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Original article

Invasion of bone cells by *Staphylococcus epidermidis*

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Abstract

Bone implants infected with *Staphylococcus epidermidis* often require surgical intervention because of the failure of antibiotic treatment. The reasons why such infections are resistant to therapy are poorly understood. We have previously reported that another bacterium, *Staphylococcus aureus*, can invade bone cells and thereby evade antimicrobial therapy. In this study we have investigated the hypothesis that *S. epidermidis* can also invade bone cells and may therefore explain the difficulties of treating infections with this organism. We found that *S. epidermidis* was capable of invading bone cells but that there were significant strain dependent differences in this capacity. A recombinant protein encompassing the D1–D4 repeat region of *S. aureus* fibronectin-binding protein B completely inhibited internalization of *S. aureus* but failed to block internalization of *S. epidermidis*. Similarly a blocking antibody to $\alpha 5\beta 1$ integrin inhibited internalization of *S. aureus* by bone cells but had no effect on the uptake of *S. epidermidis*. Therefore unlike *S. aureus*, *S. epidermidis* does not gain entrance into bone cells through a fibronectin bridge between the $\alpha 5\beta 1$ integrin and a bacterial adhesin.

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1. Introduction

Staphylococcus epidermidis is a universal skin commensal that rarely causes pathological infections in healthy people. In spite of this it is a major cause of orthopedic implant infection and is also isolated from patients with osteomyelitis and localized bone destruction [1–5]. The seriousness of such infections is evident when one considers the case of hip replacements, which are estimated at one million a year world wide [6], for which a failure rate of 20% has been reported [7] with infections causing between 10 and 15% of such failures [8,9]. After revision surgery the incidence of infection can

be as high as 40% [10]. In the case of osteomyelitis, *S. epidermidis* commonly causes chronic disease [2], the treatment of which requires prolonged antibiotic therapy and often surgical intervention. Since *S. epidermidis* is a relatively avirulent bacterium it is perhaps surprising that it causes chronic and recurrent bone diseases and it is generally thought that these diseases are the result of this organism's ability to evade host defenses and antibiotic therapy. Precisely how *S. epidermidis* escapes the host's defenses and is recalcitrant to antibiotic therapy remains to be determined.

One means by which bacteria are postulated to evade host defenses is by “hiding” within non-phagocytic cells. Our group and others have shown that the bone pathogen *Staphylococcus aureus* is internalized by bone cells [11–14] and it has been suggested that this ability enables the bacterium to avoid the host immune system and/or antibiotic treatment. In fact in vitro studies using human endothelial cells have shown that

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relatively few antibiotics penetrate into the cytoplasm of these cells and kill intracellular *S. aureus* [15].

Invasion of bone cells by *S. aureus* occurs via a receptor-mediated pathway that requires the involvement of host cell cytoskeletal elements [13,16]. The receptor on the surface of bone cells responsible for binding *S. aureus* and resulting in its internalization has not been identified. However, we have demonstrated that the *S. aureus* fibronectin-binding proteins (FnBPs) are essential in the invasion process [16]. Thus a possible mechanism of uptake by bone cells is that the *S. aureus* FnBPs bind to fibronectin which attaches to, or is attached to, the integrin $\alpha 5 \beta 1$ thus triggering internalization. Indeed the involvement of the integrin $\alpha 5 \beta 1$ in the internalization of *S. aureus* by epithelial cells has been demonstrated [17].

S. epidermidis has been shown to bind to fibronectin [18,19] leading us to speculate that bone cells may also internalize this bacterium by a receptor-mediated process. Therefore in this study we examined the capacity of different strains of *S. epidermidis* to invade bone cells. We also examined if the pathway utilized by *S. epidermidis* to invade bone cells was similar to that employed by *S. aureus*.

2. Methods

2.1. Human bone cell culture

The human bone cell line, MG63, was routinely cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM), containing 25 mM HEPES and supplemented with 10% fetal calf serum (Pierce and Warriner, Chester, UK), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma–Aldrich Ltd.). In co-culture with bacteria the supplemental antibiotics were omitted.

