Orthopedic implant infections: Incompetence of Staphylococcus epidermidis, Staphylococcus lugdunensis, and Enterococcus faecalis to invade osteoblasts

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Abstract: Septic failure is still the major complication of prosthetic implants. Entering host cells, bacteria hide from host immune defenses, shelter from extracellular antibiotics, and cause chronic infection. Staphylococcus aureus, the leading etiologic agent of orthopedic implant infections, is able to enter bone cells and induce osteoblast apoptosis, osteoclast recruitment, and highly destructive osteomyelitis. Staphylococcus epidermidis, Staphylococcus lugdunensis, and Enterococcus faecalis are opportunistic pathogens causative of implant-related infections. This study investigated the ability to internalize into osteoblastic MG63 cells of 22 S. epidermidis, 9 S. lugdunensis, and 21 E. faecalis clinical isolates from orthopedic implant infections. Isolates were categorized in clusters by ribotyping. Internalization assay was carried out by means of a microtiter plate-based method. S. epidermidis, S. lugdunensis, and E. faecalis strains turned out incompetent to enter osteoblasts, exhibiting negligible internalization into MG63 cells, nearly three orders of magnitude lower than that of S. aureus. Osteoblast invasion does not appear as a pathogenetic mechanism utilized by S. epidermidis, S. lugdunensis, or E. faecalis for infecting orthopedic implants. Moreover, it can be inferred that intracellularly active antimicrobials should not be necessary against implant infections caused by the three bacterial species. Finally, implications with the uptake of biomaterial microparticles by nonphagocytic cells are enlightened. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 788–801, 2016.

Key Words: implant infections, osteomyelitis, internalization, bone, osteoblasts, MG63 cells, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus lugdunensis, Enterococcus faecalis, ribotyping, molecular epidemiology


INTRODUCTION

The search for new therapeutic strategies and drugs to eradicate implant-associated infections is a topic of great interest in orthopedics, where periprosthesis infections often turn out elusive for the surgical cleansing, recalcitrant to the antibiotic therapy, and doomed to end up with the implant failure. Bacteria responsible for implant-related infections can survive to antibiotic therapies through different mechanisms: an equipment of genetic determinants conferring specific antibiotic resistances3; the production of a biofilm that wraps bacteria and circumvents the bactericidal effects of the antibiotics2,3; and the penetration into eukaryotic cells, where insidiously bacteria shelter and hide out creating the scenery of a chronic infection. And indeed, eukaryotic cells are impermeable to many antibiotics,5,6 which therefore cannot reach intracellular bacteria. In addition, intriguingly, once bacteria become intracellular, they themselves tend to acquire with time the ability to resist even antibiotics, such as rifampin,6 that are able to pass through the eukaryotic cell membrane. In this connection, infections caused by invasive bacteria could require antibiotic regimens effective and timely in preventing the onset of antibiotic resistances and suitable for carrying high levels of antibiotic molecules to the interior of the invaded cells. Therefore, to pave the way toward new therapeutic strategies, methods for drawing a neat distinction between bacteria able or incapable to enter eukaryotic cells are needed.

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The interaction of a bacterium with a eukaryotic cell depends not only on the molecular characteristics of the bacterial species but also on those of the specific histotype of the host cells. The assessment of the intracellular invasiveness of a given bacterial species has therefore to be performed with the cell types potentially implicated.

In past years, an increasing number of studies focused on the internalization and persistence of *Staphylococcus aureus* within eukaryotic cells, these including endothelial cells, bovine mammary epithelial cells, keratinocytes, alveolar epithelial cells, and osteoblasts. In particular, *S. aureus*, the leading etiologic agent of implant-orthopedic infections, has been found capable to enter bone cells and induce osteoblast apoptosis, osteoclast recruitment, and finally destructive osteomyelitis.

