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Abstract

The demand for joint replacement surgery is continuously increasing with rising costs for hospitals and healthcare systems. Staphylococci are the most prevalent etiological agents of orthopedic infections. After an initial adhesin-mediated implant colonization, *Staphylococcus aureus* and *Staphylococcus epidermidis* produce biofilm. Biofilm formation proceeds as a four-step process: (1) initial attachment of bacterial cells; (2) cell aggregation and accumulation in multiple cell layers; (3) biofilm maturation and (4) detachment of cells from the biofilm into a planktonic state to initiate a new cycle of biofilm formation elsewhere. The encasing of bacteria in biofilms gives rise to insuperable difficulties not only in the treatment of the infection, but also in assessing the state and the nature of the infection using traditional cultural methods. Therefore, DNA-based molecular methods have been developed to provide rapid identification of all microbial pathogens. To combat biofilm-centered implant infections, new strategies are being developed, among which anti-infective or infective-resistant materials are at the forefront. Infection-resistant materials can be based on different approaches: (i) modifying the biomaterial surface to give anti-adhesive properties, (ii) doping the material with antimicrobial substances, (iii) combining anti-adhesive and antimicrobial effects in the same coating, (iv) designing materials able to oppose biofilm formation and support bone repair.

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2.1 Projections of Increase in Arthroplasty Numbers

The demand for joint replacement surgery is continuously increasing resulting in higher and higher costs for patients, hospitals and health-care systems.

The expansion of arthroplasty surgery and the need to follow its outcome and control booming costs have promoted the institution of many registries at the regional, national and international levels.

Historical trends in total hip replacement drawn from the Swedish Hip Arthroplasty Register over the 45 year period, from 1968 to 2013, can interestingly provide some of the most accurate data in the world and can be used to provide projections of the future demand for arthroplasties (www.shpr.se). Similarly the National Joint Registry (NJR) of England, Wales and Northern Ireland, which has now published the 9th Annual Report 2012 (www.njrcentre.org.uk) provides data on all hip, knee, ankle, elbow and shoulder joint replacements across the National Health System and independent healthcare sector, thus providing trending data and providing for future projections.

Accurate statistical analyses of the data in the Swedish Hip Arthroplasty Register and projections to 2030 of total hip replacement in Sweden have been recently published (Nemes et al. 2014). The authors of this report have utilized two types of regression analysis, in order to forecast the incidence of THR operations per 10^5 Swedish residents aged 40 years or older in the decades after 2012 and to estimate the maximum incidence per 10^5 Swedish residents aged 40 years. If a Poisson regression analysis is used, which estimates the expected number of THRs per year and assumes a continuous growth, the incidence can reach, at least theoretically, 10^5 of 10^5 persons and, if the results are used for projections, unreasonably high numbers are reached. Secondly, a regression framework that assumes the existence of an upper threshold, i.e. an asymptote that depicts the forecasted maximum incidence, was adapted. Poisson regression should forecast that the incidence of

THRs would increase exponentially in the next years, with a predicted incidence of 784 total hip replacements per 10^5 Swedish residents in 2030 and 1,133 in 2040. With an expected Swedish population in 2030 of 10,660,344 persons, about 83,600 total hip replacements can be forecasted, in respect to the 16,021 THRs performed in 2013, with a fivefold increase of incidence. The projections based on asymptotic modeling, gives instead a THRs expected number of 20,152, only 1.25-fold increase of incidence in respect to 2013.

Sometimes the registries announce good news. The National Joint Registry for England and Wales, 9th annual report 2012, reports that, among the 409,096 patients operated on for primary hip replacement during 8 years, 1,743 patients died within 90 days of surgery, with a substantial decrease in mortality, from 0.56 % in 2003 to 0.29 % in 2011, even after adjustment for age, sex, and comorbidity (Hunt et al. 2013). Based on the registered clinical data, the authors ascribe the decreased mortality to several clinical factors: posterior surgical approach, mechanical thromboprophylaxis, chemical thromboprophylaxis with heparin (with or without aspirin), spinal versus general anesthetic, while the type of prosthesis was unrelated to mortality. The authors do not report data about infections, except the observation that only 19 patients had AIDS/HIV infection and none died.

A symposium report developed from the 9th Report of the National Joint Registry for England and Wales, found that a total of 8,639 hip revisions were reported in 2011, among which 87 % were single-stage revisions, 12 % were two-stage revision and less than 1 % removal of the prosthesis. The infection was the indication for revision in 12 % of cases but in 11 % of cases an adverse soft tissue reaction was recorded, confirming that the failure of metal-on-metal replacement is a rising cause of revision, while the lowest rates of revision were associated with cemented metal or ceramic on polyethylene combinations. With regard to knee replacement, a total of 5,135 revision operations were performed in 2011, the main indications being aseptic loosening (35 %) and infection (23 %).

2.2 Infection Burden for Arthroplasties

As Sculco wrote more than 30 years ago “Infection in total joint replacement is a devastating and life-threatening complication for the patient. It can also be an economic disaster for hospitals that treat large numbers of these patients” (Sculco 1993).

The infection burden, as a proportion of the total number of primary and revision total hip arthroplasties (THA), has been reported to have increased from 0.66 % in 1990 to 2.18 % in 2009 (Kurtz et al. 2008, 2012).

The incidence of post-operative infections has also been calculated for total knee arthroplasties, which are often managed with two-stage revisions, and ranges from 0.7 to 2.4 % (Kurtz et al. 2012; Whitehouse et al. 2002).

The rates of periprosthetic joint infection (PJI) after primary procedures range from 1 to 9 %, depending on the types of arthroplasty, being less than 1 % in hip and shoulder prostheses, about 2 % in knee prosthesis, and about 9 % in elbow prosthesis. The rates of PJI reach the significantly higher level of about 40 % after revision procedures (Corvec et al. 2012).

The current cumulative annual cost of revisions for periprosthetic joint infections has been estimated to exceed \$566 million in the United States and is expected to exceed \$1.6 billion by the year 2020 (Kurtz et al. 2012). According to the projections that the number of total knee arthroplasty procedures are yearly increasing, the projected cost of managing these surgical site infections is expected to become a huge problem for patients, physicians, and healthcare institutions (Kapadia et al. 2014).

