response. Minor trauma likely predisposes the infant or child to infection by producing a small hematoma, vascular obstruction, and a subsequent bone necrosis that is susceptible to inoculation from a transient bacteremia (Morrissy &

Haynes, 1989). Acute infection, which generally develops within 2 weeks of disease onset (Carek *et al.*, 2001), initially produces a local cellulitis that results in a breakdown of leukocytes, increased bone pressure, decreased pH, and decreased oxygen tension. The collective effects of these physiologic factors further compromise the medullary circulation and enhance the spread of infection. In infants, infection may spread to the joint surfaces through the vascularized growth plate (Jackson & Nelson, 1982), but in children over 1 year of age, the growth plate lacks capillaries and the infection is confined to the metaphysis and diaphysis. The joint is spared unless the metaphysis is intracapsular.

Infants and children with hematogenous osteomyelitis usually have normal soft tissue enveloping the infected bone and are capable of a very efficient metabolic response to infection. They also have the potential to absorb large sequestra and generate a significant periosteal response to the infection. If antimicrobial therapy directed at the responsible pathogen is begun before extensive bone necrosis occurs, the pediatric patient has an excellent probability for an arrest of the infection because of this resorbing ability (Berendt & Byren, 2004).

In chronic hematogenous osteomyelitis, which occurs several months after onset of the disease (Carek *et al.*, 2001), the existing cortex is usually viable. The hallmark sign of chronic osteomyelitis, the involucrum, is defined as an area of live, encasing bone that surrounds infected dead bone within a compromised soft tissue envelope (Mader *et al.*, 1980). The involucrum contains the sequestered, necrotic marrow and endosteal bone. In normal tissues, necrosis is vital as it signals granulation tissue to resorb dead bone at the junction of living and dead tissue. Some of the dead cortex will usually detach from the living bone and form a sequestrum. After complete separation, or sequestration, the dead bone is eroded by granulation tissue and destroyed. However, in some cases the area of dead bone is too large to be resorbed, or the host response is compromised. This can lead the process of resorption to be inadequate, and may cause the formation of an involucrum. The involucrum affords skeletal continuity and maintains function during the healing phase. This feature has an irregular surface and often has holes in it through which pus may move into the surrounding soft tissues and eventually drain to the skin surface, forming a draining sinus tract (Mader *et al.*, 1996). The purpose of this structure is to isolate the infection. Involucrum development occurs upon the establishment of infection, once fibrous tissue and chronic inflammatory cells surround granulations and dead bone. New bone forms, as a result of the vascular reaction to the infection, from the periosteum, endosteum, and cortex. The involucrum may continue thickening for weeks or months, and eventually form part or all of a new bone shaft.

Though the involucrum functions to contain the infection, decreases in vascularity and low oxygen tension due to this structure lead to a decreased effectiveness of the host response and chronic disease can ensue. The dead bone acts as a nonliving surface for the attachment of bacteria and the formation of biofilms. This form of infection, coupled with the host's inability to resorb the dead bone, results in a very complicated disease to treat. Therefore, débridement is often necessary for these infections to resolve.

Of all hematogenous osteomyelitis cases, 2–7% are vertebral in nature (Tyrrell *et al.*, 1999). Incidences are rising due to the increasing population of aging adults, who have risk factors for bacteremia and deteriorating spinal pathology (Berendt & Byren, 2004). In vertebral osteomyelitis (as well as in all other locations), polymorphonuclear leukocytes (PMNs) are present due to the acute inflammatory response. The enzymes released from disintegrating PMNs, as well as bacterial products and vascular ischemia, can cause an extension of the infection into the cartilaginous end-plate, disc, and/or adjacent areas. Posterior extension of the infection may lead to epidural and subdural abscesses, or even to meningitis. Extension anteriorly or laterally may lead to paravertebral, retropharyngeal, mediastinal, subphrenic, or retroperitoneal abscesses. Also, spreading to adjacent vertebral bodies may occur quickly through the rich venous networks in the spine.

Contiguous focus osteomyelitis

In the past several years there has been a decline in hematogenous osteomyelitis, with a concurrent rise in contiguous disease (Espersen *et al.*, 1991). Although the term 'contiguous focus' implies that the infection stems from an adjacent soft tissue infection, chronic contiguous focus osteomyelitis can also begin as an acute infection, with the microorganisms being directly inoculated into the bone at the time of trauma (Healy & Freedman, 2006). Infection can also be spread by nosocomial contamination during preoperative or intraoperative procedures. Contiguous focus osteomyelitis is biphasic in terms of the ages it affects, with infection occurring in younger individuals as a result of trauma and related surgery and in older adults secondary to surgical procedures and decubitus ulcers.