Many years after the studies by Koch on *Mycobacterium tuberculosis*, renewed attention to the phenomenon of the intracellular invasion as a pathogenic mechanism of chronic infections has led in recent years to recognize other pathogens competent to persist inside nonphagocytic host cells. Among which, some *Escherichia coli* strains, various species of the *Salmonella* and *Listeria* genera, *Shigella* flexneri, *Legionella pneumophila*, *Chlamydia pneumoniae*, *Yersinia pestis*, *Yersinia pseudotuberculosis* and, among Gram-positive cocci, group A, B, C, *G Streptococcus*, *Streptococcus viridans*, and *Streptococcus pneumoniae*. The ability to invade eukaryotic cells is generally associated to virulence factors termed *invasins*, often found in bacteria causing chronic infections. Most staphylococcal species, other than *S. aureus*, in the past considered mere saprophytes, are progressively emerging as opportunistic pathogens able to cause serious and persistent implant-associated infections. Even if invasion of osteoblasts by *S. aureus* and its significance in the pathogenesis of orthopedic infections is well established, the knowledge of the ability of these other staphylococcal species to gain entrance into eukaryotic cells is still rather incomplete.

Straight after *S. aureus*, *S. epidermidis* is the second staphylococcal species responsible for orthopedic infections. Till now, just very few investigations have been conducted on *S. epidermidis* internalization into osteoblasts. They analyzed a number of veterinary and clinical isolates, sometimes reporting marked strain-dependent differences in osteoblast invasiveness.

*Staphylococcus lugdunensis*, vividly called “a wolf in sheep’s clothing,” alluding to the allegoric Aeop’s Fable, is an aggressively emerging coagulase-negative pathogen responsible for orthopedic (and cardiovascular) periprosthetic infections. *S. lugdunensis* uptake by eukaryotic cells has been described with fibroblasts, epithelial, and endothelial cells, but never with osteoblasts.

*Enterococcus faecalis* is an etiologic agent of hospital infections and prosthetic-related infections exhibiting high rates of antibiotic resistances. With its large equipment of virulence factors suitable for adhering onto biomaterials and colonizing periprostheses tissues, *E. faecalis* has become a dangerous pathogen related to implant infections. The internalization of this bacterial species, enlisted among the top three nosocomial pathogens, has been up to now investigated only with enterocytes and endothelial cells.

Here, after having worked out and refined a method based on microtiter plates, suitable for screening the invasive potential of bacterial species, we aimed at assessing whether and to which extent clinical isolates of *S. epidermidis*, *S. lugdunensis*, and *E. faecalis* from implant-related orthopedic infections are capable of internalization in osteoblastoid MG63 cells. A further aim was at investigating if differences could be observed among isolates of the three species belonging to different clones identified by ribotyping.

For an adequate validation, the method was performed not only using negative and positive control strains in each plate, but even including empty wells containing just bacteria and not eukaryotic cells, to account for any background of bacterial adhesion and survival in the absence of cultured cells on the well bottom. Such approach could become a standardized method for assessing the invasive potential of different bacterial species challenged with different human cell types.

**MATERIALS AND METHODS**

**Bacterial strains**

In this study, 22 *S. epidermidis*, 9 *S. lugdunensis*, 21 *E. faecalis*, and 1 *S. aureus* clinical isolates from postsurgical orthopedic infection and the *S. aureus* reference strain ATCC25923 were analyzed. The isolates belong to the collection of the strain library of the Research Unit on Implant Infection, at the Rizzoli Orthopedic Institute (Bologna, Italy). All clinical isolates of *S. epidermidis*, *S. lugdunensis*, and *E. faecalis* were first identified by classic phenotypic methods and, subsequently, confirmed and ribotyped by automated riboprinting by a RiboPrinter® (Qualicon DuPont, Willmington, DE). EcoRI was the restriction enzyme. The isolates were taxonomically identified and automatically subtyped into ribogroups by the RiboPrinter, based on the similarity of the ribopatterns generated.

