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Differential interactions of *Streptococcus gordonii* and *Staphylococcus aureus* with cultured osteoblasts

C.E. Jauregui[1,*], J.P. Mansell[1,†], M.A. Jepson[2] and H.F. Jenkinson[1]

1 School of Oral and Dental Sciences, University of Bristol, Bristol, UK
2 School of Biochemistry, University of Bristol, Bristol, UK

Correspondence: Howard F. Jenkinson, School of Oral and Dental Sciences, University of Bristol, Lower Maudlin Street, Bristol BS1 2LY, UK Tel.: +44 117 342 4424; fax: +44 342 4313; E-mail: howard.jenkinson@bristol.ac.uk
*Present address: Center for Oral Health and Systemic Disease, School of Dentistry, University of Louisville, Louisville, KY, USA.
†Present address: Musculoskeletal Research Unit, Avon Orthopaedic Centre, School of Clinical Sciences, University of Bristol, Westbury-on-Trym, UK.

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SUMMARY

The impedance of normal osteoblast function by microorganisms is at least in part responsible for the failure of dental or orthopedic implants. *Staphylococcus aureus* is a major pathogen of bone, and exhibits high levels of adhesion and invasion of osteoblasts. In this article we show that the commensal oral bacterium *Streptococcus gordonii* also adheres to and is internalized by osteoblasts. Entry of *S. gordonii* cells had typical features of phagocytosis, similar to *S. aureus*, with membrane protrusions characterizing initial uptake, and closure of the osteoblast membrane leading to engulfment. The sensitivities of *S. gordonii* internalization to inhibitors cytochalasin D, colchicine and monensin indicated uptake through endocytosis, with requirement for actin accumulation. Internalization levels of *S. gordonii* were enhanced by expression of *S. aureus* fibronectin-binding protein A (FnBPA) on the *S. gordonii* cell surface. Lysosomal-associated membrane protein-1 phagosomal membrane marker accumulated with intracellular *S. aureus* and *S. gordonii* FnBPA, indicating trafficking of bacteria into the late endosomal/lysosomal compartment. *Streptococcus gordonii* cells did not survive intracellularly for more than 12 h, unless expressing FnBPA, whereas *S. aureus* showed extended survival times (>48 h). Both *S. aureus* and *S. gordonii* DL-1 elicited a rapid interleukin-8 response by osteoblasts, whereas *S. gordonii* FnBPA was slower. Only *S. aureus* elicited an interleukin-6 response. Hence, *S. gordonii* invades osteoblasts by a mechanism similar to that exhibited by *S. aureus*, and elicits a proinflammatory response that may promote bone resorption.

INTRODUCTION

Oral streptococci are ubiquitous in the human upper respiratory tract, and are early colonizers of the clean tooth surface (Nyvad & Kilian, 1987). The bacteria also colonize the epithelial surfaces of the tongue, buccal mucosa, gingival sulcus and nasopharynx (Frandsen *et al.*, 1991). *Streptococus gordonii* is a mitis group streptococcus associated with the formation of oral microbial communities, and is generally considered to be an oral commensal. However, outside of the normal oral environment, e.g. in the

bloodstream, *S. gordonii* has the potential to initiate infections such as infective endocarditis (Petersen *et al.*, 2010; Jung *et al.*, 2012).

Successful integration of oral implants requires osseointegration, defined as the structural and functional connection between living bone and the load-bearing surface of the implant (Brånemark *et al.*, 1985). For this to occur, new matrix materials must be deposited by healthy bone-forming cells (osteoblasts). These cells are essential to bone and tooth development, and may have an innate immune function (Ruiz *et al.*, 2003). Failure of implants can be related to microbial infection, and in the oral cavity this is often polymicrobial in etiology (Heitz-Mayfield & Lang, 2010). The microbial communities induce destructive inflammatory responses that can result in resorption of alveolar bone and implant rejection. *Staphylococcus aureus* has often been associated with oral implant failure, and is also the primary cause of osteomyelitis (Arciola *et al.*, 2012). The bacteria become internalized by human osteoblasts, using host cell cytoskeletal elements, resulting in the induction of apoptosis (Jevon *et al.*, 1999; Alexander *et al.*, 2001). In septic arthritis, *Streptococcus pyogenes* bacteria are internalized by osteoblasts, upregulating expression of receptor activator of nuclear factor-κB ligand (RANKL) and triggering bone destruction (Okahashi *et al.*, 2003). Internalization of *S. gordonii* by human umbilical vein endothelial cells (HUVECs) also depends upon eukaryotic cellular functions. Most notably, inhibition of endocytosis, particularly actin filament re-arrangement, attenuates streptococcal cell internalization (Stinson *et al.*, 2003; Nobbs *et al.*, 2007).

Osteoblasts produce fibronectin (Fn), and express the primary Fn-receptor integrin $\alpha_5\beta_1$ at all stages of differentiation (Moursi *et al.*, 1996;). Fn is required by osteoblasts for differentiation, survival, and matrix mineralization (Moursi *et al.*, 1996; Globus *et al.*, 1998; Stephansson *et al.*, 2002). A large number of different Fn-binding proteins have been identified in gram-positive bacteria (Nobbs *et al.*, 2009), and they are able to mediate bacterial cell adhesion to the host, and in some instances internalization through Fn-dependent pathways (Amelung *et al.*, 2011). *Streptococcus gordonii* expresses several proteins known to facilitate binding to immobilized Fn. Strains unable to produce cell surface proteins CshA and CshB are reduced in adherence to immobilized Fn and are abrogated in internalization by HUVECs (McNab *et al.*, 1996; Stinson *et al.*, 2003). The antigen I/II family proteins SspA and SspB are also reportedly able to interact with immobilized Fn (Nobbs *et al.*, 2007).

Various strains of *S. aureus* differ markedly in their abilities to bind Fn (Proctor *et al.*, 1982; Shinji *et al.*, 2011). Fn-binding isolates are associated with orthopedic implant failure to a greater degree than those associated with nasal carriage. Invasive *S. aureus* strains are more likely to have two, rather than one, *fnbp* genes encoding Fn-binding proteins (FnBPs) (Peacock *et al.*, 2000). Invasive potential of *S. aureus* is related to the number of FnBPs expressed and their overall affinities for Fn-binding (Massey *et al.*, 2001; Edwards *et al.*, 2011; Casillas-Ituarte *et al.*, 2012). The *S. aureus fnbp* mutants are reduced in their ability to adhere to epithelial cells, and internalization is abrogated by 60% (Brouilette *et al.*, 2003). The Fn-binding proteins FnBPA and FnBPB confer invasiveness of epithelial cells upon *Lactococcus lactis* (Sinha *et al.*, 1999). *Staphylococcus aureus* strains defective in FnBPs are impaired in invasion of epithelial cells, endothelial cells, fibroblasts (Sinha *et al.*, 1999), keratinocytes (Kintarak *et al.*, 2004) and osteoblasts (Ahmed *et al.*, 2001; Williams *et al.*, 2002). Once internalized by bronchial epithelial cells or HUVECs, *S. aureus* may be found associated with lysosomal-associated membrane protein (LAMP-1) - enriched lysosomal organelles within 1 h of infection (Jarry & Cheung, 2006). Most strains of *S. aureus* are subsequently eliminated by 24 h, but strains producing small colony variants can withstand the bactericidal activity of HUVEC lysosomes (Schröder *et al.*, 2006).