Trauma contributes to osteomyelitis infections in several ways besides direct inoculation of bacteria into the body. Damage to tissue leads to a decrease in blood supply to the area, which can cause the formation of necrotic areas of inert tissue. Bacteria are then able to bind to this tissue and infection may ensue. Also, trauma has been shown to depress the immune and inflammatory responses to bacterial invasion. The degree of severity of tissue injury seems to be correlated with the risk of infection, and the presence of bacteria in a wound alone is not sufficient to cause osteomyelitis (Ziran, 2007).

Microbial species responsible for osteomyelitis

Hematogenous osteomyelitis is generally monomicrobial in nature, that is, a single bacterial species is isolated from the infected region. Polymicrobial hematogenous osteomyelitis is rare (Lew & Waldvogel, 2004). In infants, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* are the most frequently recovered bone isolates, while in the child, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Haemophilus influenzae* are the most common organisms isolated. After the age of four, the incidence of *H. influenzae* osteomyelitis decreases. However, the overall incidence of *H. influenzae* as a cause of osteomyelitis is decreasing because of the new *H. influenzae* vaccine now given to children (De Jonghe & Glaesener, 1995). In adults, *Staphylococcus aureus* is the most common organism isolated (Shirtliff *et al.*, 1999). Overall, some other pathogenic microorganisms associated with osteomyelitis include *Enterococcus* spp., *Streptococcus* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp., *Mycobacterium* spp., as well as anaerobic and mycoid species (specifically *Candida* spp.). Each of these pathogenic species, individually, represents a very small minority of infections. The immature or compromised immune status of the host is the primary cause of initial infection and development into a persistent and chronic osteomyelitis infection by these other species. In vertebral osteomyelitis, aerobic Gram-negative rods are sometimes found, with the urinary tract or intravenous drug use as the source of infection (Berendt & Byren, 2004). *Pseudomonas aeruginosa* and *Serratia marcescens* have a high incidence in intravenous drug users (Holzman & Bishko, 1971; Sapico, 1996). It should be stressed, however, that while these varied species have been known to cause the disease, *Staphylococcus aureus* produces the vast majority of osteomyelitis infections in all age groups.

Vertebral osteomyelitis secondary to a contiguous focus is usually a polymicrobial infection in which anaerobes are often isolated. Other sources of infection include the genitourinary tract, skin, and soft tissue, respiratory tract, infected IV site, endocarditis, dental infection, as well as unknown sources (Sapico & Montgomerie, 1979; Berendt & Byren, 2004; Gasbarrini *et al.*, 2005). Positive cultures are very important for diagnosis, because other conditions such as trauma and vertebral collapse may simulate infection. Multiple organisms are found in patients with diabetic foot osteomyelitis, including *Staphylococcus aureus*, coagulase-negative *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., Gram-negative bacilli, and anaerobes (Calhoun *et al.*, 1988; Berendt & Byren, 2004; Rao & Lipsky, 2007). Aerobic Gram-negative bacilli are usually a part of mixed infection (Calhoun *et al.*, 1988).

In contrast to hematogenous osteomyelitis, multiple pathogenic species are usually isolated from the infected

bone in cases of contiguous focus osteomyelitis. Again, staphylococci contribute to the majority of cases, with *Staphylococcus aureus* and coagulase-negative staphylococci accounting for 75% of the bacterial isolates (Mader *et al.*, 1996). However, Gram-negative bacilli and anaerobic organisms are also isolated. The infection usually manifests within 1 month after inoculation of the organisms from trauma, surgery, or a soft tissue infection. Patients usually present with low-grade fever, pain, and drainage.

Diagnosis and treatment of osteomyelitis

During acute infections, if the proper antibiotic is started early, the infection will usually clear after 2–4 weeks of treatment (Berendt & Byren, 2004). However, diagnosing these infections during this early, clearable state is difficult. Acute infections usually do not feature any radiographic changes until 1–1.5 weeks after inception of the disease. Magnetic resonance imaging (MRI) is effective in diagnosing acute infections in the absence of metal implants, but features a lag time after previous surgery or infection (Berendt & Byren, 2004).