*S. epidermidis*, *S. lugdunensis*, and *E. faecalis* isolates included in the study were all sensitive to gentamicin. The clinical source of all isolates included in the study is reported in Table I. As shown in the table, the majority of the strains was isolated from implant-associated infections, predominantly from arthroprosthesis-associated (hip and knee) infections and (external/internal) bone fixation devices. All *S. epidermidis* strains, except two (one isolated from an osteomyelitis and the other one from an orthopedic postsurgical infection) were from orthopedic infections associated to implants. *S. lugdunensis* isolates, except for the clinical isolate cra1750 (picked from an orthopedic postsurgical infection nonassociated to implant), were from orthopedic infections associated to implants. Four isolates of *E. faecalis* derived from oncological patients, of whom two underwent a surgical reconstruction with both biomaterial- and bone-derived scaffolds. The *S. aureus* cra1199 laboratory strain, which had already been well characterized for its ability to invade MG63 cells, was utilized as a positive control. A further *S. aureus* isolate, the reference strain...
ATCC25923, was included in the tests for cross-comparison with coagulase-negative staphylococci (CNS) isolates. Conversely, the *S. lugdunensis* cra1750 laboratory strain used as a negative reference control should be more correctly intended as a low-internalization reference strain because it exhibited very low, but not absent, internalization.

**Preparation of the bacterial inoculum for cell invasion assays**

Bacterial isolates were thawed from frozen stock samples, stored in the bio-bank of the Rizzoli Research Unit on Implant Infections and plated on Tryptical Soy Agar (TSA, Biolife). To perform the cell invasion assays, bacteria were grown in Tryptose Broth (TB; Biolife) at 37°C for 18 h. They were pelleted by centrifugation and suspended in a medium consisting of MEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 2 mM L-glutamine (Sigma-Aldrich). Bacterial concentration was estimated by optical density at 550 nm using a Hewlett Packard G1103A spectrophotometer. Correlation curves of optical density values versus colony formation units (CFU) were made and the equations of the regression lines were applied for the esteem of the suspensions.

**MG63 cell culture**

The human osteoblast-like cell line MG63, purchased from ATCC (Rockville, MD), was routinely cultured in growth medium consisting of MEM (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Sigma-Aldrich) and penicillin/streptomycin (10,000 U/mL penicillin, 10 mg/mL streptomycin, Sigma-Aldrich). MG63 cells were regularly subcultured three times a week.

**Invasion assay of osteoblasts in 96-well plates (antibiotic protection assay)**

The microtiter plates were organized so that in each plate the positive and the negative controls were tested in triplicate and three clinical isolates were assayed in triplicate both in the presence of MG63 cells and in their absence, following the plate scheme reported in Figure 1. 96-well tissue culture microplates were purchased from Starlab Srl (Milano, Italy). For the assay, MG63 cells were seeded at a cell concentration of about 5 × 10^3 cells/well in a volume of 100 μL of cell suspension in complete culture medium without antibiotics. An equivalent number of wells were prepared adding 100 μL of culture medium without MG63 cells for appropriate control of the background of bacterial survival in the absence of internalized bacteria and confirmation of antibiotic susceptibility. The plate was cultured overnight at 37°C under standard culture conditions. At 24 h, the number of MG63 cells was found to be of about 1 × 10^3 cells/well (in three independent experiments the count was of 1.0 ± 0.8 × 10^3 cells/well). Before exposing the MG63 cells to the bacteria, the wells of each plate were washed once with 200 μL of D-PBS, 100 μL of bacterial suspension in growth medium without antibiotics were added to the wells at the desired multiplicity of infection (MOI) as reported in Figure 1.

