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Role of Biofilm in *Staphylococcus aureus* and *Staphylococcus epidermidis* Ventricular Assist Device Driveline Infections

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Abstract

Objective—Infections, especially those involving drivelines, are among the most serious complications that follow ventricular assist device implantation. Staphylococci are the most common causes of these infections. Once driveline infections are established, they may remain localized or progress as an ascending infection to cause metastatic seeding of other tissue sites. While elaboration of biofilm appears to be critical in prosthetic device infections, its role as a facilitator of staphylococcal infection and migration along the driveline and other prosthetic devices is unclear.

Methods—A mouse model of driveline infection was used to investigate staphylococcal migration along the driveline. A biofilm producing strain of *Staphylococcus epidermidis*, and a *Staphylococcus aureus* strain and its *ica* negative (biofilm deficient) isogenic mutant were used in these studies. Bacterial density on the driveline and the underlying tissue was measured over time. Scanning electron microscopy was used to examine the morphology of *S. epidermidis* biofilm formation as the infection progressed.

Results—The biofilm-deficient *S. aureus* mutant was less effective at infecting and migrating along the driveline than the wild type strain over time. However the *ica* mutation had no effect on the ability of the strain to infect underlying tissue. *S. aureus* exhibited more rapid migration than *S. epidermidis*. Scanning electron microscopy revealed the deposition of host matrix on the Dacron™ material following implantation. This was followed by elaboration of a bacterial biofilm that correlated with more rapid migration along the driveline.

Conclusion—Biofilm formation is a critical virulence determinant that facilitates the progression of drivelines infections.

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Introduction

Ventricular assist devices (VADs) are used to improve cardiac function in patients with end-stage congestive heart failure (1-3). Originally designed as a bridge-to-transplantation, the use of VADs as destination therapy (for patients who are not transplant candidates or who have refractory heart failure) is increasing (1,2). Infections are among the most common and serious complications in patients (10-40%) following VAD implantation (4). The presence of these infections may delay transplantation in bridge-to-transplant patients and is a major cause of death in destination patients. Despite a reduction in the overall number of VAD-related infections, the driveline (DL) remains a common site of infection [(21% as reported by the intermacs registry (www.intermacs.org)](3). The DL exit site is accessible to commensal flora and susceptible to trauma that can interfere with tissue integration of the DL (5). The ability of bacteria to cause these infections is due in part to their ability to adhere to and colonize both skin and device surfaces using the transcutaneous DL of VADs as a portal of entry (6,7).

The predominant pathogens associated with device infections are biofilm-forming *Staphylococcus epidermidis* and *Staphylococcus aureus*; both are part of the host commensal flora (8). Infection results from the ability of these bacteria to initially colonize the device surface and then migrate along its surface into the underlying host tissue (6,9,10). Biofilms appear to contribute to this process (5,11,12). They constitute a consortium of surface-attached bacteria encased in a self-produced extracellular polymeric substance (EPS) (5,12-14). When a VAD is implanted, a series of host molecules such as fibronectin, fibrin, collagen, lipids and inorganic ions are deposited on the different VAD surfaces creating a host tissue matrix (5,7,12-14). This matrix facilitates the attachment of biofilm-forming bacteria.

In staphylococci, the intercellular adhesion gene cluster (*ica*) comprises four genes (*icaADBC*), which directs the production of a polysaccharide that mediates intercellular adhesion and is regarded as one of the essential factors for biofilm formation (15).

While most DL infections remain localized to the entry site, they may also spread to other tissue sites. One hypothesis for this process is that there is bacterial biofilm expansion that enables bacteria to “migrate” along the DL. With this in mind, we investigated the contribution *S. aureus* and *S. epidermidis* biofilm to the initiation and progression of DL infections using an *in vivo* model of DL infection.