In chronic osteomyelitis, there are usually large areas of devitalized cortical and cancellous bone within the wound. Because antibiotics do not penetrate well into devitalized bone (Healy & Freedman, 2006), the dead areas must be completely débrided, including devitalized scar tissue, marrow, and cortex. The soft tissue covering the area of bone trauma must heal; if this does not occur, the existing infection will persist and a new infection could form. Compromise of local soft tissue is a major reason for continued drainage. Diagnosis of chronic infection can often be made by radiography. Other techniques include radionuclide scans, though these lack specificity (Berendt & Byren, 2004).

The presence of general vascular insufficiency makes suitable therapy and management of chronic contiguous osteomyelitis complicated. Most of the patients fitting this description have diabetes mellitus (Calhoun *et al.*, 1988), and range from 35 to 70 years of age. Owing to the large increase in the diabetic population, osteomyelitis in the diabetic foot is now considered the most common bone infection (Berendt & Byren, 2004). The small bones of the feet, as well as the talus, calcaneus, distal fibula, and tibia, are commonly involved in this category of infection. Often, the infection is commenced by minor trauma to the feet, such as infected nail beds, cellulitis, or trophic skin ulceration. Neuropathy in these patients impairs the proper functioning of the foot as well as protective pain responses, leading to progression of soft tissue infections into the underlying bone (Berendt & Byren, 2004).

Osteomyelitis in patients with compromised vasculature can be difficult to diagnose. The patient may present with an ingrown toenail, a perforating foot ulcer, cellulitis, or a deep

space infection. Examination shows decreased dorsal pedis and posterior tibia pulses, poor capillary refill, and decreased sensation, but fever and systemic toxicity are often absent. Although arrest of the infection is desirable, a more achievable treatment goal is to contain the infection and preserve the functional integrity of the involved limb. Débridement and ablation are often essential. The intractable character of this type of infection often leads to recurrent bone infections, even after suitable therapy. Partial removal of the infected bone is almost always necessary.

Because staphylococci are the most common infectious agent in osteomyelitis, it is advisable to begin empirical therapy upon patient presentation with a regimen that includes an antistaphylococcal agent (Berendt & Byren, 2004). Once a proper diagnosis is made, the appropriate, more specialized antibiotic can be applied. Treatment usually lasts *c.* 4 weeks and is administered intravenously, but durations can vary and are shorter in children (Jaberi *et al.*, 2002). In adults, *Staphylococcus aureus* is generally treated with penicillin [for methicillin-sensitive *Staphylococcus aureus* (MSSA)] or nafcillin [for methicillin-resistant *Staphylococcus aureus* (MRSA)], with cefazolin, clindamycin, vancomycin, ciprofloxacin, or levofloxacin being given as alternatives (Lew & Waldvogel, 2004).

Biofilms

A biofilm is defined as a microbially derived sessile community, typified by cells that are attached to a substratum, interface, or to each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression, and protein production (Donlan & Costerton, 2002). Biofilm depth can vary, from a single cell layer to a thick community of cells surrounded by a thick polymeric milieu. Structural analyses have shown that these thick biofilms possess a complex architecture in which microcolonies can exist in distinct pillar or mushroom-shaped structures (Costerton *et al.*, 1995), through which an intricate channel network runs. These channels provide access to environmental nutrients even in the deepest areas of the biofilm. Though one study shows that biofilm formation is not necessary to cause persistent infections (Kristian *et al.*, 2004), biofilms are difficult to eradicate and thus deserve special attention.

By adopting this sessile mode of life, biofilm-embedded microorganisms benefit from a number of advantages over their planktonic counterparts. One advantage is the capability of the extracellular matrix to seize and concentrate a number of environmental nutrients, such as carbon, nitrogen, and phosphate (Beveridge *et al.*, 1997). Another benefit to growing as a biofilm is the facilitation of resistance to a number of removal tactics, such as elimination by antimicrobial and antifouling agents, shear stress, host phagocytic

clearance, and host oxygen radical and protease defenses. This innate resistance to antimicrobial factors is mediated through very low metabolic levels and radically downregulated rates of cell division of the deeply entrenched microorganisms. While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors may play a role. One such feature may be the capability of biofilms to act as a diffusion barrier to slow down the infiltration of some antimicrobial agents (Xu *et al.*, 2000). For example, reactive chlorine species (such as hypochlorite, chloramines, or chlorine dioxide) in a number of antimicrobial/antifouling agents may be deactivated in the surface layers of the biofilm before they are able to disseminate into the lower layers (De Beer *et al.*, 1994). In another study, alginate (a component of *P. aeruginosa* exopolysaccharide) was shown to be able to induce an α -helical conformation in antimicrobial peptides and likely entraps these peptides, preventing their diffusion into the biofilm (Chan *et al.*, 2004a).