The authors of population-based studies hypothesize stability in the incidence of infections over the nearly 40-year time span. This hypothesized stability is tentatively ascribed to the increased patient morbidity and risk factors for infection counterbalanced by improvements in aseptic techniques, surgical skills, and infection prevention and control measures (Tsaras et al. 2012).

Whether the infection incidence per person-joint-years is increasing or not, the total number of periprosthetic infections will certainly increase in the ensuing decades, owing to the increasing number of primary implants being performed and the cumulative number of arthroplasties that remain in place (Tande and Patel 2014) for longer periods of time.

2.3 Current Classification of Prosthetic Infections

The American Association of Orthopedic Surgeons divides prosthetic infections into the following four types (Leone and Hanssen 2006):

- Type 1 (positive intraoperative culture): two intraoperative cultures turning out positive;
- Type 2 (early postoperative infection): infection occurring within the first month after surgery;
- Type 3 (acute hematogenous infection): hematogenous seeding of site of previously well-functioning prosthesis;
- Type 4 (late chronic infection): chronic indolent clinical course; infection present for more than 1 month.

A slightly different classification of prosthetic joints infections has been proposed by Zimmerli et al. (2004):

- Early (those that develop less than 3 months after surgery);
- Delayed (3–24 months after surgery), or;
- Late (more than 24 months after surgery).

For the Total Hip Arthroprosthesis three main types of postoperative surgical site infection are considered, with a classification almost superimposable to the Zimmerly-Trampuz’s classification: (1) acute postoperative (early onset), appearing within 3 months postoperatively; (2) delayed deep, appearing 3–12 months postoperatively; and (3) late hematogenous, appearing more than 1 year postoperatively (Fitzgerald 1995; Lindeque et al. 2014).

The etiopathogenic significance implied by these classifications resides in that the early and delayed infections are acquired by contamination at the time of surgery, while the delayed being caused by less virulent microorganisms so that

the onset of infection occurs not before the first 3 months. Late infections, occurring between 12 and 24 months after surgery, are often due to a haematogenous infection or, less frequently, to an indolent infection acquired at surgery time.

2.4 Epidemiology of Periprostheses Infections in Our Experience

Approximately a decade ago, we studied a collection of 1,027 clinical isolates from 699 orthopedic patients with surgical infections (Arciola et al. 2005). We compared the etiology of infections associated with medical devices (MDs) to those developed in the absence of implant materials (no MDs). MDs included infections associated to knee and hip prostheses, external and internal fractured bone fixation systems, materials for tendon and ligament reconstructions and other orthopedic implant materials.

The isolates from infections associated with medical devices accounted for over 70 % of all the bacteria consecutively isolated from orthopedic infections of patients referred to the specialized hospital Rizzoli Orthopaedic Institute. Among these microorganisms 775 (75.5 %) were identified as belonging to the *Staphylococcus* genus, 82 (8 %) to the *Enterobacteriaceae* family, 75 (7.3 %) to the *Pseudomonas* genus, 54 (5.3 %) to the *Enterococcus* genus and 20 (1.9 %) to the *Streptococcus* genus.

Staphylococcal species were found to be the most prevalent etiological agents of orthopedic infections, representing 75.5 % of all strains, ranging from 68.3 % in infections without MDs to 78.1 % of the isolates with MDs. Among the species belonging to the *Staphylococcus* genus, *S. aureus* generally exhibited the highest prevalence (35.5 % overall prevalence, 33.8 % in MDs and 40.3 % in the no MD group). The overall prevalence of *S. epidermidis* was 27.5 %, ranging from 16.5 % in infections without MDs to 31.5 % of the isolates with MDs, as in infections associated with fracture fixation devices and in pelvis surgery. No single bacterial species, except for *S. aureus* and *S. epidermidis*,

exceeded a frequency of 7 %, giving emphasis to the critical importance of these two species in the epidemiology of orthopedic infections. However, it should be noted that cultural methods often miss anaerobes, fastidious pathogens, and organisms with long interval doubling times (Costerton et al. 2011).

We have analyzed an up-dated collection of isolates recovered from 242 orthopedic patients covering the period between 2007 and 2011. The results of the new survey confirmed those of the previous epidemiological investigation. Again, the prevalence of staphylococci in the entire collection was approximately 75 %, slightly higher in the case of MDs (82.3 %) and lower for no MDs (65.4 %). *S. aureus* again represented approximately 35 % of all the isolates and *S. epidermidis* 29.9 % of all the isolates. When these data were analyzed for the presence of MDs, unlike the previous collection, a prevalence of *S. epidermidis* (39.0 %) over that of *S. aureus* (31.7 %) was found in infections associated with MDs (Montanaro et al. 2011a).

In a very recent look at epidemiologic data on orthopedic infections treated between February 2011 and May 2014 at the Rizzoli Orthopaedic Institute, the *Staphylococcus* genus is always the leading etiologic agent, but the slight prevalence of *S. epidermidis* over *S. aureus* does not seem to be confirmed. Figure 2.1 shows up-to-date findings analyzed as a function of the absence or presence of MD and the type of surgery. Infections without MD represent 31.1 % and polymicrobial infections 9.4 %.

2.5 The Steps of Infection: From First Adhesion on Implant Materials to Biofilm Production

Surface adhesion of bacteria to implant surfaces is the initial step in the pathogenesis of implant-related biofilm infections, initiating the colonization of biomaterial surfaces. During the first step, the initial interactions between bacteria and a biomaterial are nonspecific in nature and driven by different forces, as hydrophobic, electrostatic