2.2. Bacterial strains and growth media

The strains of bacteria used in this study are detailed in Table 1. Prior to assays, staphylococci were grown overnight

in 10 ml of brain heart infusion broth (Oxoid, Basingstoke, UK), aerobically in a 37 °C shaking incubator. Bacterial cell numbers were estimated spectrophotometrically at 650 nm. Bacteria were harvested by centrifugation and resuspended in assay medium to give 2.5×10^7 cfu/100 µl or 2.5×10^6 cfu/100 µl representing a multiplicity of infection (MOI) of 500:1 or 50:1, respectively, when co-cultured with bone cells.

2.3. Cloning and expression of the D1–D4 repeat region of the *S. aureus* fibronectin-binding protein

The oligonucleotides, 5'-GCATGCGAAGGTGGCCAAAA TAGCG-3' and 5'-AAGCTTTGGCGTTGGTGGCAGATTG-3', were designed to amplify a 417-bp fragment encoding the D1–D4 repeat region of the *fnbB* gene from *S. aureus* 8325-4 and contained recognition sequences (italicized) for the restriction enzymes *Sph*I and *Hind*III, respectively. The PCR fragment was initially cloned into PCR2.1-TOPO (Invitrogen) and transformed into *Escherichia coli* TOP10. The insert was extracted from PCR2.1-TOPO on an *Sph*I–*Hind*III fragment and ligated to *Sph*I and *Hind*III digested pQE30 (Qiagen Ltd., Crawley, UK). The ligation mix was transformed into *E. coli* M607(pREP4) and transformants were selected by growth at 30 °C on LB agar containing 100 µg/ml ampicillin and 25 µg/ml kanamycin.

For gene expression, positive clones were grown overnight on LB containing antibiotics after which time they were diluted 1:10 in fresh broth and incubated for a further 2 h at 30 °C. Gene expression was induced with 1 mM IPTG for 4 h at 30 °C. Cells were harvested by centrifugation at $6000 \times g$ for 20 min and then resuspended and lysed for 20 min in B-PER (Pierce and Warriner Ltd.) containing 20 mM imidazole, 1.5 mM PMSF, 1 µM pepstatin and 10 µM leupeptin. Lysates were clarified by centrifugation at $23,000 \times g$ for 10 min. Recombinant protein was purified using Ni-NTA-agarose columns under native conditions according to the manufacturer's instructions (Qiagen) except that after loading lysates onto the column an additional

Table 1
Strains of bacteria used in this study

Strains	Relevant genotype or phenotype	Reference or source
<i>E. coli</i>		
TOP10		Invitrogen
M607	Derivative of JM83 disrupted in the <i>msbB</i> gene	[21]
M607 (pREP4)	As M607 containing pREP4 (Qiagen)	This study
<i>S. aureus</i>		
8325-4	Derivative of NCTC8325, cured of known prophages	[22]
LS-1	Isolated from the swollen joint of an arthritic NZB/W mouse	[23,24]
<i>S. epidermidis</i>		
NCTC11047	Reference type strain	NCTC
K28	SdrG prototype strain	[25]
1457	Biofilm forming isolated from central venous catheter	[26]
9142	Biofilm forming strain isolated from a blood culture	[26]
19	Fibrinogen binding isolated from a patient with peritonitis	[27]
HB	Fibrinogen binding isolate from a patient with osteomyelitis	[27]

column wash consisting of 2.5 mg/ml of polymyxin B in wash buffer was introduced to remove any possible contamination by lipopolysaccharide. Finally the recombinant FnBPB[D1–D4] was dialyzed against phosphate buffered saline.

2.4. Invasion of bone cells by staphylococci

MG63 bone cells were seeded at 50,000 cells per well into 24-well tissue cultures plates (Sarstedt Ltd., Leicester, UK) in 1 ml of growth medium with antibiotics and cultured for 2 days. At the end of the first day the cells were washed twice with 1 ml of DMEM and then incubated with 1 ml of growth medium without antibiotics. Bacteria (100 µl) were added to the bone cells and incubated for 2 h in a 37 °C/5% CO₂ incubator. After 2 h of co-culture of bacteria and bone cells, cultures were washed twice with 1 ml DMEM. To each well 1 ml of fresh medium containing 100 µg/ml of gentamicin (Invitrogen) was added and the cultures were incubated for a further 2 h. The cultures were then washed three times with 1 ml of DMEM and the bacteria were harvested by adding 1 ml of 0.1% Triton X-100 to each well. Enumeration was performed by serial dilution and plate counting on 5% blood agar plates (Oxoid).