Cytokines interleukin-1β (IL-1β), IL-6 and IL-8 are pivotal in the regulation of bone turnover. Interleukin-1β, tumor necrosis factor-α and interferon-γ induce apoptosis in mouse osteoblasts, potentially implicating cytokine-induced apoptosis in the localized bone destruction seen in inflammatory bone diseases such as periodontal disease (Ozeki *et al.*, 2002). Additionally, IL-6 and IL-8 can both act on osteoclasts, bone resorbing cells. The IL-8 receptors on osteoclasts have regulatory effects on their development and activity (Fuller *et al.*, 1995). Interleukin-8 is a potent chemoattractant activator of neutrophils and is synthesized and secreted at high basal levels in osteoclasts (Rothe *et al.*, 1998). Therefore IL-6 and

IL-8 induction in osteoblasts can not only elicit a host immune response, but also lead to downstream effects on osteoclasts culminating in bone resorption. This has direct relevance in a clinical setting, with several bone-loss diseases associated with induction of IL-6 or IL-8 in osteoblasts (Siddiqi *et al.*, 1998; Steddon *et al.*, 2004). Bacterial challenge has been shown to elicit cytokine production by osteoblasts, including cytokines IL-1β and IL-6 that regulate osteoclastogenesis through RANKL (Sakurai *et al.*, 2003; Scian *et al.*, 2012). Expression of IL-6 is induced in *S. aureus*-infected mice *in vivo*, and in subjects with *S. aureus*-associated osteomyelitis (Marriott *et al.*, 2004).

In this article we have addressed the question as to whether *S. gordonii* cells interact with osteoblasts such that osseointegration might be affected. We show that commensal *S. gordonii* cells adhere to and are internalized by osteoblasts, and induce the production of proinflammatory cytokines. Internalization levels can be enhanced by expression of *S. aureus* FnBPA on the *S. gordonii* cell surface. Both *S. gordonii* and *S. aureus* cells appeared to be internalized via the classical endocytic pathway, whereas expression of FnBPA was associated with prolonged intracellular survival times.

METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains used in this study were as follows: *S. gordonii* DL-1 wild-type, *S. gordonii* (FnBPA) and *S. aureus* Col (kindly provided by Simon Foster, University of Sheffield). Culture stocks were maintained at −70°C in brain–heart infusion broth containing 10% glycerol. Plasmid pKS80-SasG was kindly provided by Tim Foster, Trinity College, Dublin. Bacterial cultures were grown statically in brain–heart infusion medium at 37°C under reduced oxygen. Bacterial cells in exponential growth phase were harvested by centrifugation (5000 ***g***, 10 min), washed twice by alternate suspension in phosphate-buffered saline (PBS) and centrifugation, and suspended in PBS at a concentration of 5.0×10^6 colony forming units (CFU) ml^{-1} for adherence and internalization assays (see below). Minimum inhibitory concentration assays were performed on *S. gordonii* and *S. aureus* with penicillin and gentamicin to determine their antibiotic sensitivities. In addition, bacteria were exposed to various Triton X-100 concentrations to determine the most appropriate concentration for eukaryotic cell lysis, without bactericidal effects, in antibiotic exclusion assays.

Cell culture

Cells of the human osteoblastic cell line MG63 were routinely cultured in conventional tissue flasks at 37°C, 5% CO_2, 100% humidity. Osteoblasts were grown statically for 72–96 h to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (4 mM), sodium pyruvate (1 mM), penicillin G (Pen) (0.1 U ml^{-1}) and streptomycin (100 ng ml^{-1}). The growth medium was also supplemented with non-essential amino acids. Unless otherwise stated, cells were maintained in this medium (DMEM-FCS). Cells were routinely seeded 1.6×10^4 cells into flasks and passaged by trypsinization twice weekly. To prepare cells for experiments (usually in 24-well plates, unless otherwise stated), 2.0×10^4 MG63 cells per well were incubated under conventional culture conditions for 72 h before achieving a confluent monolayer. For a majority of infection experiments described, DMEM minus FCS and antibiotics was used, and referred to as serum-free culture medium (SFCM).

Bacterial adherence and invasion assays

Bacterial invasion was determined using a modification of the method described by Jevon *et al.* (1999). Confluent MG63 cell monolayers were washed with PBS and then incubated with SFCM for 3 h to bring the cells to senescence. Bacterial suspension was then added [multiplicity of infection (MOI) 10 : 1] and incubation was continued for a further 2 h under conventional cell culture conditions. Samples of suspension were taken at time zero (t_0) and after 2 h of incubation (t_1), serially diluted 10-fold in PBS and spread onto brain–heart infusion agar plates to obtain numbers of CFU representing total viable counts. For adhesion assays at t_1, wells were washed three times in PBS, incubated with trypsin-EDTA (10 min) to detach the monolayer, and the cells were lysed by addition of Triton X-100 (0.2%). Numbers of bacteria (CFU) were determined by serial dilution and agar plate count. To assess levels of bacterial internaliza-

tion, medium was removed from the culture wells at t_1 and replaced with SFCM containing 5 μg penicillin ml^{-1} and 100 μg gentamicin ml^{-1}. After further incubation at 37°C for 2 h, cells were washed once in SFCM, detached and lysed as above, and bacterial CFU was determined (t_2). No CFU could be detected in the culture fluid at t_2 before cell lysis. To determine the effects of metabolic inhibitors, internalization assays were performed as described above in the presence of 0.5–1.0 μM cytochalasin D, 1–10 μM colchicine, 25–250 μM ouabain, 4–40 μM monensin, or 25–250 μM monodansylcadaverine. In each instance the inhibitor was added to the MG63 cell monolayer for 1 h before the addition of bacteria. For determination of longer-term intracellular survival of bacteria, antibiotic exclusion assays were performed as described above for up to 48 h and internalized CFU was measured by agar plate counts.

Microscopy

To visualize the effects of bacteria on monolayer integrity, infected or control (uninfected) cells on 55-mm Petri dishes were rinsed with PBS, fixed for 5 min in 95% methanol, stained with hematoxylin for 2 min, rinsed in H_2O for 5 min, and then stained with eosin for 30 s. Following a wash with distilled H_2O the cells were imaged with an Olympus CX41 microscope with a Colorview Soft Imaging System.

For scanning electron micrpscopy of the internalization process, MG63 cells were seeded into plastic wells (10^4 cells per well) containing 14 mm ASTM Grade 1 titanium disks and incubated under standard culture conditions until confluent (96 h). Following 3 h of starvation in SFCM, cells were infected at an MOI of 10 with *S. aureus* or *S. gordonii* for 1 h. Medium was then removed from the disks, and the cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 22°C. Samples were dehydrated successively for 30 min in each of the following concentrations of ethanol: 20%, 40%, 60%, 80%, Absolute. The disks were then incubated with hexamethyldisilazane for 30 min, dried, mounted on stubs, coated with a 15-nm thick layer of platinum/lead and imaged with a JEOL 6330 FEG scanning electron microscope.

Fluorescence microscopy was used to show Fn-binding by whole bacterial cells. Exponential phase bacteria were harvested by centrifugation, washed with PBS and then incubated with 2 μg ml^{-1} Fn for 30 min. Bacteria were collected by centrifugation, washed three times with PBS to remove unbound Fn, incubated for 30 min with rabbit anti-Fn primary antibody (Dako; dilution 1 : 1000) followed by a fluorescein isothiocyanate (FITC) -conjugated anti-rabbit secondary antibody (at 1 : 1000). Bacterial cells were then washed three times with PBS, suspended in PBS, and examined with an Olympus CX41 microscope with Colorview Soft Imaging System.