Methods

Bacterial strains and growth conditions

S. epidermidis strain 9491 (SE9491) (16), *S. aureus* isogenic strains RN450 (SA450) and RN450 (Δ*ica*) (SAΔica) (kindly provided by Chia Y. Lee) (17,18) were grown in tryptic soy broth [TSB, (supplemented with glucose, 0.25% for SE9491)] at 37°C with aeration (Table 1). The presence of *ica* in SA450 and SE9491 was confirmed by PCR using the following primers: *ica* forward primer: 5’-GAT TGT ATT AGC TGT AGC TAC-’3, and *ica* reverse primer: 5’-CAA CTG CAG CTG ATT TCG CCC ACC GCG TG-’3). For plate counts, mannitol salt agar plates or tryptic soy agar (TSA) plates were incubated overnight at 37°C. A growth curve (TSB at 37°C) was performed to assess the effect of the *ica* deletion on the SAΔica. No difference was found in the rate of growth between the isogenic pair.

In vivo Infection Model

The murine model of DL infection has been previously described (7,19). Briefly, 15 mm (in length) Dacron™ (kindly provided by Thoratec, Pleasanton, CA) coated DLs were

implanted transcutaneously in the back of C57BL/6J mice The model is illustrated in Figure 1. The internal (subcutaneous) part of the DLs was 10 mm (in length). The day of implantation was considered day -2. Two days after implantation (day 0), the skin surrounding the exposed DL was inoculated with a bacterial suspension (50μl) containing 5×10^7 colony forming units (CFU) of SE9491 or 5×10^6 CFU of *S. aureus* (suspended in PBS). No perioperative antibiotics were administered to the mice. Two days after bacterial challenge (day 2), the DLs were explanted, the external portion sterilely separated and the internal portions processed (see below). These studies were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee.

The DLs were washed twice with PBS (1mL). Then, adherent bacteria were harvested by vortexing in 0.05% trypsin-EDTA (0.5mL). Dilutions of the samples were plated onto mannitol salt agar plates and bacterial cell counts determined. The tissue underlying the DL was also collected from each mouse and weighed. The homogenized samples were weighed then plated onto mannitol salt agar plates, bacterial counts determined and then normalized by sample weight. Two independent experiments were performed using a total of 20 mice per strain.

For the bacterial migration studies the DL size was increased to 20 mm (in length), increasing the subcutaneous portion to 15 mm (in length). To measure bacterial migration along the DL over time, we divided the DL into three 5mm segments. The three subcutaneous sections were defined as: Proximal (P), Medial (M) and Distal (D) based on their position with respect to the transcutaneous entry point. Infection of the DL was carried out as outlined above. The bacterial inoculum was increased to 1×10^7 CFU. DLs were explanted at days 0, 2, 6, 9, 12. Each section (P, M, D) were processed separately. Results were expressed as a ratio by comparing the bacterial counts of each section (P, M, D) to the total number of bacteria found per DL (P+M+D).

To assess for possible metastatic seeding tissue samples of kidney, liver and spleen were collected and processed in a similar manner as the underlying muscle tissue described above,

Scanning electron microscopy

Sections of explanted DLs were fixed with glutaraldehyde (2%) in cacodylate buffer as previously described (20). A JEOL JSM6400 Scanning Electron Microscope (Peabody MA) was used. Three micrographs where obtained from each section of two different DLs. Images of control DL samples (non-implanted and implanted but non-infected) were compared to images obtained from infected DLs at different time points (day 6 and day 12 after infection).

Statistical Analysis

Data were analyzed using an unpaired Student *t* test. A value of $p<0.05$ was considered to be statistically significant.

Results

The role of biofilm in DL infection

The role that biofilm contributes to bacterial density on the DLs and in the surrounding tissue was assessed using both the isogenic strain of *S. aureus* anda biofilm positive *S. epidermidis* strain. The average bacterial counts on the explanted DLs, for the biofilm forming strain SA450 was 5×10^4 CFU/DL; this was significantly higher than those for the biofilm negative strain SAΔica (2×10^4/DL) (Figure 2) ($p<0.05$). Despite the difference in

adherence to the DL, the presence of bacteria in the underlying tissue was similar for the isogenic strains ($p>0.05$) (Figure 2). Although biofilm positive, the SE9491 had fewer bacterial counts than SA450 on both the DLs (3×10^4/DL) and in the underlying tissue (1×10^4 CFU/gram) ($p<0.05$) (Figure 2). This was despite the use of a higher initial bacterial inoculum.