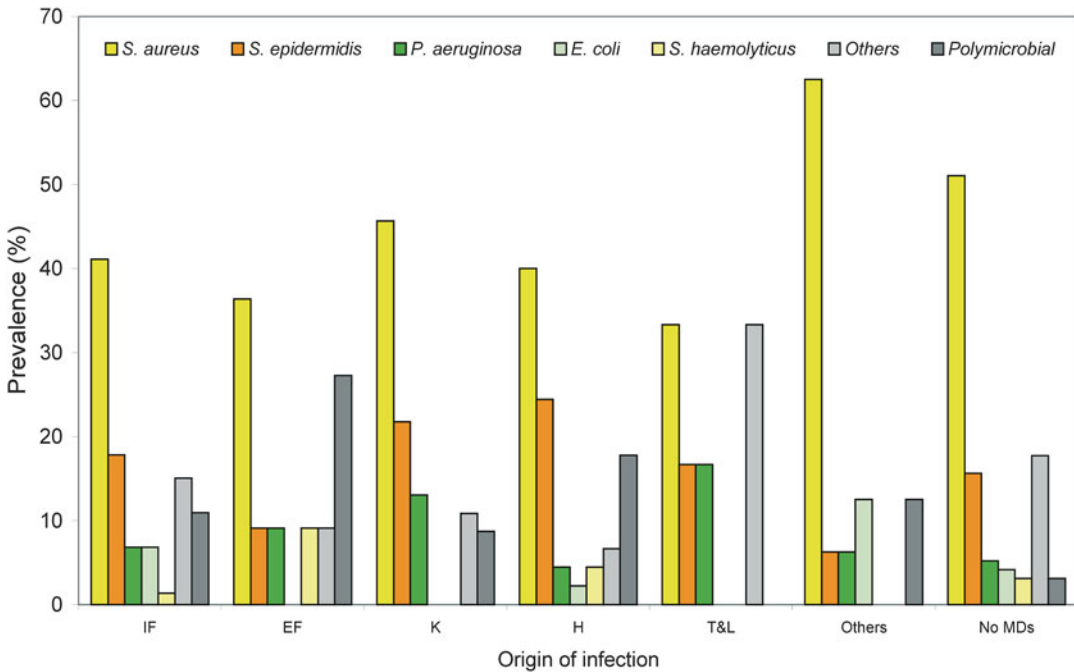


Fig. 2.1 Prevalence of the most frequent pathogens as a function of the origin of the orthopedic infection in a collection of 338 clinical isolates obtained from 309 patients in the period February 2011–May 2014 (29 polymicrobial infections) (*IF* Internal fixation, *EF* External fixation, *K*

Knee, *H* Hip, *T&L* tendons and ligaments, *Others* other medical devices, *No MDs* no explicitly reported presence of medical devices at the site of infection (Arciola CR, Campoccia D, Cangini I, Montanaro L, unpublished results))

and Van der Waals forces. In this phase, bacteria are therefore passively adsorbed onto the material surfaces. In addition to this passive adhesion, specific proteins have been identified that mediate the binding to the abiotic surfaces, the autolysins, first described by Heilmann et al. (1997). Autolysins possess a double function: enzymatic (being peptidoglycan hydrolases) and adhesive. In *S. epidermidis*, the major autolysin/adhesin is AtIE, a 148 kDa protein, which mediates attachment to polystyrene. In *S. aureus*, the autolysin/adhesin is AtIA, a 137 kDa protein, highly homologous to AtIE (Foster 1995). Both AtIA and AtIE, in particular their glycine-tryptophane dipeptide repeats, are involved not only in surface association and biofilm production but also in a novel mechanism of staphylococcal internalization by host cells (Hirschhausen et al. 2010).

Passive bacterial adsorption spontaneously occurs on material surfaces, but active stable anchorage of the bacterial cells is established

by adhesins, which bind to host proteins adsorbed on the implant surface following exposure to physiologic fluids. Therefore, in the early phases of infection, adhesins play a primary role, acting even as invasins and, furthermore, intervene in the process of bacterial internalization into host cells.

S. aureus harbors approximately 50 accessory genes, encoding for factors either secreted or expressed on the bacterial surface, all having a function in pathogenesis (Sittka and Vogel 2008). Among them, adhesins are an important group of virulence factors responsible for interactions between microbial cells and host cells and extracellular matrix (ECM).

S. aureus adhesins comprise the cell wall-anchored microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Patti et al. 1994a; Speziale et al. 2009), as well as the secretable expanded repertoire adhesive molecules (SERAMs), ionically rather than covalently

associated to the bacterial cell wall (Chavakis et al. 2005). MSCRAMMs are receptorial proteins anchored to the bacterial cell wall through a typical cell wall signal LPXTG motif. The *S. aureus* enzyme sortase A, located on the extracellular side of the membrane, cleaves the LPXTG anchor motif, covalently anchoring the adhesin to the cell wall peptidoglycan. Bound to the peptidoglycan and exposed on the bacterial surfaces, MSCRAMMs recognize specific host extracellular matrix (ECM) proteins. Through the interaction with ECM proteins, certain MSCRAMMs, acting as invasins, can facilitate the process of internalization into host cells. Other MSCRAMMs mediate bacterial cell accumulation, contributing to biofilm formation by means of accretion as opposed to the classical formation via elaboration of exopolysaccharides typical of most *S. aureus* strains and associated with the expression of an *ica* locus encoding for the polysaccharide intercellular adhesin (PIA).

2.6 Prevalence of Adhesin Genes in Collections of Clinical Isolates from Periprosthetic Infections

S. aureus MSCRAMMs play an important role in various processes of infection pathogenesis such as tropism, invasion, intracellular penetration; and, in the peri-implant tissues, bacterial adhesion on biomaterials coated by host extracellular matrix (ECM) proteins. Many adhesins involved in adhesion on indwelling devices appear multifunctional, as fibronectin-binding proteins A and B (FnBPA and FnBPB) and clumping factors A and B (ClfA and ClfB) (Greene et al. 1995; Herrmann et al. 1993), which bind more than one specific ligand. FnBPA in addition to fibronectin can bind fibrinogen and elastin, and ClfA binds fibrin besides the fibrinogen γ -chain. Moreover, binding of fibronectin by FnBPs was found to be crucial in the invasion of eukaryotic cells, where the ECM protein serves as a bridging molecule between the adhesin and the integrin $\alpha_5\beta_1$ (Sinha et al. 1999; Hauck and Ohlsen 2006), enabling the internalization of the bacteria within the cells.

The FnBP-fibronectin internalization mechanism into osteoblast cells is thought to trigger apoptosis, osteolysis and, ultimately, destructive osteomyelitis (Arciola et al. 2012a; Montanaro et al. 2011b).