For confocal experiments, bone cells were seeded onto 19 mm glass coverslips. Subconfluent cells were infected with FITC-labeled *S. epidermidis* strain 19 at an MOI of 200:1 for 2 h, washed three times with PBS before a 10-min fixation with 4% paraformaldehyde. To visualize filamentous actin, the bone cells were stained with 1 U/ml of rhodamine-conjugated phalloidin (Cambridge BioSciences Ltd.) for 20 min in the dark followed by three washes with PBS. The distribution of fluorescent *S. epidermidis* and F-actin was monitored with a Leica TCS-NT confocal laser scanning microscope using standard filter settings and sequential scanning mode to avoid overlap of emission from the fluorophores. The thickness of the optical sections was set to 0.6 µm and for some images the X–Y sections were added together with the extended focus option of Leica TCS-NT software and edited with Adobe Photoshop software.

2.5. Inhibition of staphylococcal invasion of bone cells

MG63 bone cells were cultured as described above for the invasion assay. One hour prior to adding bacteria to the bone cells the inhibitors (at the concentrations detailed below) were added separately to triplicate wells. To examine the role of staphylococcal binding to fibronectin and the α5β1 integrin in the invasion process, recombinant FnBPB[D1–D4] was used at a concentration of 1 µM and the blocking antibody, JBS5, to α5β1 (Chemicon Ltd., Hampshire, UK) was used at 2 µg/ml.

2.6. Statistics

All data are shown as the mean ± the standard deviation. Data were compared using Student's *t*-test.

3. Results

3.1. Invasion of bone cells by *S. epidermidis*

Given that *S. epidermidis* is a commensal opportunistic pathogen and it is generally accepted that infections are caused by the strain a patient is colonized with (or possibly clinical staff), we conducted initial studies with the type strain of *S. epidermidis*, NCTC11047, which is not associated with infection, to determine if this bacterium could invade bone cells. Fig. 1 shows that the type strain NCTC11047 invaded bone cells and that the number of intracellular bacteria was dependent on the multiplicity of infection (MOI), with greater numbers of *S. epidermidis* being taken up as the MOI increased from 10:1 to 500:1, at a higher MOI of 1000:1 the numbers of intracellular bacteria recovered began to decline.

To determine if there were strain differences in the capacity of *S. epidermidis* to invade bone cells we compared the invasive capacity of strains isolated from heterologous sites/infections. Comparison of the capacity of six different strains of *S. epidermidis* invading bone cells revealed that three strains, 1457, 9142 and K28, were not taken up by bone cells over the period of the experiment (Fig. 2). Three strains of *S. epidermidis*, NCTC11047, HB and 19, invaded bone cells to varying degrees with one strain (strain 19) invading at significantly higher levels than the other strains (Fig. 2). Examination of the invasion of bone cells by strain 19 at different MOIs revealed a similar pattern to that obtained with strain NCTC11047 (Fig. 1), i.e. an increase in intracellular bacteria recovered up to about an MOI of 600:1 after which it began to plateau (data not shown). The number

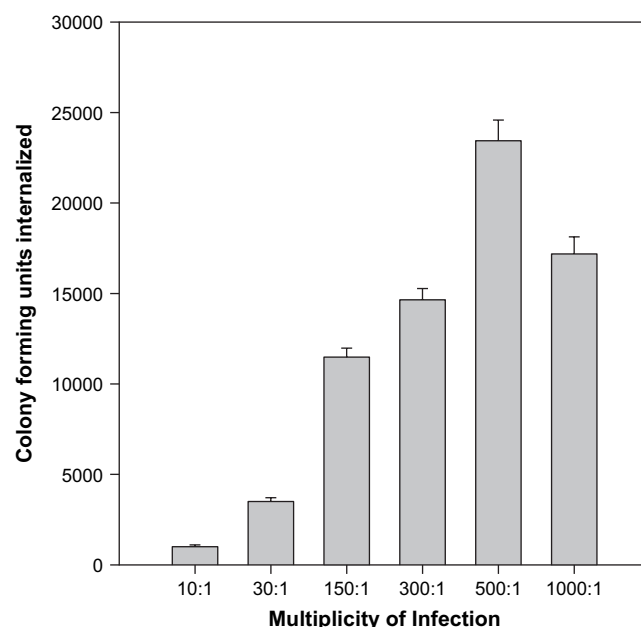


Fig. 1. Invasion of bone cells by the type strain of *S. epidermidis*, NCTC11047. Strain NCTC11047 was co-cultured with bone cells at MOIs of between 10:1 and 1000:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