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**TABLE I. Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain Names</th>
<th>No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control strains (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> cra1199</td>
<td>1</td>
<td>Infection associated to external bone fixation device</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> cra1750</td>
<td>1</td>
<td>Orthopedic infection nonassociated to medical device</td>
</tr>
<tr>
<td>Reference strain (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC25923</td>
<td>1</td>
<td>Clinical strain isolated in 1945</td>
</tr>
<tr>
<td><em>S. epidermidis</em> orthopaedic clinical isolates (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Hip prosthesis-associated infection</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Knee prosthesis-associated infection</td>
<td></td>
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<tr>
<td>11</td>
<td>Infection associated to internal bone fixation device</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Infection associated to external bone fixation device</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Osteomyelitis nonassociated to medical device</td>
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<tr>
<td>1</td>
<td>Postsurgical infection</td>
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<td><em>S. lugdunensis</em> orthopaedic clinical isolates (8)</td>
<td></td>
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<tr>
<td>3</td>
<td>Infection associated to internal bone fixation device</td>
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<tr>
<td>2</td>
<td>Knee prosthesis-associated infection</td>
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<td>2</td>
<td>Hip prosthesis-associated infection</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Infection associated to external bone fixation device</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> orthopaedic clinical isolates (21)</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>Infection associated to internal bone fixation device</td>
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<tr>
<td>3</td>
<td>Hip prosthesis-associated infection</td>
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<tr>
<td>2</td>
<td>Infection associated to external bone fixation device</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Infection associated to medical device following reconstructive surgery in bone oncology</td>
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<tr>
<td>2</td>
<td>Infection nonassociated to medical device following radical surgery in bone oncology</td>
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<tr>
<td>2</td>
<td>Postsurgical infection associated to medical device</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Postsurgical infection nonassociated to medical device</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Infection associated to medical device following tendon reconstruction</td>
<td></td>
</tr>
</tbody>
</table>
In past studies by Khalil et al.,\textsuperscript{19} a MOI of about 500:1 was found to be a convenient inoculum size because approaching a plateau in terms of internalization into osteoblasts of the reference strain \textit{S. epidermidis} NCTC11047. In the present study, the concentrations of the inocula prepared with the different isolates were approximated by measuring the optical density. The concentration of the bacterial suspensions was calculated from the O.D. versus CFU equation obtained using two staphylococcal strains, respectively, \textit{S. epidermidis} reference strain RP62A and \textit{S. lugdunensis} cra1750. The data obtained from three independent experiments for both strains were joined together. Nevertheless, the size of each inoculum was always verified \textit{a posteriori} by CFU counting. Slight variations were anyway observed even among bacterial strains of the same species. Therefore, in this study the confirmed MOI was always expressed in order to know the inoculum sizes effectively reached. For antibiotic-sensitive \textit{S. epidermidis}, real verified concentrations of bacteria were found to correspond to an average MOI of 560:1. Conversely, for \textit{S. lugdunensis} and \textit{E. faecalis} clinical isolates, the average size of the inoculum was confirmed to be, respectively, of 1844 and 1642:1, thus slightly exceeding the target MOI of 1000:1 suggested in past studies with other cell types.\textsuperscript{22,28} Only in the case of the negative control, the \textit{S. lugdunensis} cra1750 strain, and of the positive control consisting of \textit{S. aureus} cra1199 a MOI of about 100:1 was used. Among others, the negative control had the important function of excluding plate contamination with aminoglycoside-resistant bacteria during plate processing.

After 2 h of incubation at 37°C during which bacteria were allowed to invade the osteoblasts, each well was washed four times with 200 \(\mu\)L of D-PBS and then treated with MEM medium supplemented with 100 \(\mu\)g/mL of gentamicin (Sigma-Aldrich) and 20 \(\mu\)g/mL of lysostaphin (Sigma-Aldrich) to kill all extracellular bacteria. For \textit{E. faecalis}, on account of its intrinsic greater resistance, the concentration of gentamicin was increased to 200 \(\mu\)g/mL. Gentamicin is an aminoglycoside antibiotic that does not penetrate eukaryotic cells.\textsuperscript{31} Lysostaphin is an antimicrobial agent unable to enter eukaryotic cells, effective at eradicating extracellular organisms\textsuperscript{32} and active even on aminoglycoside-resistant strains. The multiwall plate was incubated for 2 h at 37°C under standard culture conditions. At the end of the incubation each well was washed once with 200 \(\mu\)L of D-PBS and then treated with 150 \(\mu\)L of 0.1% Triton X-100 for 5 min at 37°C to lyse the eukaryotic cells and release all intracellular bacteria. To quantify internalized bacteria, the cell lysates were processed for CFU counts, by plating serial dilutions on TSA plates and reading the CFU after 1 day of incubation at 37°C (Fig. 1).
Statistics
To assess the statistical significance of the results, the internalization of the different isolates was cross-compared by Bonferroni/Dunn test (significance level: 5%), using the StatView (ver. 5.0.1) software.