An interesting subgroup of MSCRAMMs characterized by the Serine-Aspartate repeat (Sdr) proteins, among which SdrE, and the bone sialoprotein-binding protein (Bbp). Bone sialoprotein, the binding target of Bbp, is an ECM highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues (Ganss et al. 1999), where it represents 10 % of the non-collagenous proteins of the matrix, being mostly synthesized in osseous tissue. Bone sialoprotein-binding capacity, together with collagen-binding capacity, was found in all staphylococci associated with septic arthritis (Patti et al. 1994b; Ryde'n et al. 1997), thus suggesting that Bbp and Cna could represent important virulence factors.

In our collection of 200 *S. aureus* isolates from orthopedic implant-associated infections, categorized by genotyping by a RiboPrinter® and dendrogram analysis, an epidemic cluster has been identified. In this predominant ribogroup, consisting of 27 isolates, the *bbp* gene encoding bone sialoprotein-binding protein appeared to be an important virulence trait, found in 93 % of the isolates. The *bbp* gene was instead found in just 10 % of the remaining isolates of the collection. In this epidemic cluster, co-presence of *bbp* with the *cna* gene, encoding collagen adhesion, was a pattern consistently observed (Campoccia et al. 2009).

The same collection of 200 *S. aureus* isolates from orthopedic implant infections was also typed for their *agr* groups, and screened for the presence of adhesin and leukotoxin genes. Interestingly, specific virulence gene patterns emerged in association with *agr* groups. The *agr* groups I and II, were associated with the presence of *sdrE*, *fib* (*agr* II more than *agr* I), *fnbB* (*agr* I more than *agr* II), and *lukE/lukD* (*agr* II more than *agr* I). The third most frequent *agr* group, *agr* III, differed clearly from *agr* I and II, exhibiting high prevalence of *bbp*, generally not harbored by *agr* I and II, and copresence of *bbp* with *cna* (Montanaro et al. 2010). These studies

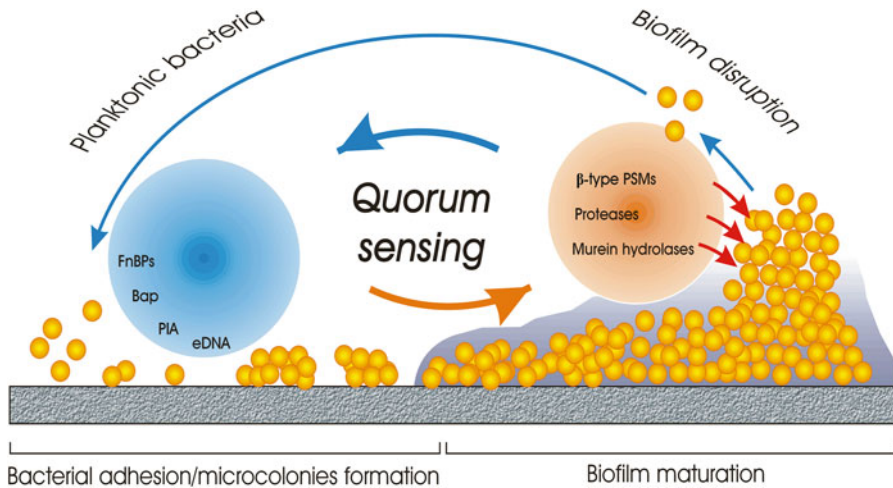


Fig. 2.2 Simplified view of the four-step process of biofilm formation in *Staphylococcus aureus*. Planktonic cells adhere and anchor to the material substrate through adhesins such as the fibronectin-binding proteins (FnBPs), also implicated in the following steps of biofilm formation. Factors playing a fundamental role in biofilm formation include the biofilm-associated protein (Bap), the intercellular polysaccharide adhesin (PIA) and a series of other

polymeric extracellular substances among which extracellular-DNA (eDNA). Once a mature biofilm has formed, under the fine control of the *Quorum sensing* system, enzymes such as proteases and murein hydrolases, and phenol-soluble modulins (β -type PSMs) with surfactant-like properties determine the disruption and detachment of the biofilm leading to the release of bacteria in the planktonic phase

indicate that specific adhesins may synergistically act in the onset of implant-related infections and that anti-adhesin strategies should be usefully targeted to adhesins conjointly present.

2.7 Biofilm: Role of Biofilm in Implant Infections

After the initial adhesin-mediated implant colonization, *S. aureus* and *S. epidermidis* produce biofilm. Biofilm is a structured consortium of bacteria, which encase themselves in an extracellular matrix and firmly stick them to the implant surface (Fig. 2.2).

Biofilm formation is classically viewed as a four-step process: (1) initial attachment of bacterial cells; (2) cell aggregation and accumulation in multiple cell layers; (3) matrix elaboration and biofilm maturation and (4) detachment of cells or rafts from the biofilm into a planktonic state or flock to initiate a new cycle of biofilm formation elsewhere (Costerton et al. 2005; Mack et al. 2004).

During the second step, the biofilm is progressively established on the colonized surface. Then, in the subsequent step, the maturation of biofilm takes place and characteristic structural features of the biofilm, specific for the bacterial species, are developed. During the final stage, the bacteria previously encased and protected in the biofilm structure return to their initial planktonic form of life, ready for a new invasive phase. Bacterial detachment and dispersion therefore characterize this final step of the bacterial life cycle (Arciola et al. 2012b).

2.8 Biofilm Structural Components: The Extracellular Polymeric Substance

Composition, structure, formation and regulation of the *Staphylococcus* biofilms have been illustrated and discussed by Arciola et al. (2012b) and by Speziale et al. (2008) in dedicated reviews to which the readers could be refer for an in-depth treatise.

In staphylococcal orthopedic infections, the extracellular polymeric substance of the biofilm is composed of polysaccharides, proteins, and extracellular DNA.

2.8.1 PIA and *ica* Locus

The principal polysaccharide of the staphylococcal biofilm matrix is a linear homoglycan composed of at least 130 residues of β -1,6-linked *N*-acetylglucosamine, partially deacetylated (15–20 % of the residues) and therefore positively charged. This polysaccharide was initially discovered and characterized in *S. epidermidis* (Mack et al. 1996) where its biosynthesis is encoded by the intercellular adhesion (*icaADBC*) locus (Heilmann et al. 1996). For a long time the *ica* locus was considered a virulence determinant peculiar to *S. epidermidis* strains responsible for catheter- or indwelling device-related infections. Later the presence of the *ica* locus was documented in the *S. aureus* species (Cramton et al. 1999), and recognized also in clinical isolates of *S. aureus* from catheter-associated infections (Arciola et al. 2001).