The role of biofilm as a facilitator of migration along the DL

Bacterial migration along the DL was studied over time (Figure 3). With SA450, total bacterial density went from 2.6×10^4 CFU/DL at day 2 to 4.8×10^5 CFU/DL by day 12, almost doubling every two days (Figure 4A). In contrast, the bacterial density for SAΔica, although increasing throughout the assay from 9×10^3 CFU/DL (day 2) to 5.4×10^4 CFU/DL (day 12), was 10-fold lower than SA450 (Figure 4B).

At an early stage of infection (day 2), 60% of SA450 was located on the P section, 30% in the M section and 10% on the D section. By day 6, SA450 was uniformly distributed among the three DL sections ($p>0.05$) (Figure 3A). Although SAΔica was also found in all three sections (Figure 3B), this biofilm-deficient mutant never achieved an even distribution along the DLs ($p<0.05$). Even by day 12, 64% of the CFU/DL of SAΔica was still limited to the P section of the CFU/DL (Figure 3B).

The biofilm forming strain SE9491 was also evaluated in the *in vivo* DL assay. In contrast with *S. aureus*, the total bacterial counts declined over the 12 day study suggesting a more indolent infection. The bacterial density of SE9491 started at 8.6×10^4 CFU/DL (day 2) and decreased to 3.8×10^3 CFU/DL by days 9 and 12, nearly 100-fold lower than SA450 bacterial density at the end of the experiment. Despite this, a similar pattern to that of SA450 was observed regarding bacterial distribution. By day 12 bacteria were evenly distributed in all three DL sections (Figure 3C) ($p>0.05$).

No bacteria were found in samples taken from the kidney, liver or spleen.

Scanning electron microscopy

SEM was used to visualize and characterize the progression of the DL infection over time. The Dacron™ fibers examined prior to implantation appeared smooth and uniform in size (Figure 5). Electron micrographs of implanted, uninfected DLs taken at day 6 revealed extracellular matrix components and host cells coating the Dacron™ fibers (Figure 5). Micrographs of SE9491-infected DLs at day 6 showed formation of biofilm in the P and M sections. The D sections of these DLs were almost free of SE9491 and showed no biofilm structures at day 6 (Figure 5). By day 12, uninfected DLs were covered with an extensive extracellular matrix populated with adherent cells distributed throughout the implanted material while infected DLs showed a more developed biofilm structure in both P and M sections than at day 6 (Figure 5). The bacterial biofilm was far more extensively developed with an increased number of bacteria visible within its structure. In addition by day 12 the biofilm extended to the D section of the infected DLs (Figure 5).

Discussion

DL infections continue to be the single most common infectious complication of VAD implantation. Fifteen to twenty percent of patients develop these infections and, in contrast with other VAD-related infections, they occur over the entire lifetime of the VAD (3). There is limited understanding of the pathogenesis of these infections (4,7).

In this study an *in vivo* mouse model was used to examine the role of biofilm formation in the pathogenesis of DL infections and in particular the role of biofilm in bacterial migration

along prosthetic devices. Infection was initiated in this model 48 hours after implantation of the driveline. This time frame was selected because staphylococcal infections in particular often occur shortly after surgery at a time when the internal portions of the device remain accessible to skin commensal flora.

For staphylococci the *ica* gene cluster is essential for biofilm formation (17,21,22). The intercellular adhesion gene cluster (*ica*) contains four genes (*icaADBC*) that are responsible for the synthesis of poly-N-acetyl glucosamine, the molecule that mediates intercellular adhesion, facilitating biofilm formation. Biofilm provides a potential sanctuary for bacteria protecting against innate host defense mechanisms such as complement, white blood cells and immunoglobulin, as well as reducing the efficacy of antibiotics (8,11,12,14,15). Less well established is whether biofilm also facilitates the migration of bacteria along catheters, or in this instance DLs, enabling infection to progress to deeper tissue structures or to spread to other organs. This question was addressed in the present study.