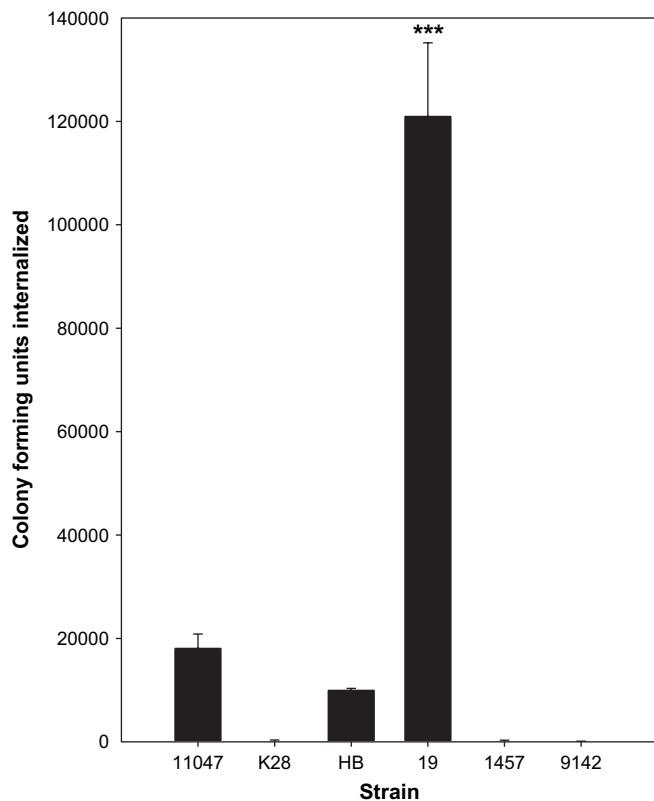


Fig. 2. The capacity of six strains of *S. epidermidis* to invade bone cells. Strains NCTC11047, K28, HB, 19, 1457 and 9142 were co-cultured with bone cells at an MOI of 500:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. The invasiveness of *S. epidermidis* strains was compared to that of strain 11047 using Student's *t*-test. *** $P < 0.001$.

of colony forming units of *S. epidermidis* strain 19, at an MOI of 300:1, internalized by bone cells was of the same order of magnitude as *S. aureus* strains 8325-4 and LS-1 at an MOI of 50:1 (Fig. 3). Interestingly when we investigated the invasion of bone cells by *S. epidermidis* strain 19 at a low MOI (1:1) it was possible to detect intracellular replication of the bacterium over a 12-h time period (data not shown). Further evidence that *S. epidermidis* can invade bone cells is presented in the confocal microscopic images of Fig. 4 showing FITC-labeled staphylococci within bone cells stained with rhodamine–phalloidin to detect actin.

3.2. Inhibition of staphylococcal invasion of osteoblasts

Since *S. epidermidis* is known to bind to fibronectin it was possible that the invasion of bone cells was mediated through this molecule. Fig. 5 shows the effect of pre-incubating bone cells with either recombinant FnBPB[D1–D4] or the antibody JBS5 on the internalization of staphylococci. Recombinant FnBPB[D1–D4] blocked invasion of bone cells by *S. aureus* but had no effect on the invasion by *S. epidermidis*. Similarly the $\alpha 5\beta 1$ -blocking antibody, JBS5, inhibited invasion of bone cells by *S. aureus* but had no effect on the invasion of *S. epidermidis* (Fig. 5).