RESULTS
Characterization of bacterial strains
S. epidermidis ribotyping. By ribotyping, ribogroup cra-63-S-7 turned out the most frequent epidemic clone including five strains (cra1231, cra1330, cra1341, cra1362, and cra1379). In order of epidemic prevalence the other ribogroups were: the ribogroup cra-119-S-4 (two strains, cra1378 and cra1324); the ribogroup cra123-S-5 (a single strain, cra1282); the ribogroup cra-63-S-4 (three strains: cra1275, cra1298, and cra1182); the ribogroup cra-80-S-1 (the strain cra1428); the ribogroup cra-122-S-2 and the ribogroup cra-92-S-5 (the former: cra1138, cra1145, cra1187, and cra1198; the latter: cra1141, cra1186, cra1305, and cra1423); and, finally, cra93-S-3 (two strains: cra1131 and cra1404). The ribopatterns characterizing the diverse ribogroups considered in this study are shown in Figure 2.

S. lugdunensis ribotyping. Four S. lugdunensis strains, namely cra1871, cra1363, cra2847, and cra2653, were comprised in the ribogroup cra-62-S-2. Four S. lugdunensis isolates belonged to another prevalent ribogroup, named cra-64-S-8. The strain cra1750 belonged to the ribogroup cra-74-S-5.

Figure 3 illustrates the profiles of the three S. lugdunensis ribogroups automatically identified by the Riboprinter system.

E. faecalis ribotyping. The 21 E. faecalis strains investigated in the present study turned out to belong to seven...
distinct ribogroups: the ribogroup cra-115-S-2 (including five strains: cra1983, cra2397, cra1590, cra1165, and cra1524); cra-115-S-8 (cra1705, cra2175, cra1806, and cra2586); cra-116-S-1 (cra1648, cra2168, and cra2174); cra-116-S-7 (cra1884, cra1970, and cra2184); cra-118-S-1 (cra1527 and cra1349); cra-118-S-2 (cra2341 and cra1365); and cra-115-S-4 (cra1571 and cra1244/1).

Figure 4 shows the ribopatterns of the seven ribogroups.

**Microplate-based cell invasion assay**

The microplate-based method was found to be an advantageous technique to screen bacteria for assessing their invasiveness, with good reproducibility of the results and adequate sensitivity, being capable to theoretically detect an invasion rate of as low as around two internalized bacteria per well or per $1 \times 10^4$ eukaryotic cells, that is, 0.02%. In practice, the isolate of *S. lugdunensis* that was introduced as a negative (low internalization) control demonstrated a rate of invasion of about 0.16 ± 0.18%, that is, 16 CFU per $10^4$ bone cells (approximately 0.002% of the inoculum), while empty wells never showed the presence of viable bacteria proving the efficient total destruction of all extracellular bacteria. It should be noted that, for the negative as well as for the positive controls, wells without MG63 cells always gave a count of 0 CFU (Fig. 1).

On the contrary, for the positive control, the CFU counts showed that *S. aureus* cra1199 always exceeded the valid plate counts of a 1:10 dilution and, when quantified, its rate of invasion resulted $49.5 \pm 11.7 \times 10^3$ CFU per $1 \times 10^4$ cells, in percent 495%, meaning that an average of five bacteria invaded each osteoblast or otherwise that about 7.5% of the inoculum was internalized.