For co-localization studies glass cover slips (in plastic wells) were seeded with 50 μl of cell suspension of freshly harvested MG63 cells, incubated for 45 min under conventional culture conditions to allow cells to attach and then incubated for 16 h in DMEM-FCS. Infection with bacterial strains was performed as described and at t_1 or t_2 cover slips were removed, washed four times in PBS, fixed in 95% methanol for 20 min at 4°C, and then air-dried. Cover slips were blocked with 5% fetal bovine serum in PBS for 30 min at 22°C and incubated with mouse anti-human LAMP-1/CD107a antibodies (B & D Pharmingen, San Diego, CA) and rabbit anti-*S. gordonii* antibodies diluted 1 : 50 in PBS for 50 min at 22°C. The *S. gordonii* antibodies also bound to *S. aureus* via staphylococcal protein A. Following four washes in PBS, cover slips were incubated with Alexa 594-conjugated anti-mouse antibodies and FITC-conjugated anti-rabbit antibodies (diluted 1 : 100) for 45 min in the dark. Cover slips were washed five times with PBS, mounted on glass slides using Vectashield Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Labs. Inc., Burlingame, CA), and visualized by confocal laser scanning microscopy. A Leica SP2-AOBS confocal laser scanning microscope attached to a Leica DMIRE2 inverted microscope was employed, using a 100 × objective lens with imaging parameters selected using LEICA LCS software to optimize resolution.

Genetic techniques

Molecular cloning and expression work was undertaken with approval from the University of Bristol Genetic Manipulation and Biosafety Committee. Chromosomal DNA was isolated from *S. aureus* Col using GenElute Bacterial Genomic DNA kit (Sigma, St Louis, MO) with lysozyme (2×10^6 U ml^{-1}) and lysostaphin (200 U ml^{-1}). Primers specific to the

upstream start and downstream of *fnbpA* (see Supplementary material, Table S1) were used in polymerase chain reaction (PCR) amplification with the proof-reading enzyme *pfx* under the following conditions: initial denaturation step (2 min at 94°C), 34 cycles of denaturation (15 s at 94°C), annealing (30 s at 55°C) and extension (3 min at 68°C). The 3057-base-pair product corresponding to the full-length *fnbpA* coding region was digested with a combination and *Bam*HI and *Nsi*I, and cloned into pKS80 (Roche *et al*., 2003) digested with *Bam*HI and *Pst*I. This was then transformed into *S. gordonii* DL-1 as described by Haisman & Jenkinson (1991) to generate *S. gordonii* (FnBPA) expressing FnBPA on the cell surface. The presence of pKS80-derived plasmids in *S. gordonii* was confirmed by PCR with relevant primers (see Supporting information, Table S1).

Analysis of mRNA expression

MG63 cells were grown to confluence in cell culture flasks and antibiotic exclusion assays were performed as described previously. At various times up to 48 h postinfection, cell monolayers were detached by trypsinization, the cells were harvested by centrifugation (900 ***g***, 10 min) and the RNA was isolated from the cell pellets using a Qiagen RNeasy plus kit according to the manufacturer's instructions. Cells were homogenized by passing samples twice through QIA Shredder columns (Qiagen, Hilden, Germany) and genomic DNA was removed using gDNA Eliminator columns (Qiagen). The amount and purity of samples was assessed by the ratio of absorbances at 260 nm and 280 nm. The quality of RNA was confirmed by gel electrophoresis. RNA samples (1 μg) were reverse transcribed with oligo-dT primers using BioRad iScript cDNA synthesis kit (BioRad, Hercules, CA). The PCR was performed on the cDNA with IL-specific primers (see Supplmentary material, Table S1) under the following conditions (35 cycles): denaturation, 94°C for 15 s; annealing, 55°C for 30 s (IL-1β, IL-8) or 61°C for 30 s (IL-6), and extension at 68°C for 10 s. TATA-box binding protein (TBP) was also amplified to ensure equal RNA inputs and reverse transcription (RT) efficiency. Primers specific to TBP worked at both annealing temperatures used. Following PCR, 10% of each amplified product was electrophoresed through an agarose gel containing ethidium bromide and visualized using UV illumination.

Measurement of cytokine secretion

Cytokine secretion into infected or noninfected culture fluid (supernatant) was determined with a Quantikine kit (R & D Systems, Minneapolis, MN). Supernatant samples, standards and appropriate controls were added to microtitre plate wells pre-coated with IL-6 or IL-8 capture antibodies and incubated for 2 h at 22°C. Wells were washed four times in assay buffer and then horseradish peroxidase-linked antibody specific to IL-6 or IL-8 was added to wells and incubated for up to 2 h at 22°C. Bound antibody was detected with tetramethylbenzidine and hydrogen peroxide and absorbance at 450 nm was recorded. Cytokine concentrations were calculated from respective standard curve data, and experiments were repeated on three separate occasions.

RESULTS

S. gordonii interactions with osteoblasts

Initial experiments were designed to investigate if *S. gordonii* DL-1 cells adhered to and were internalized by cultured MG63 osteoblasts. Approximately 10% of input (1×10^7 CFU) *S. gordonii* DL-1 were found to be strongly associated (adhered plus internalized) with MG63 cells (10^5) after 2 h of incubation at 37°C (Fig. 1A). By the antibiotic protection assay (see Methods) it was estimated that 0.01% of the associated bacteria were intracellular at 2 h (Fig. 1A).

To determine the effects of *S. gordonii* internalization on MG63 cell morphology, infected cells were incubated for up to 16 h, in the presence of the antibiotics penicillin and gentamicin, and were examined using hematoxylin & eosin staining. Internalized numbers of *S. gordonii* decreased nearly 10-fold over this time, but the presence of intracellular bacteria caused no visible changes in osteoblast morphology (Fig. 1B) compared with uninfected controls. Bacteria could be clearly seen associated with MG63 cells, with very few bacteria bound to the intercellular spaces (Fig. 1B).

S. aureus interactions with osteoblasts

In contrast to the results obtained with *S. gordonii* DL-1, under the same assay conditions, numbers of *S. aureus* Col internalized at 2 h were at least 100-fold higher (Fig. 2A). The much larger numbers