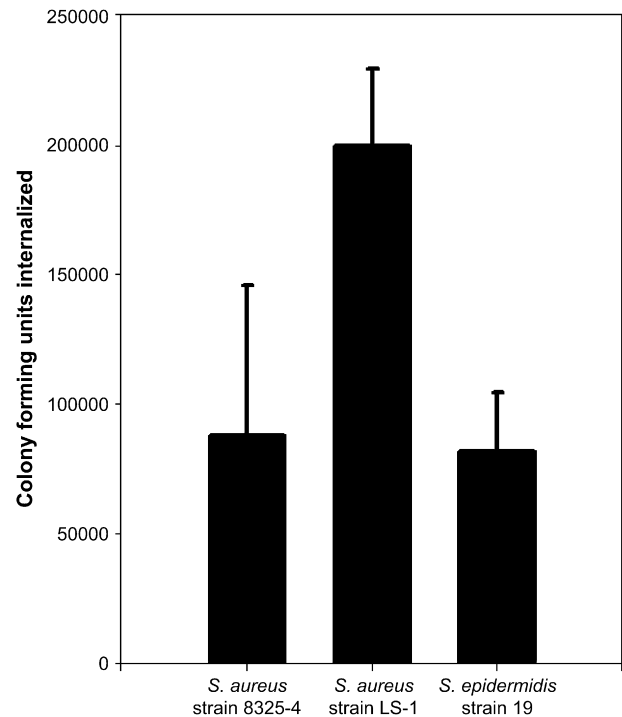


Fig. 3. Comparison of the ability of *S. epidermidis* strain 19, *S. aureus* 8325-4 and LS-1 to invade bone cells. Staphylococci were co-cultured with bone cells at an MOI of 300:1 in the case of *S. epidermidis* or 50:1 in the case of *S. aureus*. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

4. Discussion

There is now a significant body of evidence showing that *S. aureus* is not exclusively an extracellular pathogen and is taken up by a variety of cell types including bone cells [11–14,16]. This led us to examine the possibility that *S. epidermidis* may also be taken up by bone cells.

We found that a reference type strain of *S. epidermidis*, NCTC11047, could invade bone cells. The number of bacteria that gained entrance into the osteoblasts was dependent on the infectious dose with an MOI of 500:1 resulting in about 3.5-fold more *S. epidermidis* being internalized than an MOI of 50:1. To ascertain if there were differences in the capacity of *S. epidermidis* strains to invade bone cells, a trait we found in strains of *S. aureus* [13,16], a total of six strains of *S. epidermidis* were tested in the invasion assay. Because *S. epidermidis* is a commensal opportunistic pathogen and the expression of specific virulence traits is not necessarily related to the site of infection, the strains chosen were from distinct infections/sites. The results of this assay demonstrated that *S. epidermidis* strains have very different capacities to invade bone cells. Three of the strains examined did not invade bone cells to any appreciable level, whilst three strains were significantly invasive although to different extents.

The capacity of *S. epidermidis* strains to invade bone cells did not correlate with the clinical source of the strain as demonstrated by the fact that strain 19, from a patient with peritonitis, was internalized to a greater extent than strain HB which

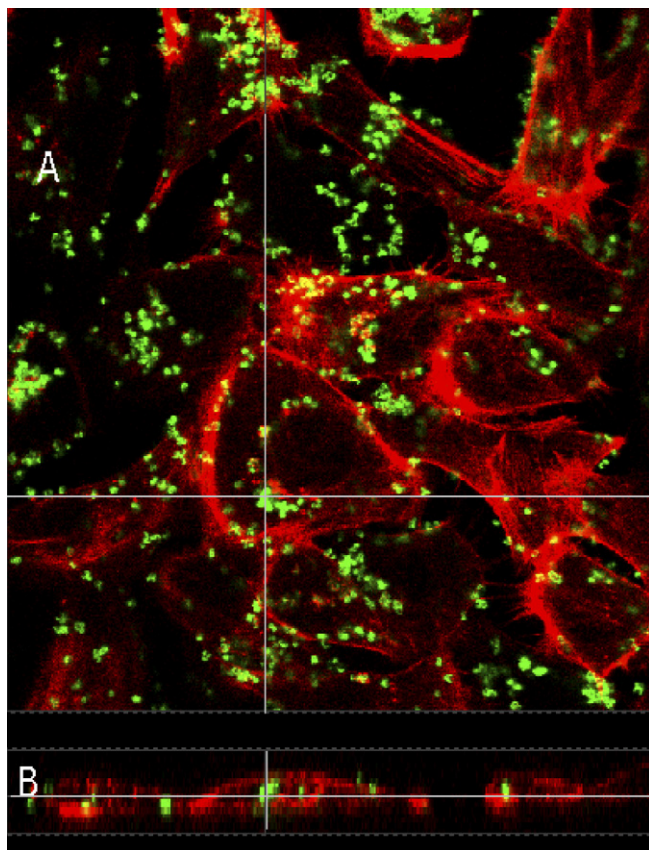


Fig. 4. Confocal microscopic examination of bone cells after co-culture with *S. epidermidis* strain 19. Bone cell actin filaments were stained with rhodamine-conjugated phalloidin (shown in red) and FITC-labeled *S. epidermidis* (shown in green). The panels depict an X–Y confocal image through an area of the co-culture and an X–Z image of the same area. The cross hair provides a line of reference. Panel (A) shows an image of the X–Y plane through a representative area of the co-culture. Panel (B) is an X–Z image reconstructed from 0.5 μ m optical sections through the area of the co-culture shown in panel A.