The study found that both biofilm-producing SA450 and a biofilm positive SE9491 strain migrated along the Dacron™ coated DL and achieved an even distribution along the DL. The biofilm deficient strain SAΔica was less capable of infecting the implanted DLs and remained unevenly distributed. The biofilm positive SE9491 while establishing a higher initial count on the DL (a higher initial bacterial inoculum was used) had a lower bacterial density on the DLs and, in contrast with *S. aureus*, lost density over time. This suggests that the strain had diminished virulence when compared with *S. aureus.* Overall the results suggest that *ica* dependent biofilm formation played an important role in bacterial migration along the DL. Interestingly biofilm formation appeared to have no effect on the ability of the strains to infect the underlying tissue, illustrating the selective virulence of biofilm for prosthetic device infections.

The SEMs demonstrated the orderly progression of events leading to infection. Bacteria contaminating the skin migrate across the transcutaneous DL site, adhere to the host matrix material coating the Dacron™, replicate and elaborate a biofilm allowing the bacteria to both colonize the DL surface and, as the biofilm expands, to migrate along the DL. The biofilm architecture was similar to that observed on infected catheters (23,24). Control images of uninfected DL showed the matrix coating of DLs after implantation. Host cells, associated with the DL surface, deposit fibers and other host molecules that permit integration of the implant into the host tissue. These same molecules (*e.g.*, fibronectin and fibrinogen) are also recognized by bacteria as sites for attachment (12,16,25).

While this study illustrates the role of biofilm in DL infection it is limited by the use of a single isogenic pair of *S. aureus* and a biofilm producing strain of *S. epidermidis* without an isogenic *ica* mutant. Creating a biofilm negative *S. epidermidis* mutant has proven technically difficult due to the limited susceptibility of these strains to transduction and electroporation. While spread of infection was investigated using the DL model, metastatic seeding to other organs (*i.e.* spleen, kidney, liver) was not detected. The subcutaneous site of DL implantation in this DL model made the likelihood of seeding other tissue sites unlikely.

The present study suggests that biofilm, in addition to providing a sanctuary that protects bacteria from host defenses, also facilitates migration of bacteria along prosthetic devices. Access to the underlying tissue enhances the likelihood of a local infection as well as the potential for spread to other tissue sites. Further studies will need to address host-tissue interactions that facilitate integration of prosthetic materials such as Dacron™ into host tissues and reduce access of bacteria to the underlying tissues. Understanding particular properties of a pathogen that leads to infection is critical to the design of devices that are less

susceptible to infection. These determinants may then potentially be used as targets for either preventative or therapeutic interventions.

New therapies that address DL design, incorporate antimicrobials into prosthetic materials or that reduce biofilm formation may help reduce the frequency of these infections.

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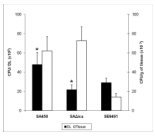

Figure 1.
Description of the driveline model. Upper figure displays a schematic representation of placement of the driveline into the back of the mouse. Lower figure displays the driveline implanted into a mouse. The inset provides a more detailed view of the implanted driveline. This figure is reproduced with the permission of the Journal of Heart and Lung Transplantation, Elsevier Press.