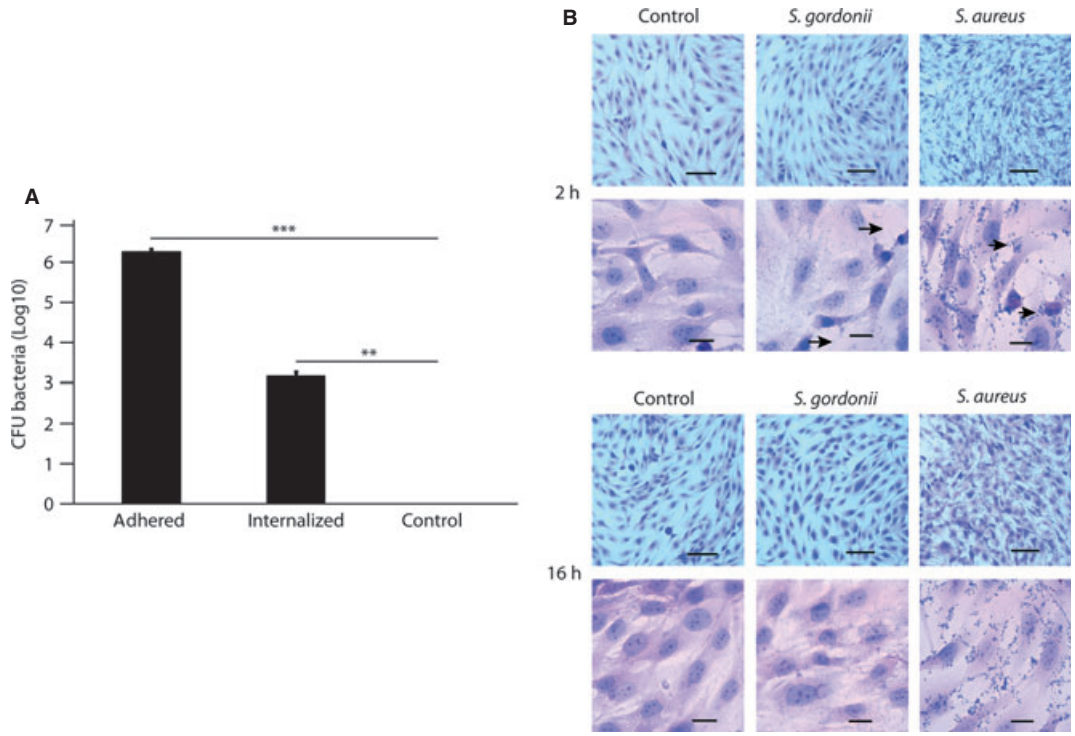

Figure 1 Adhesion and internalization of *Streptococcus gordonii*, and osteoblast morphologies in the presence or absence of bacteria. (A) MG63 osteoblast cells were incubated with *S. gordonii* for 2 h (adhesion) and for a further 2 h in the presence of antibiotics (internalization). The *S. gordonii* colony-forming units (CFU) were determined following lysis of the cell monolayers as described in Methods. Data are representative of three independently performed assays, and error bars indicate standard deviation from the mean ($n = 3$). Unpaired *t*-test: ***$P = 0.0007$; **$P = 0.0033$. (B) MG63 cells were grown on chamber slides, inoculated with 10^6 CFU bacteria (multiplicity of infection 10 : 1) and stained with hematoxylin & eosin after 2 h or 16 h. Scale bars: Upper rows = 200 μm, bottom rows = 50 μm. Bacteria were associated with the osteoblasts and were not adhered to intercellular regions (arrows, 2 h lower panel). *Staphylococcus aureus* was included for comparison and many more cells can be seen associated with the osteoblasts at early times (arrows, 2 h lower panel).

of *S. aureus* associated with MG63 cells compared with *S. gordonii* could be clearly visualized (Fig. 1B). Following 16 h incubation of infected cells with antibiotics, as before, there was only a two- to three-fold reduction in numbers of intracellular *S. aureus* CFU. Much larger numbers of viable *S. aureus* cells were retained intracellularly and morphological changes to the osteoblast monolayer began to appear (Fig. 1B) with the cells becoming more elongated and the cytoplasm shrinking.

Function of fibronectin-binding in internalization

Expression of FnBPA by *S. aureus* facilitates internalization of bacteria by keratinocytes and epithelial cells, and is linked with invasive disease (Sinha *et al.*, 1999; Peacock *et al.*, 2000; Kintarak *et al.*, 2004). Inactivation of the *fnbp* genes in *S. aureus* LS1 inhibited internalization of staphylococci by MG63 cells (Ahmed *et al.*, 2001). On the other hand, *S. gordonii* has a low affinity for Fn, interacting with immobilized Fn but not fluid phase Fn (McNab *et al.*, 1996). To determine if adhesion or invasion levels of *S. gordonii* would be influenced by increased ability to bind Fn, the *fnbpA* gene was isolated from *S. aureus* Col, ligated into a replicative plasmid vector pKS80, and transformed into *S. gordonii* DL-1. As control, pKS80-expressing *S. aureus* SasG, a biofilm-mediating adhesin (Corrigan *et al.*, 2007; Geoghegan *et al.*, 2010), was introduced into *S. gordonii* DL-1. Expression of SasG did not significantly affect *S. gordonii* internalization levels (data not shown). However, >500-fold numbers of intracellular bacteria were obtained for *S. gordonii* expressing FnBPA ($P < 0.001$) (Fig. 2A) compared with *S. gordonii* DL-1 wild-type. The *S. gordonii* DL-1 has previously been shown to bind immobilized, but not soluble, Fn (McNab *et al.*, 1996), whereas FnBPA binds soluble

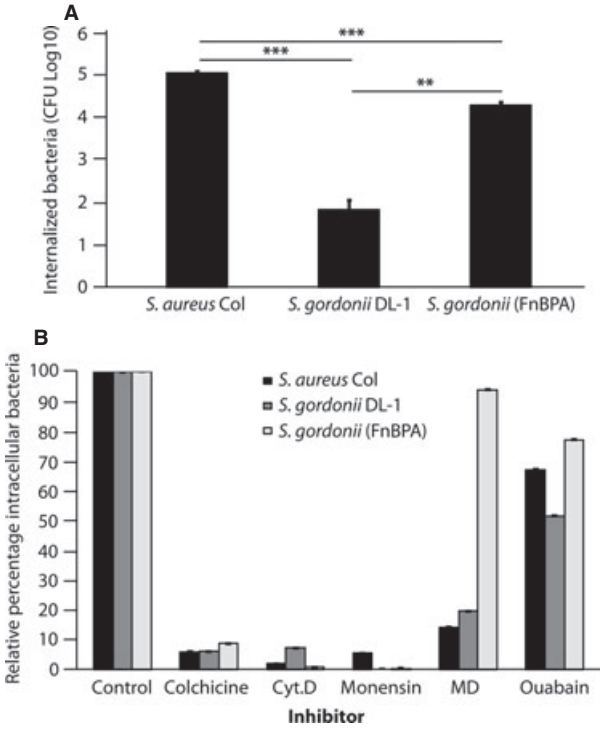

Figure 2 Internalization of *Streptococcus gordonii* and effects of microfilament or microtubule inhibitors. (A) Internalization levels of bacteria by MG63 cells in antibiotic exclusion assays [multiplicity of infection (MOI) 10 : 1]. Results shown are representative of two independent assays. (B) Bacterial internalization by MG63 cells in the presence of various inhibitors. MG63 cells were treated with inhibitors for 1 h before internalization assays being performed: 10 μM colchicine; 1 μM cytochalasin D (Cyt.D); 40 μM monensin; 250 μM monodansylcadaverine (MD); 250 μM ouabain. Initial bacterial inoculum was 10^6 colony-forming units (CFU) ml^{-1} (MOI 10 : 1). Results shown are averaged data from assays run in triplicate ($n = 4$/assay). Error bars indicate standard deviation from the mean ($n = 12$). Tukey–Kramer Multiple Comparison Test: $P < 0.001$. All inhibitors significantly reduced numbers of intracellular bacteria compared with control.

Fn (Sinha *et al.*, 1999). In confirmation of this, *S. gordonii* cells expressing FnBPA clearly bound soluble Fn as detected by immunofluorescence microscopy (Fig. 3), whereas strain DL-1 cells, and various antibody controls, showed no fluorescence.

Bacterial internalization by osteoblasts grown on titanium

For successful osseointegration of titanium implants to occur after surgery, osteoblasts must attach to and grow upon the implant surface. In the laboratory, we have been able to grow MG63 cells on titanium disks and obtain confluent monolayers after 96 h, compared with 72 h on plastic surfaces. The morphologies of titanium-grown cells were similar to those grown on plastic (data not shown). In addition, internalization levels of *S. gordonii* DL-1, *S. aureus* Col and *S. gordonii* (FnBPA) by osteoblasts grown on titanium (MOI 10 : 1), were not statistically different from those determined for osteoblasts grown on plastic (not shown). In scanning electron microscopy images of osteoblasts infected for 1 h with bacteria, fibrillar or pseudopodic projections were visible on MG63 cells (Fig. 4) . These projections could be seen as tethering *S. gordonii* or *S. aureus* to the eukaryotic cell surface (Fig. 4A,B,D,F). Bacterial cells also appeared to be sinking into the cell membrane, consistent with their being engulfed and internalized (Fig. 4C,E,G,H). There were no obvious morphological differences associated with internalization of *S. gordonii* DL-1, *S. gordonii* (FnBP), or *S. aureus* Col.

Internalization mechanisms for *S. gordonii*

To investigate possible differences in the modes of uptake of *S. aureus* or *S. gordonii* by MG63 cells, the effects of various inhibitors of eukaryotic cell functions were determined. In these experiments, antibiotic exclusion assays were performed after treatment of MG63 cells for 3 h with inhibitors (2 h of which comprised the bacterial infection process). MG63 cell viability following inhibitor treatment was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) : PMS (phenazine methosulfate; cell metabolism) and pNPP (p-nitrophenyl phosphate) assays, and there were no significant differences between treated and control (untreated) groups (data not shown).