The product of the *icaA* gene is a transmembrane protein with a *N*-acetylglucosaminyltransferase activity that synthesizes short PIA oligomers from UDP-*N*-acetylglucosamine as substrate. The product of the *icaD* gene is required for the optimal efficiency of IcaA. The product of *icaC* is involved in externalization of the nascent polysaccharide. The product of *icaB* is an *N*-deacetylase, responsible for the partial deacetylation of the *N*-acetylglucosamine polymer.

2.8.2 *ica*-Independent Biofilm Production

Besides the demonstration of the important role of the *icaADBC* operon and of the PIA components in the biofilm extracellular polymeric substance, new evidence highlights the existence of *ica*-independent mechanisms involved in biofilm formation both in *S. aureus* and in coagulase-negative staphylococci, in particular *S. epidermidis* and *S. lugdunensis* (O’Gara 2007).

This alternative mechanism of biofilm synthesis relies on the ability of *S. aureus* to express a variety of adhesion proteins that favor the attachment of bacterial cells to many different surfaces. These proteins, which are anchored to the cell-wall of *S. aureus*, maintain a cell-to-cell interaction inside the biofilm. Among the adhesive proteins that are implicated in biofilm formation, an important role is played by a biofilm-associated protein termed Bap, which was demonstrated to be essential both for both initial adherence and for intercellular accumulation during biofilm development of *S. aureus* strains isolated from bovine chronic mastitis infections (Cucarella et al. 2001).

The *bap* gene is present in other *Staphylococcus* species, including *S. epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus simulans*, and *Staphylococcus hyicus* (Tormo et al. 2005).

While the *S. aureus bap* gene has been detected only in strains isolated from bovine mastitis and never in strains isolated from human infections, in coagulase negative staphylococci (CoNS) the presence of the *bap* gene has been found in clinical isolates from human nosocomial infections in Brazilian hospitals (Potter et al. 2009). Thus, the role of Bap in human infections, at present, seems to be limited to CoNS species and its presence in *S. aureus* strains isolated from human infections has not been confirmed.

With regard to orthopedic infections, Rohde et al. have investigated the presence and expression of biofilm-associated genes in clinical isolates of *S. aureus* and *S. epidermidis* from total hip and total knee infected arthroplasties. All *S. aureus* strains and nearly 70 % of *S. epidermidis* strains produced biofilm. Among the *S. epidermidis* biofilm-producing strains, 27 % were PIA-independent and at least in part involved the expression of the accumulation associated protein (Aap) (Rohde et al. 2007).

In *S. aureus* other surface proteins are involved in the formation of biofilm. Among these SasG has been shown to promote formation of biofilm. This protein exerts its action during the biofilm accumulation phase when, in the presence of physiological concentrations of Zn^{2+} , it supports cell-to-cell interactions (Geoghegan et al. 2010).

Moreover, the fibronectin-binding proteins (FnBPs) were demonstrated to be part of the proteinaceous component of biofilm formed in the presence of glucose, while a PIA/PNAG-dependent biofilm was shown to be produced under osmotic stress conditions (Vergara-Irigaray et al. 2009; Houston et al. 2011). There is therefore evidence that *S. aureus* can modulate its metabolism switching from the production of a proteinaceous to an exopolysaccharidic biofilm matrix, as an adaptation to the external conditions.

2.8.3 Extracellular DNA in Biofilm

Another biofilm matrix component, recently attracting attention, is the extracellular DNA (eDNA), which has been shown to be important for biofilm structural stability. Starting from the observations of Arciola et al. on strong biofilm production by epidemic clones of *Enterococcus faecalis* (Arciola et al. 2008), Thomas et al. have described the relationship between DNA release, role of proteases and biofilm production in *E. faecalis* (Thomas et al. 2008).

After having given evidence that the mechanisms underlying eDNA production is autolysis, they advanced the concept of two modes of autolysis: an altruistic suicide and a fratricide killing of different sub-populations of bacterial cells. In *S. aureus* altruistic suicide predominates, in which *altruist* cells commit suicide by programmed cell death (a process similar to apoptosis in eukaryotic cells), for the common sake of the larger community with salvage of *survivor* cells. In *E. faecalis*, *Bacillus subtilis* and *Streptococcus pneumoniae* the fratricide mechanism prevails: *attacker* cells release killing factors (a process similar to necrosis in eukaryotic cells) that destroy *target* cells. The *attackers* themselves are protected from self-destruction by specific immunity proteins they express (Thomas and Hancock 2009).

The mechanisms of eDNA production have been thoroughly investigated in *Pseudomonas aeruginosa*, in which eDNA originates by lysis of a bacterial subpopulation. Lysis is controlled by *quo-*

rum sensing systems, based on acyl homoserine lactone (AHL) and on *Pseudomonas* quinolone signaling (PQS) (Allesen-Holm et al. 2006). In *S. epidermidis*, eDNA is a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development. In this case too, eDNA originates from lysis of a small subpopulation of the *S. epidermidis* bacteria. DNA release from *S. epidermidis* appears to be mainly mediated by the autolysin protein AtlE, since inactivation of *atlE* drastically reduced DNA release (Qin et al. 2007).