The final benefit to the biofilm manner of growth is the potential for dispersion via detachment. Microcolonies may detach under the direction of mechanical fluid shear or through a genetically programmed response that mediates the detachment process (Boyd & Chakrabarty, 1994). Under the direction of fluid flow, this microcolony travels to other regions of the host or water system to attach and promote biofilm formation in previously uninfected areas. In addition, detachment and seeding of virgin surfaces may be accomplished by the migration of single, motile cells from the cores of attached microcolonies (Sauer *et al.*, 2002). Therefore, this advantage allows an enduring bacterial source population that is resilient against antimicrobial agents and the host immune response, while simultaneously enabling continuous shedding to encourage bacterial spread.

Staphylococcus biofilms

Staphylococcus aureus is a Gram-positive, ubiquitous bacterial species. *Staphylococcus aureus* is a normal commensal of the human nostrils; *c.* 20% of the population is consistently colonized with this bacterium, while 60% of the population are transient carriers (Kluytmans *et al.*, 1997). While colonization with *Staphylococcus aureus* does not usually lead to illness, if the mucosal or epithelial surfaces are breached, serious infection can ensue (Fitzpatrick *et al.*, 2005b). Owing to the escalating participation of *Staphylococcus aureus* in foreign body-related infections, the swift development and exhibition of multiple-antibiotic resistance, and their predilection to transform from an acute infection to one that is persistent, chronic, and recurrent, this pathogen continues to receive considerable attention.

Staphylococcus spp. can produce a multilayered biofilm embedded within a glycocalyx, or slime layer. The glycocalyx

develops on devitalized tissue and bone, or on medically implanted devices, to produce an infection (Akiyama *et al.*, 1993; Ziran, 2007). The presence of implants are a predisposing factor in the development of infection because they are coated in host proteins soon after implantation, and this host protein coating provides an excellent source of attachment for any bacteria introduced or remaining after débridement surgery (Herrmann *et al.*, 1988). Once attached, the bacteria can form the glycocalyx.

Early studies described the solid component of the glycocalyx as primarily composed of teichoic acids (80%) and staphylococcal and host proteins (Hussain *et al.*, 1993). Host-derived proteins, such as fibrin, may be derived from the conversion of fibrinogen by the staphylococcal coagulase–prothrombin complex (Akiyama *et al.*, 1997). In later studies, a specific polysaccharide antigen named polysaccharide intercellular antigen (PIA) was isolated. PIA is composed of β -1,6-linked *N*-acetylglucosamine residues (80–85%) and an anionic fraction with a lower content of non-*N*-acetylated *D*-glucosaminyl residues that contains phosphate and ester-linked succinate (15–20%) (Mack *et al.*, 1996). PIA is a polymer of *c.* 130 residues, but other sizes of this β -1,6-linked *N*-acetylglucosamine have been identified, termed PNAG-I (the immunogenic 460 kDa compound), II (100 kDa), and III (21 kDa) (Maira-Litran *et al.*, 2002).

PIA is produced *in vitro* from UDP-*N*-acetylglucosamine via products of the intercellular adhesion (*ica*) locus (Cramton *et al.*, 1999). The genes and products of the *ica* locus [*icaR* (regulatory) and *icaADBC* (biosynthetic) genes] have been demonstrated to be necessary for biofilm formation and virulence, and are up-regulated in response to anaerobic growth, such as the conditions seen in the biofilm environment (Cramton *et al.*, 2001). In the homologous *Staphylococcus epidermidis* locus, regulation of *ica* can occur via reversible inactivation by insertion sequence (IS256) phase variation in 25–33% of variants (Conlon *et al.*, 2002), and this has been observed in some *Staphylococcus aureus* strains as well (Kiem *et al.*, 2004). Additional levels of control are accomplished through IcaR-mediated transcriptional repression (relieved by ethanol stress) and the *sigB* operon product σ^B in *Staphylococcus epidermidis* (regulated by operon genes *rsbU* and *rsbV*). However, in *Staphylococcus aureus*, *sigB* does not seem to play such a role, as a *sigB* mutant is not defective in biofilm formation (Valle *et al.*, 2003). Jefferson *et al.* (2003) discovered that IcaR binds to a 42-bp region just upstream of *icaA* and hypothesize that its role is to sterically hinder the binding of σ^B , thus preventing activation of the *ica* locus. Environmental factors can also play a role in regulation of *ica*, including glucose, ethanol, osmolarity, temperature, oxygen levels, and antibiotics such as tetracycline (Fitzpatrick *et al.*, 2005b). Other factors necessary for the early stages of biofilm formation, including