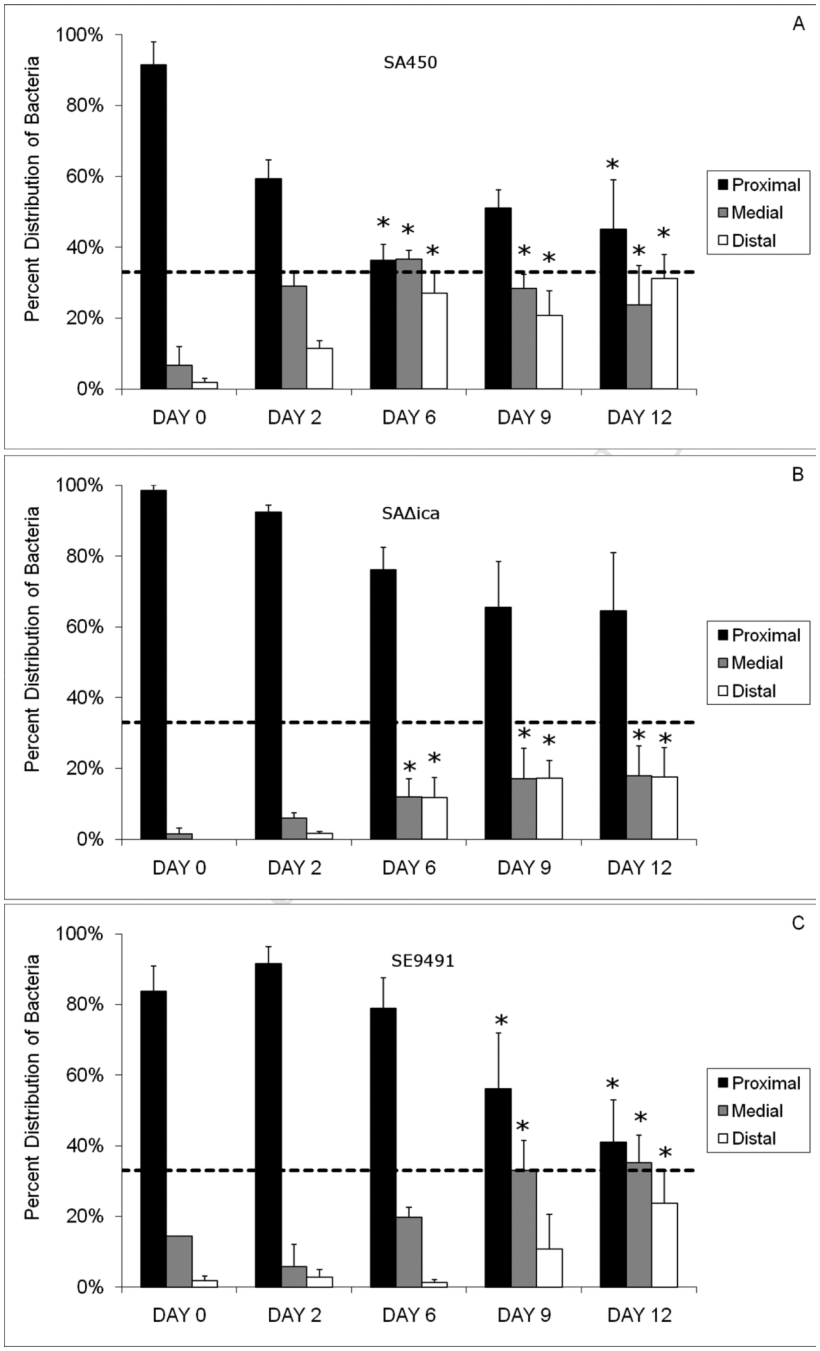

Figure 2.

Bacterial density on DLs and in tissue harvested from mice infected with *S. aureus.* Mice with implanted DLs were infected with SA450 (n=20) or SAΔica (N=20). The DLs and samples of the underlying muscle were collected at 48h. Cells were enumerated by plate counting. There was a statistically higher number of bacteria adherent to the SA450 infected DL than with SAΔica ($p<0.05$). There was no difference in bacterial density in the underlying tissue.

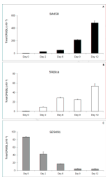

Figure 3.