**Cell invasiveness of aminoglycoside-sensitive *S. epidermidis* isolates**

The analysis of the capacity of invasiveness of gentamicin-sensitive clinical isolates of *S. epidermidis* showed that for all 22 strains investigated no bacteria survived in wells where osteoblasts had not been seeded. However, even in presence of MG63 cells, the number of CFU counted per well was extremely low and, anyway, non comparable with that observed for either the positive control *S. aureus* cra1199 strain or the *S. aureus* ATCC25923 reference strain, respectively, exhibiting a count $5.0 \times 10^4$ and $1.9 \times 10^4$ per seeded well (Fig. 5). Indeed, the highest count of CFU recorded for *S. epidermidis* was observed with the isolate cra1231 and was of 106 CFU/well, roughly corresponding to about 1 internalized bacterium every 100 osteoblasts exposed to over 50,000 bacteria (Fig. 6).

This low number of bacteria that survived to the microbicidal mix of gentamicin and lysostaphin in the presence of osteoblasts does not fully support a significant ability of this opportunistic pathogen to gain entrance into osteoblastic cells. In view of the high inoculum consisting of about 500 bacteria per MG63 cell (note that for *S. aureus* the needed inoculum was as low as 100:1 MOI), this extent of bacterial survival appeared rather marginal. The curve MOI versus internalization was explored with the isolate cra1231. Figure 7 shows as a plateau of internalization was reached with inocula higher than the 500:1 MOI, which had been previously established for the reference strain NCTC11047 by Khalil et al., and approaching 1000:1 MOI. This slight variation could depend on differences in the test method or, alternatively, suggest that the curve of internalization is strain-specific, being influenced by a series of variables, such as strain adhesiveness to osteoblasts and, at least in the case of NCTC11047, a particular repertoire of mechanisms of internalization.

Figure 6 includes the information of the ribogroup of the isolates. Due to the low numerosity of the isolates within each single ribogroup and some variability of the verified inoculum size, it would be excessive to establish tout court possible clonal associations with the degree of internalization. Even so, some trends could be observed when considering similar inocula sizes, for example, those >500:1 MOI, within the same ribogroup and among ribogroups. For instance the ribogroup cra-63-S-7 included three strains that showed relatively high internalization (>50 CFU), while ribogroup cra-122-S-2 included three strains all exhibiting a level of internalization lower than 50 CFU. Furthermore, some ribogroups appear homogeneous in
terms of CFU internalized, independently from the inoculum size, while others exhibit some heterogeneity in spite of similar inoculum levels.

**Cell invasiveness of *S. lugdunensis* clinical isolates**

Nine *S. lugdunensis* clinical isolates were tested for their potential of invasiveness into MG63 cells. All the strains tested were sensitive to the treatment with gentamicin and, for all of them, the wells without MG63 cells showed a count of 0 CFU as expected, except for the isolate cra2363 in which case there was a single CFU in one of the three replica wells.

The cra1750 isolate was tested with two different inocula, ranging from a MOI of 270–495 (see Fig. 8). In spite of the differing size of the inocula, internalization of cra1750 did not show substantial differences and was anyway marginal (just 0.0004% of the inoculum was internalized at 270:1 MOI). All four *S. lugdunensis* isolates of the epidemic ribogroup cra-62-S-1 were tested with a relatively high inoculum and showed very low levels of internalization, similar than those observed for the cra1750 strain. The most internalizing isolate of the series was the isolate cra2653 that, tested at a MOI of 321:1, exhibited an internalization of just 0.0007% of the inoculum and only 0.2% of the MG63 cells (about 25 CFU/well) internalized bacteria. Despite the huge inoculum exceeding 1000:1 MOI, the internalization was even lower for the remaining isolates of the ribogroup, namely cra1871, cra1363, and cra2847.

The four *S. lugdunensis* strains of ribogroup cra-64-S-8 were tested at a MOI approaching and more frequently exceeding a ratio of 1000:1. The count of CFU internalized per well ranged from 17 for the cra3006 isolate to 62 for the most internalizing *S. lugdunensis* strain, cra2501/1. The minor differences observed when cross-comparing the *S. lugdunensis* ribogroups analyzed in this study do not support the conclusion of distinctive strain behavior associated

**FIGURE 4.** Grayscale and waveform ribopatterns of the ribogroups of the 21 *E. faecalis* isolates.
**FIGURE 5.** Bacterial internalization exhibited by the different orthopedic clinical *S. epidermidis* isolates in comparison to the positive (*S. aureus* cra1199) and negative control (*S. lugdunensis* cra1750) and the *S. aureus* ATCC25923 reference strain. The levels of *S. epidermidis* intracellular invasiveness appear marginal with respect to those of the positive control consisting of the internal strain *S. aureus* cra1199 and of the ATCC25923 reference strain.