The presence of eDNA in biofilms accomplishes three important roles, which are treated in (Montanaro et al. 2011c):

- (i) Stabilization of the biofilm matrix, as demonstrated by the effect of DNase I in preventing the formation of a stable biofilm and in impairing the attachment of bacterial cells to culture flow-chambers.
- (ii) Part of gene-transfer mechanisms. Extracellular DNA present in bacterial biofilm communities constitutes a dynamic gene pool from which bacteria competent for natural transformation can derive genetic information by horizontal gene transfer (Ehrlich et al. 2005, 2010). The impact of horizontal gene transfer is exemplified by bacterial acquisition of virulence traits and antimicrobial drug resistance.
- (iii) Conditioning of the innate immune response, prevention of phagocytosis, and attenuation of inflammation. The components of biofilm matrix (eDNA, proteins and exopolysaccharides) are microbial structural motifs recognized by the innate immune system via the TLR family of pattern recognition receptors (PRRs). Upon phagocytosis and digestion of *S. aureus* in the phagosome, bacterial DNA is liberated and engages TLR9. TLR9-dependent activation can be triggered not only by phagocytosis of whole *S. aureus* cells but also by that of extracellular DNA, extensively contained in the biofilm matrix. After TLRs engagement, the behavior of immune response appears different between biofilm-encased and planktonic bacteria. Turlow et al. have demonstrated that *S.*

aureus biofilms actively attenuate classical antibacterial immune responses, inducing a significant reduction in cytokine/chemokine production in biofilm infected tissues (Thurlow et al. 2011).

2.9 The Problem of Etiological Diagnosis in Biofilm Infections

The encasing of bacteria in biofilms gives rise to insuperable difficulties not only in the treatment of the infection owing to the high antibiotic resistance of bacteria embedded in biofilm, but even in assessing the state and the nature of the infection (Costerton et al. 2003). The traditional culture methods turn out often to be inefficacious in reaching a proper diagnosis of the microbial species responsible for the infection (Ehrlich et al. 2012). The only laboratory techniques approved by the U.S. Food and Drug Administration to detect and identify bacteria responsible for human infections are cultures, which necessarily depend on the ability of bacteria to grow and produce visible colonies when seeded on the surfaces of appropriate agar plates. However, this 100-year-old technology is able to detect, under ideal circumstances, only one or two out of dozens of bacterial species that may be present in a wound. The agar plate culture technique may fail completely in the detection of bacteria present in very large numbers in orthopedic infections (Wolcott and Dowd 2011; Costerton and DeMeo 2011).

In implant infections, particularly in orthopedics, a rapid and sensitive identification of the etiological agents is mandatory for undertaking efficacious therapeutic measures. The accurate assessment of the infecting pathogen and its identification at species- and strain-level are needed (Ehrlich and Post 2013) to establish the virulence potential, the antibiotic resistance profiles, and to predict the biofilm-forming capacity in order to optimize appropriate therapeutic approaches. Therapeutic measures can go from local and systemic antibiotic therapy, to surgical debridement, and lastly to the removal and replacement of the implant.

Classically, methods used to diagnose prosthesis-related infections start with the *in vitro* culture of bioptic samples taken from periprosthetic tissues, to ascertain any bacterial growth. The definite characterization of an infection as a biofilm infection should be based on the microscopic demonstration of matrix-embedded bacterial colonies in affected tissues, but, for routine clinical use, this diagnostic procedure is invasive, costly and time intensive.

In the field of biofilm-centered implant infections, these classical culture methods, developed for acute infective diseases caused by planktonic bacteria, have encountered rising skepticism. Etiological diagnosis is seriously limited by the frequent failures in detaching and collecting biofilm cells from infected tissues and in culturing them on agar, since planktonic bacteria produce colonies on agar, whereas biofilm-forming bacteria do not.

The difficulties or even the impossibility to isolate the bacterium responsible for an implant infection often leads to the greatly abused diagnosis of “aseptic loosening”, even in cases in which clinical signs of infection clearly exist, with the serious consequence to fail rational basis for the therapy (Jacovides et al. 2012).

Thus, DNA-based molecular methods not relying on cultural methods have been developed to provide rapid identification of all microbial pathogens.

Benefits and limits of molecular methods for etiological diagnosis and for identification of virulent strains have been discussed by Arciola et al. (2011).

The new advanced technologies for rapid bacteriological identification demonstrate a shift from the traditional biochemical and molecular testing methods towards those using mass spectrometry (MS) for the analysis of microbial proteins and genetic elements (Ehrlich et al. 2014).

Costerton and his colleagues have in-depth reviewed the plethora of molecular techniques that could replace cultures in the diagnosis of bacterial diseases and have identified the new IBIS technique that is based on base ratios (not base sequences), as the molecular system most likely to fulfill the requirements of routine diagnosis in orthopedic surgery (Costerton et al. 2011).

Another mass spectroscopy-based technology, MALDI-TOF, has earned some diagnostic interest, but this technology, while rapid and useful for species identification (Harris et al. 2010), nevertheless requires, in the first step of analysis, a colony plating and, thus, suffers from all of the disadvantages of the microbial culture approach (Arciola et al. 2011).

2.10 Clinical Diagnosis of Periprosthetic Infections

Together with diagnostic molecular methods, highly sensitive and specific biochemical and hematological markers are searched, which can be applied to both serum and joint fluid aspirate for early diagnosis.

Recent reviews have scrutinized current research efforts in the field of these markers, to evaluate their features and their positive or negative predictive values in diagnosing implant infections (Rak et al. 2013; Hansen et al. 2012). C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cells (WBC), and leukocyte esterase (LE) are the markers studied for their sensitivity, specificity, and positive and negative predictive values in diagnosing periprosthetic joint infections (PJI) (Parvizi et al. 2011).

According to the majority of Authors, a clinical diagnosis of PJI can be suspected when at least of one of the following criteria is present: (i) cutaneous sinus tract communicating with the prosthesis, (ii) visible purulence around the prosthesis, (iii) histopathological characteristics of acute inflammation, (iv) increased leukocyte count and differential in the synovial fluid, or (v) positive culture of the synovial fluid, periprosthetic tissue or sonication fluid cultures (Osmon et al. 2013).

None of the routine blood tests, including WBC, ESR, CRP and procalcitonin is sufficiently sensitive or specific enough to diagnose or exclude a PJI with high accuracy. In particular, normal values of ESR or CRP do not exclude PJI, especially in cases of low-grade infection. Moreover, ESR and CRP usually increase after surgery and reflect

post-interventional inflammation. Therefore, rather than a single value, serial post-operative measurements are needed for accurate interpretation (Zimmerli et al. 2004).

The investigation of synovial fluid is more helpful than blood. The synovial-fluid leukocyte count is highly sensitive and specific for infection. Additional tests in synovial fluid, such as glucose, lactate or CRP were not shown to bring additional information regarding the diagnosis of infection (Schinsky et al. 2008).