The role of biofilm in bacterial migration along the DL. Mice were implanted with 20mm DLs and exposed to SA450 (A), SAΔica (B) or SE9491 (C). DLs were harvested at different time points following implantation and the number of bacteria adherent to different segments of the DL (P, proximal; M, medial; and D distal) determined. A ratio of adherence was determined [# bacteria adherent to the DL segment/total # bacteria on DL (P+M+D)]. The experiments were done in duplicate with six mice per treatment. Similar ratios are noted with a star ($p>0.05$). The horizontal line indicates what would represent an even distribution of adherent bacteria/section. When the distribution of the bacteria along the catheter of the isogenic pair are compared SA450 (A) versus SAΔica (B) there is a significantly higher number of bacteria found distally in the wild type SA450 ($p<0.05$)

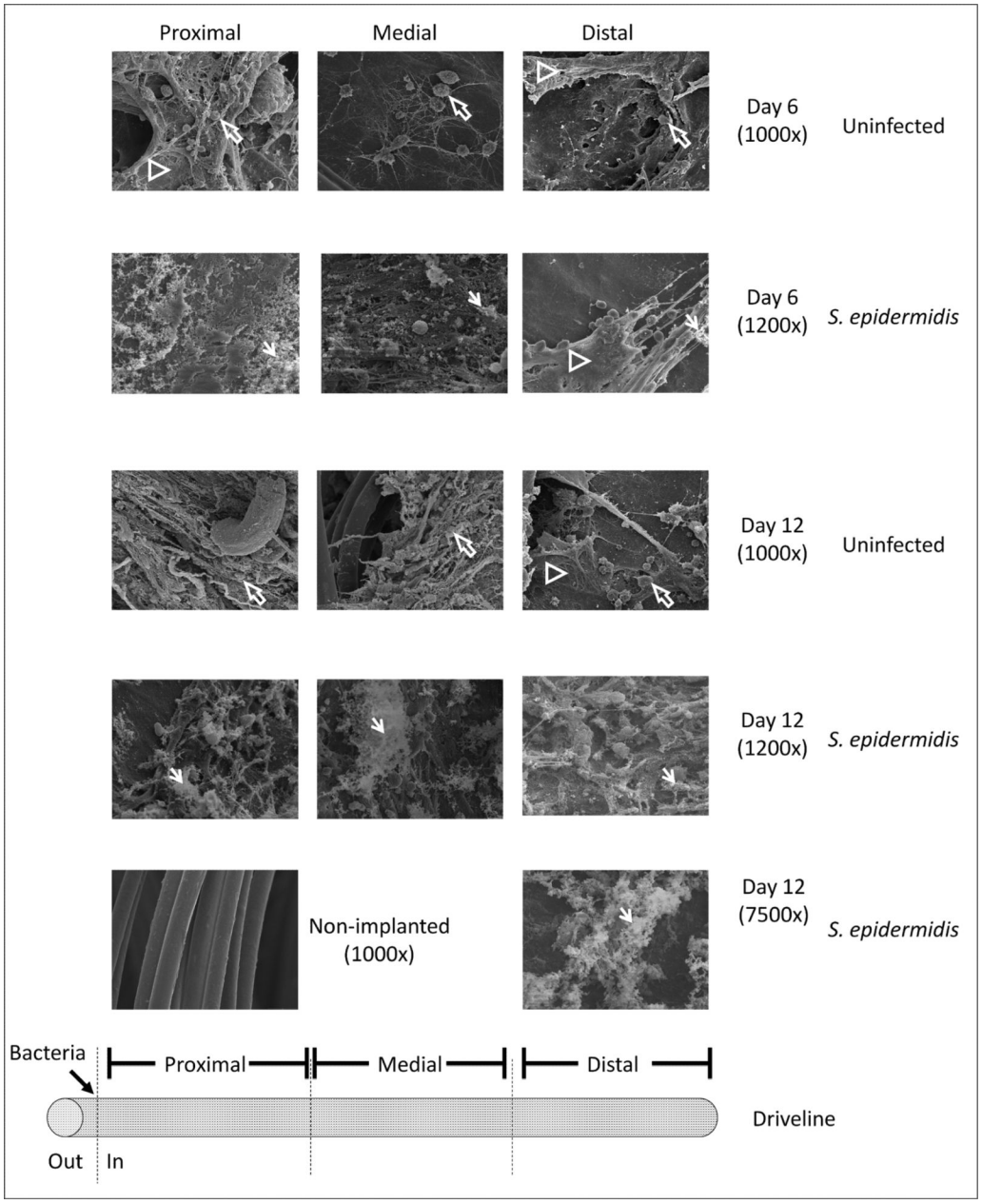

Figure 4.