clumping factor, fibronectin binding protein A (FnbpA), and coagulase, are all up-regulated by σ^B (Nicholas *et al.*, 1999; Nair *et al.*, 2003).

In more recent work, however, the role of PIA in biofilm formation has been questioned. In *Staphylococcus epidermidis*, the *ica* gene cluster is not present in all strains (Ziebuhr *et al.*, 1997). Moreover, up to 30% of *Staphylococcus epidermidis* biofilms are found to be PIA-negative (Rohde *et al.*, 2007), and another study that looked at prosthetic hip infection found that only one third of patients infected with *Staphylococcus epidermidis* carried an *ica*-positive isolate (Nilsdotter-Augustinsson *et al.*, 2007). While the *ica* cluster is commonly found in *Staphylococcus aureus* isolates, the correlation between PIA production and virulence is not cut and dried. For example, in a guinea pig model of biofilm infection, deletion of *ica* and, thus, lack of PIA production caused no decrease in virulence (Francois *et al.*, 2003), and deletion of *ica* in the clinical isolate UAMS-1 (University of Arkansas Medical System-1) did not lead to lesser biofilm formation either *in vitro* or in an *in vivo* mouse model of catheter infection (Beenken *et al.*, 2004). Also, several clinical isolates of MRSA (that were *ica*-positive) have been identified in which biofilm production is independent of *ica*, as increased transcription of the operon was not seen during glucose-mediated biofilm growth. Moreover, under NaCl-induced biofilm growth, though *ica* transcription was increased, levels of biofilm production were not similarly heightened (Fitzpatrick *et al.*, 2005a). Deletion of the *ica* locus in one of these isolates did not lead to a lessened ability to form a biofilm; however, the same deletion in a laboratory strain of *Staphylococcus aureus* did abrogate biofilm formation (Fitzpatrick *et al.*, 2005a). Other studies support this idea and show that, in 114 MRSA clinical isolates, PIA production did not correlate with biofilm production, and deletion of the *ica* locus in six of these isolates did not lead to lessened biofilm formation (O'Neill *et al.*, 2007). However, in MSSA, there was a correlation between PIA production and biofilm formation, and deletion of *ica* abolished biofilm formation (O'Neill *et al.*, 2007). Thus, it seems likely that the *ica* locus's contribution to biofilm development is strain- and environment-dependent, and that there are different mechanisms of biofilm development in MRSA vs. MSSA strains. In those *Staphylococcus epidermidis* and *Staphylococcus aureus* strains in which biofilm formation is not dependent on PIA expression, protein adhesin(s) seem to be the most important factors. For example, in *Staphylococcus epidermidis*, the accumulation-associated protein (Aap) is found at heightened levels in the proteinaceous biofilm (Hennig *et al.*, 2007; Rohde *et al.*, 2007). Clearly, more work must be done to elucidate the alternative mechanisms to biofilm formation in these species.

Another important component of the staphylococcal biofilm is extracellular DNA (eDNA). The discovery that

this substance is an important component of biofilms was recently made in *P. aeruginosa* (Whitchurch *et al.*, 2002; Steinberger & Holden, 2005; Allesen-Holm *et al.*, 2006). This is supported by the usage of DNase, concurrently with antibiotic therapy, in the treatment of cystic fibrosis patients (Gibson *et al.*, 2003), and the finding that DNase found on skin cells can lessen biofilm formation (Eckhart *et al.*, 2007). Rice *et al.* (2007) very recently showed that eDNA is important for biofilm formation and adherence in *Staphylococcus aureus*, and that this DNA release seems to be, at least in part, mediated through the *cidA* murein hydrolase. This gene has been shown to be a holin homologue involved in cell lysis, and it is thought that this gene allows *Staphylococcus aureus* biofilm cells to lyse and release DNA into the extracellular milieu. Other factors that may be involved in this process include autolysins such as Atl, or the induction of prophages that lead to lysis (Webb *et al.*, 2003). In fact, in *Staphylococcus epidermidis*, the autolysin AtlE was shown to be important in the release of chromosomal DNA and subsequent initial attachment during early biofilm formation, as an *atlE* mutant did not release DNA and was less able to form biofilms (Qin *et al.*, 2007).