2.11 Sonication of Removed Implants

Sonication of the removed prosthesis, followed by culture of the sonication fluid is a method of diagnosing implant-associated infections reported few years ago. Sonication of explanted prosthetic components in bags for diagnosis of PJI was first associated with risk of contamination due to bag leakage and subsequent risk of microbial contamination, especially due to non-fermentative, Gram-negative bacilli (Trampuz et al. 2006).

Nevertheless, the culture sensitivity of sonication fluid is superior to that of standard periprosthetic tissue (75 % versus 54 %, respectively (Corvec et al. 2012).

A cutoff of 50 colony-forming units (CFU)/ml of sonication fluid yields a sensitivity of 79 % and a specificity of 99 % for the diagnosis of PJI based on a study involving 331 patients with total knee prostheses or hip prostheses (Puig-Verdié et al. 2013).

Sonication is mainly recommended when an implant failure does not have clear signs of infection and in patients with delayed implant failure. In early failure, culture of fluid obtained by sonication is not superior to culture of peri-implant tissues for the diagnosis of infection and, therefore, is not recommended as a routine diagnostic test in these patients (Esteban et al. 2014).

In conclusion, sonication of the implant increases the sensitivity of the culture of periprosthetic tissues and is being increasingly adopted by many centers. Molecular diagnostic

methods compared with intraoperative tissue culture, especially if combined with sonication, have a higher sensitivity, a faster turnaround time and are not influenced by previous antimicrobial therapy. However, molecular methods still lack a system for detection of antimicrobial susceptibility, which is crucial for an optimized and less toxic therapy of periprosthetic joint infections (Esteban et al. 2014).

Recently, Parvizi et al. have discussed the problem of negative results of culture methods in the diagnosis of periprosthetic joint infections, when many clinical signs indicate an infection. According to these Authors, the most important reason is the administration of antibiotics prior to obtaining culture samples. In the presence of a suspect of infection, antibiotics should not be given until the diagnosis is confirmed. Alternatively, aspiration of the joint should be delayed for at least 2 weeks after the last dose of antibiotics. Different and appropriate technical suggestions are given in the cited Parvizi's article in order to enhance the likelihood of obtaining a positive result, including biomarkers and molecular techniques (Parvizi et al. 2014).

How the embedding of causative microorganisms in a biofilm is responsible for the negative culture diagnosis comes from the clear lesson of Bill Costerton, and his suggestion to recourse to advanced molecular methods instead of culture procedures has been highlighted by Ehrlich and Arciola (2012).

2.12 Future Perspectives: Infection-Resistant Materials

Among the new strategies to combat biofilm-centered implant infections, antibiofilm agents, able to inhibit biofilm formation or disrupt formed biofilm, are subjects of extensive researches, and this item is treated in other chapters of the book.

Especially in orthopedics, the recourse to anti-infective or infective-resistant materials is at the forefront in the biomaterial science.

Achievement of infection-resistant materials can be based on different approaches: (i) modifica-

tion of the biomaterial surface to give anti-adhesive properties, (ii) doping the material with antimicrobial substances, (iii) combining anti-adhesive and antimicrobial effects in the same coating, (iv) realization of materials able to oppose biofilm formation and, at the same time, to support bone repair (Fig. 2.3).

Two recent reviews have surveyed the different approaches for obtaining efficacious infection-resistant materials and the reader could be referred to them for an extensive treatise of this subject (Arciola et al. 2012b; Campoccia et al. 2013).

The first approach is based on adsorption of molecules conferring hydrophilic properties to the material surface and competing with the interaction between bacteria and host matrix proteins that film the implant. Heparin, with its strong hydrophilic properties, has been proposed long ago to be able to hamper adhesion of bacterial cells. Besides acting by increasing hydrophilicity, forming a highly hydrated layer between the bacteria and the surface (Arciola et al. 1993, 1994, 1995, 1998; Legeay et al. 2006), heparin has been proved that can interfere with *S. epidermidis* adhesion by specifically inhibiting the binding of bacterial adhesins FnBPs to fibronectin that film the biomaterial surfaces (Arciola et al. 2003; Bustanji et al. 2003). Bacterial adhesion on implant surfaces can be inhibited by hydrophilic polymeric brushes based on poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO). A coating of these highly hydrated polymer chains on a surface inhibits protein and bacterial adhesion (Neoh and Kang 2011).

The second approach is mainly based on the local delivery of antimicrobial agents, in particular antibiotics, through carrier biomaterials. The risk of inducing antibiotic resistance is an intrinsic drawback of the antibiotic-loaded materials (Campoccia et al. 2010). Another antibacterial substance that avoid the limits of antibiotic-loading is the natural cationic polysaccharide chitosan, which besides having an antibacterial action, is a promising biopolymers for tissue engineering.

Recent results by Zhao et al. indicate that chitosan-lauric acid may be successfully