First, inhibitors of cytoskeletal rearrangement were investigated. Treatment with cytochalasin, an actin filament depolymerizer, resulted in inhibition of *S. aureus* Col, *S. gordonii* DL-1 and *S. gordonii* (FnBPA) internalization ($P < 0.001$). Internalization of all three bacterial strains was reduced >80% by 1 μM cytochalasin D (Fig. 2B). Colchicine treatment, which prevents microtubule polymerization, also curtailed internalization levels (Fig. 2B), all strains exhibiting >80% lower numbers of internalized bacteria with 10 μM colchicine. These data suggested a major cytoskeletal involvement in internalization of *S. gordonii* and *S. aureus* cells by MG63 osteoblasts.

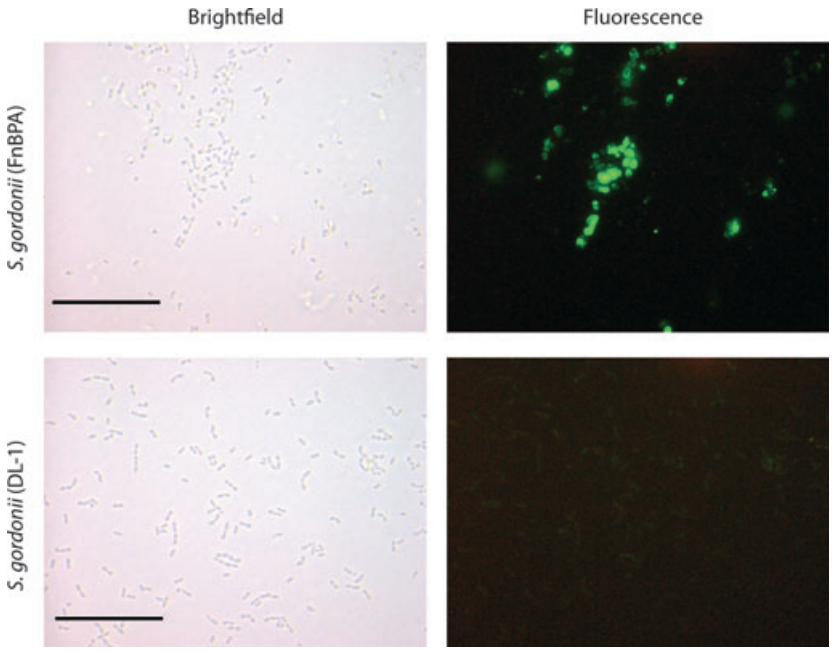

Figure 3 Binding of fibronectin (Fn) by *Streptococcus gordonii* (FnBPA). Fluorescence microscopy images of *S. gordonii* cells following incubation with Fn and reaction with FITC-conjugated anti-Fn antibodies. Upper panels: *S. gordonii* DL-1 (FnBPA); Lower panels: *S. gordonii* DL-1. Bars = 20 μM.

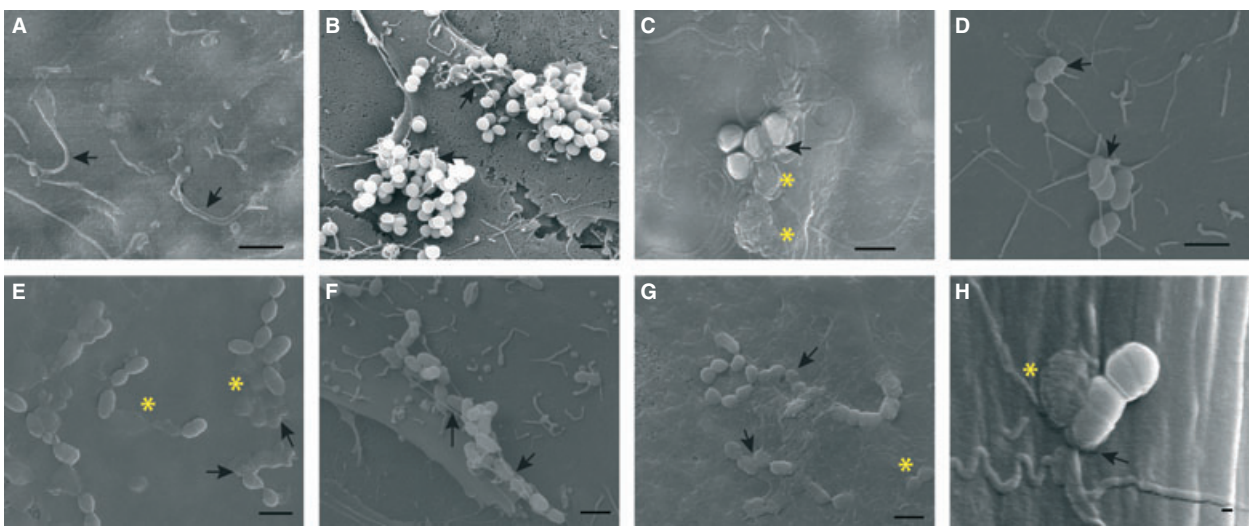

Figure 4 Visualization by scanning electron microscopy of bacterial interactions with osteoblasts. MG63 cells were grown on titanium disks, incubated with bacteria for 1 h, then fixed, dehydrated and coated with platinum before scanning electron microscopy. (A) No bacteria control; (B, C) *Staphylococcus aureus* Col; (D, E) *Streptococcus gordonii* DL-1; (F–H) *S. gordonii* DL-1 (FnBPA). Filopodia-like projections are apparent on the osteoblast cell surface (arrows panel A). In the presence of *S. aureus* (B) or *S. gordonii* DL-1 (D, F) these appear to tether the bacteria to the cell surface (arrows). *S. aureus* (C), *S. gordonii* DL-1 (E) and *S. gordonii* (FnPBA) (G. H) all appear to be disappearing into the surface of the osteoblasts and become engulfed within the cell membrane. In (C, E, G, H) the asterisks indicate internalized bacterial cells. Scale bars = 1 μM.

Inhibitors of endocytosis were also investigated. Monodansylcadaverine (MD) inhibits the formation of coated pits and interferes with receptor recycling. This inhibitor significantly inhibited both *S. aureus* Col and *S. gordonii* DL-1 internalization by MG63 cells in a dose-dependent manner ($P < 0.001$) (Fig. 2B).

Numbers of intracellular *S. aureus* Col were reduced >10-fold in 250 μM MD. Interestingly, there was little effect of MD on internalization levels of *S. gordonii* (FnBPA). Ouabain treatment, which blocks Na^+/K^+-ATPase and arrests pit formation, had little effect on *S. gordonii* (FnBPA) internalization but both *S. aureus* Col and *S. gordonii* DL-1 were significantly inhibited. Internalization levels of *S. gordonii* (FnBPA) were reduced by ≈ 20% in the presence of 250 μM ouabain, whereas *S. aureus* Col and *S. gordonii* DL-1 internalization were both reduced >30% ($P < 0.01$). Monensin interferes with endosome acidification and inhibits low-density receptor recycling. Treatment with this inhibitor resulted in attenuated internalization for all three bacterial strains. Numbers of intracellular *S. aureus* Col decreased >10-fold in the presence of 40 μM monensin ($P < 0.001$). Inhibition was more pronounced for *S. gordonii*, with internalization levels of *S. gordonii* DL-1 and *S. gordonii* (FnBPA) >100-fold reduced with 4 μM monensin ($P < 0.001$, data not shown) and >1000-fold in 40 μM monensin ($P < 0.001$). These data suggest a primary role for endocytosis, endosome acidification and receptor cycling, in the internalization of *S. gordonii*. On the other hand, *S. aureus* Col showed less sensitivity to endocytic pathway inhibitors, suggesting that more than one internalization pathway may exist for *S. aureus* (Jevon *et al.*, 1999).