In *Staphylococcus aureus*, several virulence factors are σ^B -regulated genes, including clumping factor, fibronectin binding protein A, and coagulase (Nicholas *et al.*, 1999), all of which are positively controlled, as well as α - and β -hemolysin, enterotoxin B, SplA (a serine protease), cysteine protease (SplB), the metalloprotease Aur, staphopain, and leukotoxin D, all of which are negatively regulated (Kullik & Giachino, 1997). Thus, the genes needed for attachment and biofilm formation are up-regulated by σ^B . This is exemplified in the case of the laboratory strain RN6390, which lacks σ^B and is deficient in biofilm formation (Cassat *et al.*, 2006). Also, it has been shown that IcaR is a strong negative regulator of the *ica* locus, as deletion of *icaR* augmented PIA production by nearly 10-fold, and increased transcription of the *ica* locus *c.* 100-fold (Jefferson *et al.*, 2004). Another gene, *rbf*, has recently been identified by transposon mutagenesis. The Rbf protein was shown to be important in multicellular aggregation during biofilm formation, and also in the induction of biofilm formation by NaCl and glucose, but had no effect on *ica* transcription (Lim *et al.*, 2004). Spx is a regulator that was recently shown to have a negative impact on biofilm formation, seemingly by modulating IcaR (Pamp *et al.*, 2006).

Several other regulators also play a role in biofilm formation. The Agr quorum sensing (QS) system, a central regulator of virulence, has been shown to down-regulate genes of cell wall-associated adherence factors (Chan *et al.*, 2004b). This would lead to lesser adherence and thus, indirectly, decreased initial biofilm formation. As well, the Agr system has been shown to up-regulate the expression of detergent-like peptides that seem to increase biofilm detach-

ment (Kong *et al.*, 2006), and mutation of the system leads to increased biofilm growth (Vuong *et al.*, 2000, 2003; Otto, 2004). Another regulatory system, Target of RAP (TRAP), has been implicated in biofilm formation, with its secreted factor [RNAIII activating peptide (RAP)] increasing biofilm growth and its antagonistic peptide [RNAIII inhibitory peptide (RIP)] inhibiting it (Balaban *et al.*, 2000; Korem *et al.*, 2005). TRAP is believed to work through the Agr system, activating RNAIII production (the effector of the Agr response) when RAP levels are high (Balaban & Novick, 1995). However, controversy surrounds this system. Other authors have shown RIP to be inactive, and the concentrations needed to see the effects of RIP are high (Novick, 2003). Also, the mechanism of secretion of RAP (as it lacks a signal sequence) is questioned, as is the mechanism of its interaction with TRAP, which is cytoplasmic (Harraghy *et al.*, 2007). Furthermore, two recent papers attest that they found no role for the TRAP system in *agr* expression, biofilm formation, or virulence (Shaw *et al.*, 2007; Tsang *et al.*, 2007).

The Staphylococcal accessory regulator (SarA) is also important as a *sarA* mutant of both *Staphylococcus aureus* and *Staphylococcus epidermidis* are defective in biofilm formation (Valle *et al.*, 2003; Conlon *et al.*, 2004). A two-component regulatory gene locus encoded by *arlRS*, a member of the OmpR-PhoB family of response regulators, is regulated by the *agr* and *sarA* loci (Fournier *et al.*, 2001). When up-regulated, the product of *arlS* prevents biofilm formation and may mediate attachment to polymer surfaces by affecting peptidoglycan hydrolase activity.