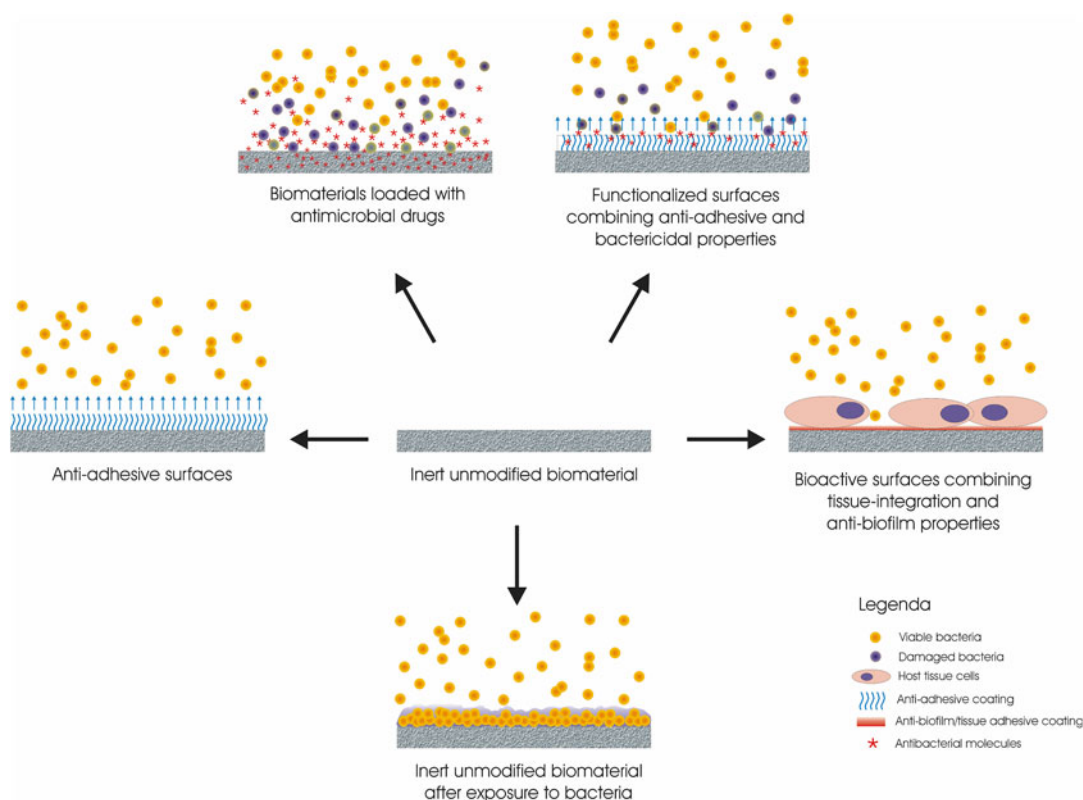


Fig. 2.3 Different strategies to achieve infection-resistant biomaterial surfaces

immobilized onto the surfaces of Ti substrates. The chitosan-functionalized titanium promotes osteoblast cell adhesion, cell viability, intracellular alkaline phosphatase activity and mineralization capacity of osteoblasts. Antibacterial assays against *S. aureus* and *P. aeruginosa* showed that titanium functionalized with chitosan-lauric acid conjugate efficiently inhibited the adhesion and growth of bacteria. The Zhao's study represents a promising approach to fabricate functional Ti-based orthopedic implants, since this surface-modified material enhances the biological functions of osteoblasts and concurrently reduces bacteria adhesion (Zhao et al. 2014).

To the specific aim designed to search for new biomaterials having intrinsic antibacterial properties, able to hamper the formation of a biofilm, new quaternised chitosan derivatives appear promising. PMMA loaded with quaternised chitosan-loaded inhibits surface biofilm formation by antibiotic-resistant staphylococci, more

strongly than PMMA alone, gentamicin-loaded PMMA and chitosan-loaded PMMA. Moreover the quaternised chitosan-loaded PMMA markedly down-regulates expression of *ica locus* genes, encoding essential enzymes for biofilm biosynthesis, and also down-regulates the expression of *mecA*, which is responsible for methicillin resistance (Tan et al. 2012).

The third approach is illustrated, as an example, by the multilayer film constructed by assembling layer-by-layer heparin and chitosan, obtaining an antiadhesive and antibacterial biomaterial. This new multilayer material not only reduced the bacterial adhesion but also killed the bacteria adhered onto the surface, proving to be a powerful anti-infective coating (Fu et al. 2005).

The possibility to combine anti-adhesive and antimicrobial effects in the same coating, without recurring to antibiotic-loading is offered by the new evidence of the antimicrobial activity of cationic antimicrobial peptides. These peptides

are an important component of innate immune defenses and have been shown to kill a broad variety of Gram-negative and Gram-positive bacteria and are promising tools to treat multidrug-resistant bacteria (Kang et al. 2012). Different biomaterials can be employed as surface supports for immobilizing cationic antimicrobial peptides, such as resin beads, gold surfaces, polymer brushes, cellulose membranes and block copolymers. The antimicrobial peptides immobilized onto a hydrophilic polymer has been proved to give a robust coating with antiadhesive and antimicrobial properties, highly effective in combating biofilm formation. Since polymer brushes with immobilized antimicrobial peptides can be synthesized on most of the current implant material surfaces, the coating will be widely applicable for combating implant-associated infections (Hancock and Sahl 2006; Bagheri et al. 2012).

The fourth approach, the achievement of materials able to oppose biofilm formation and, at the same time, to support bone repair, is of outstanding interest in orthopedics. Hydroxyapatite, besides its properties as infection-resistant material (Arciola et al. 1999), have been proposed as a coating surface undergoing slow in vivo degradation and as a stable interface for osseointegration and bone fixation (Campoccia et al. 2003).

An innovative osteointegrative and antibacterial biomimetic coating on titanium has been obtained by Anodic Spark Deposition (ASD) treatment. The anodization treatment creates a chemically and morphologically modified titanium oxide layer, characterized by a microporous morphology enriched by calcium, silicon, phosphorous, and silver. A biological characterization of this coating has shown an optimal adhesion of osteogenic SAOS-2 and proliferation as well as a strong antibacterial effect (Della Valle et al. 2012).

Bioglasses are of wide interest since they spontaneously bond and integrate with living bone in the body. By varying the glass chemistry and/or by adding some dopants, it is possible to improve their clinical applications. A bioglass doped with gold nanoparticles has been developed, which showed efficient antibacterial properties against *S. aureus*, in addition to its bone reconstruction property (Grandi et al. 2011).

2.13 Conclusion

The significant worldwide impact of periprosthetic joint infections and the loss of efficacy of antibiotic-based conventional therapies urgently demand new preventive strategies able to effectively limit the infection burden that parallels the increasing total number of primary and revision arthroplasties.

Many categories of anti-infective biomaterials are currently available and new ones are rapidly advancing. The use of materials coated with immobilized antibacterial substances, particularly cationic antimicrobial peptides, appears very innovative and promising. Nanotechnologies and nanomaterials in medical research have created new therapeutic horizons and are rapidly growing.

The biomaterial science offers powerful and valuable tools. Their potential is often well proved in vitro and in preclinical models. However, clinical trials, appropriately designed at multicenter scale, together with well-implemented international registries are necessary to obtain evidence-based data on the benefits of the scientific advancements in the field. In this way, we may reach the aim at identifying the most effective anti-infective strategies.

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