Intracellular survival of commensal bacteria

Intracellular survival levels of internalized *S. aureus* Col, *S. gordonii* (FnBPA) and *S. gordonii* DL-1 were determined at 2, 24 and 48 h following antibiotic exclusion assay. Numbers of *S. gordonii* DL-1 declined 10-fold over 24 h following infection (Fig. 5), and at 48 h no viable cells of *S. gordonii* DL1 could be recovered. Viable cell numbers of *S. gordonii* (FnBPA) also showed 10-fold reduction at 24 h incubation ($P < 0.001$), but at 48 h viable bacteria were still present (Fig. 5). Numbers of internalized *S. aureus* Col declined three-fold over 24 h ($P < 0.001$) and by a further 10-fold after 48 h ($P < 0.001$). These results suggest that FnBPA confers upon *S. gordonii* increased levels of internalization and longer intracellular survival times. The *S. aureus* would appear to express factors in addition to FnBPA that confer more prolonged intracellular survival compared with *S. gordonii* (FnBPA).

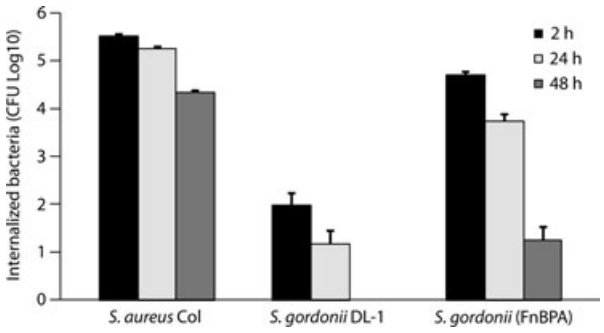

Figure 5 Longer-term survival of intracellular bacteria within osteoblasts. Following incubation of bacteria with osteoblasts (multiplicity of infection 10 : 1) for 2 h, antibiotics were added to the medium and colony-forming units (CFU) internalized were determined at 24 h and 48 h following infections. Error bars indicate standard deviation from the mean ($n = 4$).

Intracellular location of bacteria

We next investigated the intracellular locations of *S. aureus* or *S. gordonii* cells, in particular whether intracellular bacteria were found within host cell late endosomes or lysosomes, as indicated by co-localization with LAMP-1/CD107a. MG63 cells expressing LAMP-1 were seeded on to glass cover slips within plastic wells and incubated for 24 h before infection with bacterial strains (MOI 10 : 1). Cover slips were prepared for microscopy by incubation with mouse anti-human LAMP-1 and rabbit anti-*S. gordonii* (diluted 1 : 50) followed by Alexa 594-conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies. At early stages of infection (2 h), *S. aureus* Col was found associated with host cell late endosomes/lysosomes, as indicated by co-localization with LAMP-1 (Fig. 6). Clusters of *S. aureus* cells were visible around host cell nuclei (arrowed, Fig. 6, bottom left panel). The *S. gordonii* (FnBPA) also co-localized with LAMP-1 (Fig. 6 arrow, bottom right panel) but there were no obvious clusters of intracellular bacteria. Any association of wild-type *S. gordonii* DL-1 with LAMP-1 could not be detected.

Effects of bacteria on cytokine expression by osteoblasts

A number of recent studies have reported a role of osteoblasts in the initiation of an inflammatory immune response at sites of bacterial challenge. Accordingly, we measured production of the proinflammatory cytokines IL-6 and IL-8 by osteoblasts

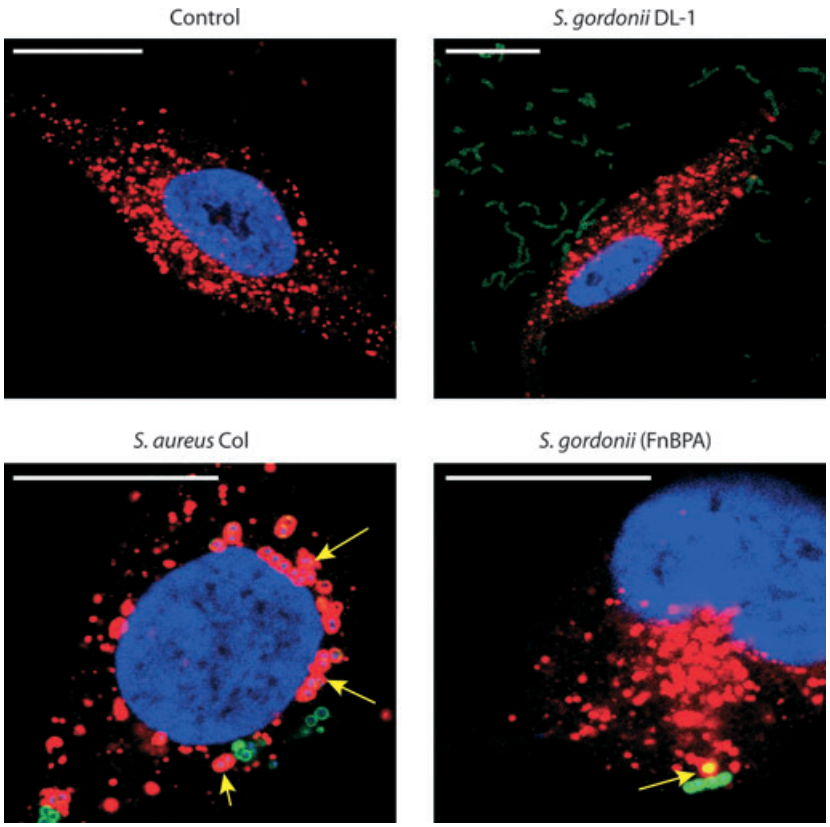

Figure 6 Co-localization of bacterial cells with lysosomal-associated membrane protein (LAMP-1) -enriched host cell vesicles. Confocal laser scanning microscopy images of MG63 cells infected with bacteria detected by FITC-conjugated antibody (appearing green) and LAMP-1 detected using Alexa 594-conjugated antibody (appearing red). Nucleic acid material was stained using DAPI (appearing blue; large spheres are host cell nuclei, small spheres are bacterial DNA). Intracellular *Staphylococcus aureus* and *Streptococcus gordonii* were located in host cell LAMP-1-enriched organelles from 2 h postinfection (arrows, lower panels). Clusters of *S. aureus* Col were located within lysosomes/late endosomes close to the host cell nuclei (arrows, lower left panel.). Intracellular *S. gordonii* DL-1 (FnBPA) cells were found within lysosomes, but no obvious clustering was apparent (arrow, lower right panel). No co-localization between *S. gordonii* DL-1 and LAMP-1 was found (upper left). Image has been merged from individual channels (see Supplementary material, Fig. S1). Scale bars = 50 μM.

carrying internalized *S. gordonii* or *S. aureus*. Following infection with bacterial strains for 2 h, MG63 cells were incubated in the presence of antibiotic medium for 24 h or 48 h at which times RNA was isolated. RT-PCR, using primers specific to IL-6 and IL-8, was performed to estimate the transcript levels of these genes. *Staphylococcus aureus* Col infection resulted in increased expression levels of IL-6 mRNA whereas *S. gordonii* infection did not (Fig. 7A). In *S. aureus*-infected or *S. gordonii*-infected osteoblasts, IL-8 mRNA expression levels were elevated at 24 h and 48 h post-infection, whereas no increase in IL-8 expression was observed for non-infected controls.

Secretion of IL-6 or IL-8 by infected MG63 osteoblasts was also quantified by ELISA (Fig. 7B). Antibiotic exclusion assays were performed, as previously described, with infected MG63 cells incubated for 6, 24 or 48 h. Cell culture supernatants were then collected and assayed by quantitative sandwich enzyme immunoassay, using antibodies specific to human IL-6 (monoclonal) or IL-8 (polyclonal). Expression of IL-6 was not detectable at 6 h postinfection. However, *S. aureus* Col infection for 24 h resulted in significantly increased IL-6 secretion by MG63 cells ($P < 0.01$) (Fig. 7B), and >10-fold upregulation at 48 h compared with 24 h ($P < 0.001$), reaching levels of >100 pg ml^{-1}. Conversely, infection with *S. gordonii* did not result in elevated IL-6 secretion by MG63 cells.