was isolated from a patient with osteomyelitis, which was actually internalized to a lesser extent than NCTC11047 a common laboratory strain isolated from the nose. This finding is unsurprising given that *S. epidermidis* is an opportunistic pathogen which gains access to the site of infection through a local breach of the mucosal barrier. However, the differences in the capacities of *S. epidermidis* strains to invade bone cells may affect the prognosis of disease.

We have previously shown that in the case of *S. aureus* the fibronectin-binding proteins FnBPA and/or FnBPB are essential for the invasion of bone cells [16] and that *S. epidermidis* can also bind to fibronectin [19]. Although our own studies show that the predominant binding site on fibronectin is in the cell-binding domain we have also found binding to the same region of fibronectin as bound by *S. aureus*. This suggested that *S. epidermidis* might gain entry into host cells in a similar manner to *S. aureus* by binding to fibronectin which attaches to, or is attached to, the integrin $\alpha 5 \beta 1$ thus triggering internalization. To investigate this the possibility of the effect of a recombinant fragment of the *S. aureus* fibronectin-binding protein, FnBPB[D1–D4], on the capacity of *S. epidermidis* to

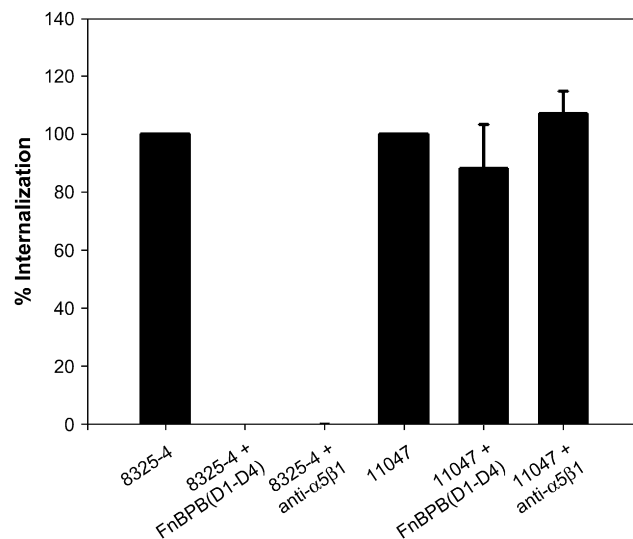


Fig. 5. The effect of pre-incubating bone cells with either recombinant FnBPB[D1–D4] or the $\alpha 5 \beta 1$ -blocking antibody, JBS5, on the internalization of staphylococci. Bone cells were incubated with 1 μ M FnBPB[D1–D4] or 2 μ g/ml of JBS5 1 h prior to co-culture with staphylococci. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

invade bone cells was examined. Whilst FnBPB[D1–D4] inhibited the invasion of bone cells by *S. aureus* it had no effect on the invasive capacity of *S. epidermidis*. This suggests that the invasion of bone cells by *S. epidermidis* does not involve binding to fibronectin or that *S. epidermidis* binds to a different region of fibronectin from that bound by *S. aureus*. Using a blocking antibody to the integrin $\alpha 5 \beta 1$, we have demonstrated that this fibronectin binding integrin is required for the invasion of bone cells by *S. aureus* but not *S. epidermidis*. Thus the invasion of bone cells by *S. aureus* occurs via a fibronectin bridge to the $\alpha 5 \beta 1$ integrin. However, invasion of bone cells by *S. epidermidis* is not dependent on this receptor.

The studies reported here demonstrate that *S. epidermidis* can invade bone cells. However, the host cell processes involved in this process differ significantly from those utilized by *S. aureus*. The data presented herein demonstrate that the invasion of bone cells by *S. epidermidis* is not via the $\alpha 5 \beta 1$ receptor. This is in contrast to the findings for a number of Gram-positive organisms which gain access into the host cell cytoplasm by a mechanism involving binding to fibronectin and bridging to the $\alpha 5 \beta 1$ receptor, e.g. *S. aureus* and *Streptococcus pyogenes* [20].

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