Bacterial density on DLs infected with *S. aureus* or *S. epidermidis* over time. Mice were implanted with 20mm. DLs and exposed to SA450 (A), biofilm-deficient SAΔica (B) and SE9491 (C). At different time points DLs were explanted and the number of bacteria adherent to the three sections (Proximal, P; Medial, M; Distal, D) counted. The results display the total number of bacteria found on each DL. The total number of bacteria increased in both *S. aureus* strains while there was a decrease in the number of *S. epidermidis* strain SE9491 on the infected DLs, suggesting diminished virulence for the latter.

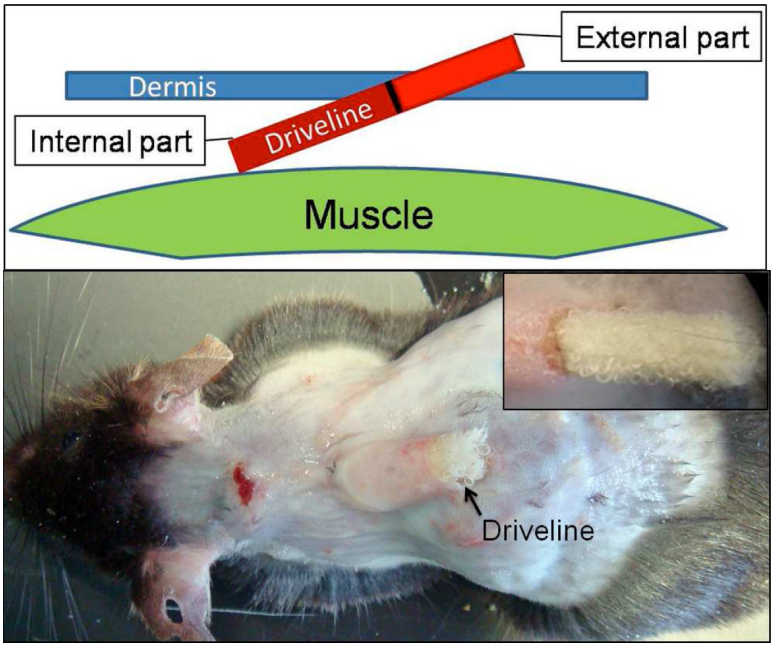

Figure 5.

Scanning Electron Microscopy of DL (Proximal, P; Medial, M; Distal, D) explanted at different time points after infection with *S. epidermidis* strain SE9491. Dacron™ fibers from nonimplanted material revealed a smooth surface (bottom left). By day 6, images of the uninfected, implanted DL showed the Dacron™ coated with matrix material (arrowhead) and host cells (outlined arrows). The SE9491 infected DLs demonstrate bacteria organized in a biofilm (full arrows) in the P and M sections while the D sections were mostly free of bacteria. By day 12, uninfected DLs were covered with a dense tissue matrix with adherent cells (outlined arrows). The infected DL showed extensive biofilm formation extending to the D section (full arrows). The images demonstrate that there is a progression that is characterized by the formation of a scaffold of matrix molecules and cells that is first formed along the DL followed by the adherence of bacteria and elaboration of a biofilm that allows bacterial migration along the DL.

Table 1

Description of bacterial strains used in this study

Species	Strain	Phenotype	Reference
Staphylococcus aureus	RN450 SA450	Biofilm forming wild type	Chia Y. Lee, University of Arkansas
	RN450 (Δica) SAΔica	Biofilm deficient strain	Chia Y. Lee, University of Arkansas
Staphylococcus epidermidis	9491 (SE9491)	Biofilm positive laboratory strain	McCrea, 2000