Interleukin-8 secretion was detectable in MG63 cells at 6 h postinfection with *S. aureus* Col ($P < 0.01$) or *S. gordonii* DL-1 ($P < 0.001$) (Fig. 7B).

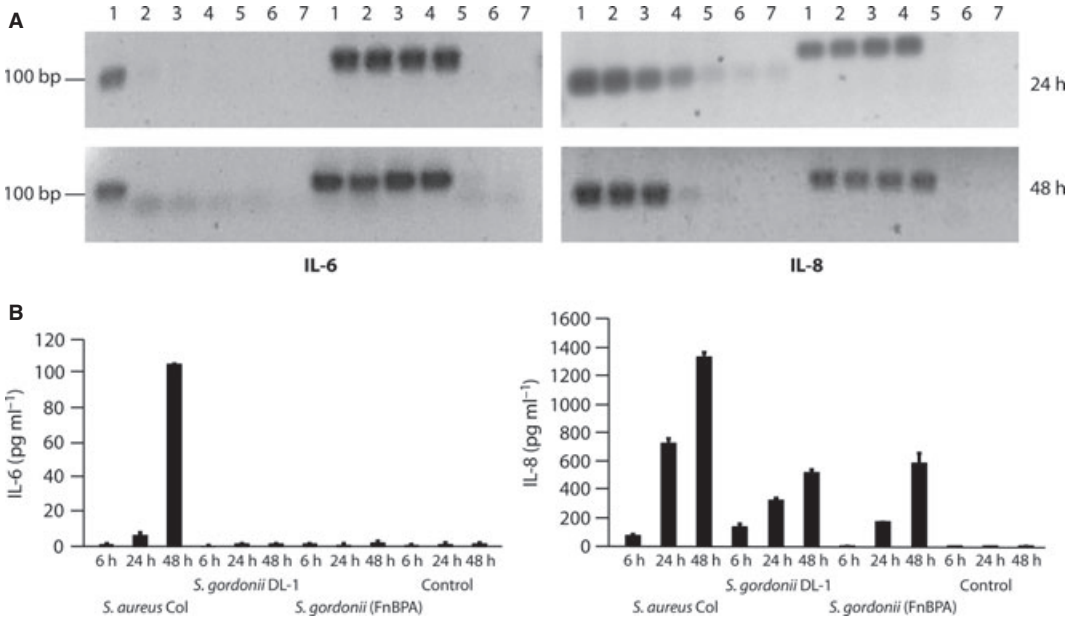

Figure 7 Interleukin-6 (IL-6) and IL-8 expression and secretion by MG63 cells. (A) MG63 cells were incubated for 24 h (upper panels) or 48 h (lower panels) with or without bacteria, and equal quantities of extracted RNA were used for cDNA synthesis, before performing PCR with gene-specific primers (see Supplementary material, Table S1). TATA-box-binding protein (TBP) was used as a positive control housekeeping gene (right-hand lanes 1–5). Lanes: 1, *Staphylococcus aureus* Col-infected; 2, *Streptococcus gordonii* DL-1 (FnBPA)-infected; 3, *S. gordonii* DL-1-infected; 4, No bacteria control; 5, -RT control; 6, cDNA H_2O control; 7, PCR H_2O control. Results shown are representative of at least two independently performed assays. (B) MG63 cells were infected with *S. aureus* Col, *S. gordonii* DL-1 (FnBPA) or *S. gordonii* DL-1 at a multiplicity of infection 10 : 1 for 2 h, followed by the removal of extracellular bacteria. Culture supernatants were taken at various time-points and sandwich ELISAs were performed to quantify cytokine secretion. Results shown are representative of at least two independently performed assays. Error bars indicate standard deviation from the mean ($n = 3$).

Upregulation was apparent in all infected groups compared with noninfected controls. Infection with *S. aureus* Col resulted in 10-fold (24 h) and 20-fold (48 h) increases in IL-8 secretion levels to concentrations exceeding 1300 pg ml^{-1}. In MG63 cells infected with *S. gordonii* (FnBPA) IL-8 became detectable at 24 h with a further more than two-fold increase in IL-8 levels at 48 h ($P < 0.001$) to a maximum concentration approximately 600 pg ml^{-1} ($P < 0.001$). *Streptococcus gordonii* DL1-infected cells also secreted significantly less IL-8 compared with *S. aureus*-infected cells ($P < 0.001$). However, compared with the FnBPA-expressing strain, IL-8 levels induced by strain DL-1 were detectable at 6 h and were approximately two-fold higher at 24 h. Following 48 h of incubation, MG63 cells infected with *S. gordonii* DL-1 had secreted approximately 500 pg ml^{-1} IL-8. These results suggest that IL-6 expression and secretion is associated with *S. aureus* internalization, but not with *S. gordonii* internalization. Moreover, *S. gordonii* infection induced lower levels of IL-8 than *S. aureus* infection. Despite higher levels of internalization associated with FnBPA expression by *S. gordonii*, the levels of IL-8 produced by MG63 cells infected with wild-type strain DL-1 or strain DL-1 expressing FnBPA were similar.

DISCUSSION

Failures of dental or orthopedic implants are of relatively low incidence (Sakka & Coulthard, 2011) but are clinically very serious and are principally associated with microbial infections. Microorganisms such as coliforms, *S. aureus* and *Candida albicans* have been cited as frequent pathogens in failing oral implants (Leonhardt *et al.*, 1999). In addition, oral bacteria have been implicated in the development of dental implant infections (Pye *et al.*, 2009), and species of *Fusobacterium*, *Porphyromonas*, *Treponema* and *Streptococcus* are of high incidence in various implantitis communities (Al-Radha *et al.*, 2012; Kumar *et al.*, 2012). Infections prevent osteoblasts from

properly integrating the implant into the recipient tissues, therefore leading to inflammation and rejection. The studies described in this article were initiated because there is increasing evidence that bacteria in the oral cavity, regarded as part of the normal microbiota, may cause low to high level infections themselves or prime host cells for subsequent infection by single or multiple species of bacteria (Hajishengallis *et al.*, 2011). Phenotypic features of *S. aureus* that are believed to lead to this bacterium being the major etiological agent of orthopedic implant infections include: (i) avidity of adherence to osteoblasts involving synergy of multiple surface proteins (Testoni *et al.*, 2011), (ii) internalization through mainly an Fn-based mechanism (Jevon *et al.*, 1999), and (iii) activity of Protein A inducing apoptosis, inhibiting mineralization and initiating bone resorption (Claro *et al.*, 2011).

Internalization of bacteria by non-professional phagocytes presents a mechanism by which bacteria are potentially able to replicate intracellularly and spread to adjacent cells and tissues. A number of oral bacteria are able to infect cells in this way, e.g. *Porphyromonas gingivalis* (Yilmaz *et al.*, 2006), *Filifactor alocis* (Moffatt *et al.*, 2011), *Aggregatibacter actinomycetemcomitans* (Meyer *et al.*, 1999), causing various responses and effects including modulation of cytokine production (Dickinson *et al.*, 2011) and apoptosis (Wang *et al.*, 2010). We have shown previously that *S. gordonii* is able to adhere to and become internalized by epithelial cells, and that this involves direct interaction of surface molecules of *S. gordonii* with integrin β_1 (Nobbs *et al.*, 2007). The present studies were designed to determine the ability of *S. gordonii* to interact with osteoblasts and therefore potentially influence their functions at an early stage of osseointegration.