In addition to regulatory systems, a number of other genes and their products have proved vital in the development of staphylococcal biofilms. There is evidence that attachment of bacterial cells to a polymer surface, a prerequisite for biofilm formation, may be promoted by an autolysin of *Staphylococcus epidermidis* (Heilmann *et al.*, 1997); the homologue in *Staphylococcus aureus* (*atl*) may also function in this manner, perhaps through DNA release as discussed above. Teichoic acid structure is also crucial in the development of biofilms. Specifically, the addition of D-alanine esters to teichoic acids via *dltA* may be an important factor in imparting the proper charge balance on the Gram-positive cell surface, enabling initial attachment and subsequent biofilm formation. Another *Staphylococcus aureus* gene, biofilm-associated protein (Bap), which was required for biofilm formation on inert surfaces, was discovered via transposon mutagenesis. However, because only 5% of bovine mastitis isolates and none of the 75 clinical isolates tested possessed the coding sequence for Bap, the *in vivo* significance of this protein may be doubtful. In another study, the differential gene expression in planktonic (shaken) vs. biofilm (static) *Staphylococcus aureus* cultures was evaluated, and five genes whose expression were increased in biofilms were identified (Becker *et al.*, 2001). These included

the gene encoding threonyl-tRNA synthetase (up-regulated by amino acid starvation), three oxygen starvation response genes, and a stress response gene that encodes the ATPase ClpC. Another study using microarrays to study differential gene expression between these conditions found 48 genes that were enhanced at least twofold in the biofilm compared with planktonic conditions (Beenken *et al.*, 2004). Overall, it seems that genes involved in cell wall synthesis and pH balance are important in biofilms, whereas toxin and protease production is at higher levels during planktonic growth (Beenken *et al.*, 2004; Resch *et al.*, 2006).

Treatment of *Staphylococcus aureus* biofilm infections

Biofilms are recalcitrant to clearance by antimicrobials. One of the biggest reasons for this is that the antibiotics currently in use are chosen because they are able to kill planktonic cells (Fitzpatrick *et al.*, 2005b). As mentioned above, the intrinsic ability of biofilm cells to avoid this clearance can be due to several factors, including altered metabolic states within the biofilm and lessened diffusion of the antibiotic into the biofilm. Moreover, the increased incidences of antibiotic-resistant clinical isolates make treating these already difficult to eradicate infections even more complicated. It is quite obvious that new means of treating these infections must be developed. Recent approaches include using anti-PIA antibodies to prevent attachment or the formation of PIA in general (McKenney *et al.*, 1999; Mair-Litran *et al.*, 2005; Kelly-Quintos *et al.*, 2006). Another option is to coat medical devices before implantation. An enzyme made by *Actinobacillus actinomycetemcomitans* termed 'dispersin B' or DspB has been found to cleave PIA (Itoh *et al.*, 2005). However, as mentioned, many clinical isolates do not express the PIA polysaccharide. Finally, Balaban *et al.* (2003a, b, 2005, 2007) have discussed the idea of using the RIP heptapeptide, which is proposed to inhibit RNAPIII-activated virulence factors, in the treatment of biofilm-associated infections (Giacometti *et al.*, 2003). They attest that inhibition of QS in *Staphylococcus aureus* will lead to a lessened biofilm that is more treatable by antibiotics. However, other authors have shown that the *agr* system works to increase levels of biofilm detachment and that disruption of the QS system leads to increased biofilm formation (Vuong *et al.*, 2000, 2003, 2004; Otto, 2004; Kong *et al.*, 2006). Therefore, whether or not RIP will truly be an effective antibiofilm agent is questionable. Thus, there are very limited upcoming options for novel therapies that could be effective against all clinical isolates of *Staphylococcus aureus*. Surgical interventions remain the most effective means of treatment of biofilm-associated infections. In osteomyelitis infections, this means débridement of all infected bone.

Conclusion

Osteomyelitis, or infection of the bone, is a prevalent issue facing clinicians. These infections commonly occur in the long bones of the legs, but may also be found in nearly every bone of the body. Contiguous focus osteomyelitis is commonly seen in patients with diabetes mellitus, and often involves multi-species infections in the feet, while haematogenous osteomyelitis often occurs in the long bones, commonly in children. *Staphylococcus aureus* is, by far, the most commonly isolated organism in monomicrobial osteomyelitis cases. The prevalence of *Staphylococcus aureus* in these infections is likely due to its wide array of virulence factors, including secreted products for host damage and immune avoidance, as well as adherence factors. Probably the most important factor in the development of the chronic form of this disease, however, is its ability to form a biofilm. Biofilm formation allows for immune evasion as well as resistance of antimicrobial agents, so that the only way to successfully treat such infections is to remove the diseased tissue. Because this species relies on biofilm formation for persistent infection, therapeutics targeting this phenomenon could prove to be promising candidates in the treatment of osteomyelitis.

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