In our studies, *S. gordonii* exhibited high-level adhesion to MG63 cultured osteoblasts, and 500–1000-fold lower levels of internalization compared with adhesion. *S. aureus* adhered at similar levels but showed much higher invasion levels (approximately 10% bacteria adhered). However, invasion levels of *S. gordonii* could be increased 100–300-fold by expression of the *S. aureus* Fn-binding protein FnBPA on the streptococcal cell surface. This suggests that osteoblasts are open to be infected more readily by bacteria that produce high-affinity Fn-binding proteins, e.g. *S. aureus*, *S. pyogenes*. The osteoblasts in our cultures produced Fn, which was sufficient for maximal bacterial adherence and internalization, because addition of exogenous fibronectin had no increased effects (data not shown).

The responses of the invasion mechanisms of *S. gordonii* and *S. aureus* to various inhibitors of coated pit (MD and ouabain), receptor recycling (monensin) and microfilament dysregulation (cytochalasin D) provide evidence for subtle differences in the pathways of uptake. The sensitivities of the bacteria to inhibition by cytochalasin D, colchicine and monensin indicate uptake through an endocytosis pathway with requirement for actin accumulation. However, the lack of sensitivity of *S. gordonii* expressing FnBPA to MD suggests that a clathrin-independent pathway may operate in respect of FnBPA recognition. This is wholly consistent with recent evidence showing that endocytosis and turnover of fibronectin via integrin $\alpha_5\beta_1$ is linked to caveolin-1 and occurs in a clathrin-independent manner (Shi & Sottile, 2008; Hoffmann *et al.*, 2010). On the other hand, *S. aureus* internalization was much less sensitive to ouabain, which blocks Na^+/K^+-ATPase with arrest of coated pit formation. This has been observed previously (Jevon *et al.*, 1999) and it was suggested that inhibition of transglutaminase by MD might have some alternative effect.

Entry of *S. gordonii* and *S. aureus* showed typical features of phagocytosis, with membrane protrusions interacting with bacterial cells and closure of the osteoblast membrane leading to engulfment and internalization. These are reminiscent of the images of *S. pyogenes* invading endothelial cells (Amelung *et al.*, 2011), with subsequent accumulation of actin. The later stages of intracellular trafficking were then probed by determining localization of the late endosomal/lysosomal marker LAMP-1 in the phagosomal membrane. LAMP-1 accumulated around intracellular *S. aureus* and around *S. gordonii*, indicating trafficking of bacteria into the late endosomal/lysosomal compartment.

Integrin $\alpha_v\beta_3$, the collagen-binding integrin $\alpha_s\beta_1$, and fibronectin-binding integrin $\alpha_5\beta_1$ are taken up together with their ligands, thereby contributing to the turnover of the extracellular matrix (Lee *et al.*, 1996; Memmo & McKeown-Longo, 1998; Shi & Sottile, 2008). Several pathogenic bacteria exploit this endocytotic capacity of integrins to promote internalization by non-professional phagocytes (Hauck *et al.*, 2006). Bacterial proteins such as the *Yersinia enterocolitica*

invasin directly bind to integrins and, upon receptor clustering, induce a zipper-style uptake mechanism that involves integrin-associated protein tyrosine kinase signalling and reorganization of the actin cytoskeleton (Alrutz & Isberg, 1998; Dersch & Isberg, 1999). The *S. gordonii* cells also bind directly to integrin β_1 (Nobbs *et al.*, 2007), but the mechanisms by which *S. gordonii* cells adhere to and invade osteoblasts are not yet defined and are under investigation.

With regard to intracellular survival, *S. gordonii* CFU declined quickly, suggesting prolonged exposure within lysosomes, whereas *S. aureus* CFU were relatively well sustained over 48 h. This indicates that the bacteria were able to escape from the effects of acidic pH and hydrolytic enzymes within the lysosome (Jarry & Cheung, 2006), possibly through release into the cytoplasm or by endogenous resistance to lysosomal destruction (Schröder *et al.*, 2006; Zander *et al.*, 2008). *Streptococcus gordonii* FnBPA survived longer than *S. gordonii* DL-1, suggesting that expression of FnBPA somehow endowed the bacteria with a survival advantage. If FnBPA directed bacterial internalization through a caveolae-like pathway, as opposed to other endocytic pathways, then bacteria may have been able to survive longer within the caveosome. This pathway avoids fusion with lysosomes, thereby protecting bacteria from enzymatic degradation. Caveolin 1 is expressed by osteoblasts (Solomon *et al.*, 2000) and we were able to confirm this by immunofluorescence with anti-caveolin-1 antibodies. However, caveolin-1 was expressed at high levels and distributed all over the cell surface, so attempted co-localization experiments with labelled bacteria were inconclusive (data not shown).

The differential release of cytokines from host cells in response to pathogenic or commensal species of bacteria is important to the progression of disease. The cytokines IL-1β, IL-6 and IL-8 are crucial in bone turnover (Pantouli *et al.*, 2005) and they can elicit responses from osteoclasts resulting in bone resorption (Rothe *et al.*, 1998). Following *S. aureus* infection, osteoblasts have been shown to secrete proinflammatory cytokines (Wright & Friedland, 2004), express receptors on their surface that directly interact with T lymphocytes (Schrum *et al.*, 2003a,b) and produce factors known to initiate bone resorption (Bost *et al.*, 2000). Therefore IL-6 and IL-8 induction in osteoblasts can not only elicit a host immune response, but also lead to downstream effects on osteoclasts culminating in bone resorption.

In our studies, we have shown that MG63 cells produced IL-6 following infection with *S. aureus* Col, confirming previous *in vitro* (Bost *et al.*, 2000) and *in vivo* (Marriott *et al.*, 2004) findings. However, *S. gordonii* did not elicit an IL-6 response, even when expressing FnBPA. The IL-6 response would have a stimulatory effect on B and T cells (Balto *et al.*, 2001) and enhance bone resorption. Intracellular *S. aureus* might therefore stimulate cytokines that negatively affect bone remodeling following surgery or trauma. On the other hand, intracellular *S. gordonii* elicited no IL-6 response, suggesting that osteoblasts might be able to tolerate challenge by this commensal bacteriium. It should be noted that, in these experiments, the interactions of *S. gordonii* and *S. aureus* with osteoblasts were compared at similar MOIs. It is likely that osteoblasts would encounter much higher numbers of *S. gordonii* than *S. aureus in vivo*, because *S. gordonii* is a significant component of the normal microbiota.

Infection of MG63 cells by *S. aureus* Col or *S. gordonii* DL-1 led to rapid (6-h) induction of IL-8 secretion, but ultimately IL-8 levels were lower at 24 h for *S. gordonii* DL-1 than for *S. aureus*. Although *S. gordonii* (FnBPA) did not elicit an IL-8 response as rapidly as *S. gordonii* DL-1, the response was long lived and similar to that for *S. aureus*. This might support the notion that FnBPA directs *S. gordonii* to become internalized by an alternative pathway so delaying the IL-8 response. Overall our results show that osteoblasts growing on plastic or titanium surfaces have the ability to internalize both *S. gordonii* and *S. aureus*. These organisms are internalized through an endoycytic pathway, triggering cytoskeletal rearrangements and subsequently leading to trafficking to LAMP-1-positive compartments. Expression of FnBPA on the surface of *S. gordonii* (and by *S. aureus*) leads to higher numbers of bacteria being internalized and longer survival times. One possibility is that FnBPA-mediated internalization directs the bacteria through a caveosome-based pathway so avoiding cellular degradation mechanisms and favoring shorter-term survival. Of importance in future work would be to determine the effects of commensal or pathogenic oral bacteria on the initial attachment of osteoblasts to titanium surfaces, to understand better the processes occurring in a clinical setting

immediately following the introduction of an implant within alveolar bone.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bacteria co-localization with LAMP-1-enriched host cell vesicles.

Table S1. Oligonucleotide primers used in this study.