**FIGURE 6.** Internalization of aminoglycoside-sensitive *S. epidermidis* orthopaedic clinical isolates. Verified MOI values are reported on the top of each bar. The internalization of the different isolates was cross-compared by Bonferroni/Dunn test (significance level: 5%). Comparisons were significant only with *p* values < 0.0002. Legend: a significant difference was found with: *(a)* all strains except for cra1298, cra1275, cra1428, and cra1330; *(b)* cra1324, cra1378, cra1198, cra1305, cra1362, and cra1379; *(c)* cra1278, cra1305, cra1324, and cra1378; *(d)* cra1279 and 1324; *(e)* cra1379; *(f)* cra1379; and *(g)* cra1231.
to a specific ribogroup, even if the two most internalized strains belonged to ribogroup cra-64-S-8. Overall, *S. lugdunensis* strains were found to exhibit a rather low number of internalized CFU, suggestive of a bacterial uptake similar, or even lower in view of the higher inocula tested, than that observed for the *S. epidermidis* clinical isolates of the present collection. The Bonferroni/Dunn test did not demonstrate any significant difference between the strains of *S. lugdunensis* investigated.

**Cell invasiveness of *E. faecalis* clinical isolates**

Five of the 21 strains of *E. faecalis* showed a marked increase of internalization with respect to *S. epidermidis* and *S. lugdunensis* strains. In particular, three ribogroups, cra-116-S-1, cra-118-S-2, and cra-115-S-4, included the strains exhibiting higher internalization (Fig. 9). Nonetheless, taking into account the conspicuous inocula frequently exceeding a ratio of 1500 bacteria per MG63 cell (3–4 times that used for *S. epidermidis*), internalization should be considered overall poor and corresponding to a quote, at most, of about 0.002% of the inoculum used. For the strain showing the highest intracellular invasiveness, the proportion of internalized bacteria-MG63 cells reached about 4%, that is, two orders of magnitude lower than that of *S. aureus* cra1199.

FIGURE 7. MOI versus CFU relationship curve for the *S. epidermidis* cra1231 strain. It may be noticed that a plateau seems to be reached at inocula higher than 1000:1 MOI, this finding differing from what previously observed in literature by other investigators using the *S. epidermidis* NCTC11047 reference strain. Excluding differences associated with the different experimental model (our data on *S. aureus* provided a curve equivalent to that reported in literature with a MOI value approaching the plateau of 100:1), these results indicate that a curve of MOI should ideally be calculated for each single isolate. In fact, variables such as adhesion on eukaryotic cell surfaces (determined either by passive adhesion or by repertoire of adhesive factors influencing bacterial anchorage on cell membrane proteins), and virulence traits capable of cell transduction signals facilitating bacterial internalization could determine differently shaped MOI versus CFU curves.

FIGURE 8. *S. lugdunensis* cell invasiveness. The bar-graph illustrates the internalization of nine different *S. lugdunensis* isolates belonging to three distinct ribogroups. On the top of each bar the verified values of MOI are reported. The *S. lugdunensis* cra1750 isolate was obtained from an infection non-associated with medical devices and was analyzed at two different inocula. Despite the different MOI tested, no substantial variation could be observed in the extent of internalization, which remained anyway very low. Conversely, for each of the ribogroups cra-62-S-1 and cra-64-S-8, four different *S. lugdunensis* isolates derived from implant-related infections were tested. In spite of the high MOI employed, the internalization always remained very low. The cra2601/1 isolate within the cra-64-S-8 ribogroup showed the highest internalization rate consisting of about 62 CFU/well containing approximately 10^4 MG63 cells. Also in view of the high MOI tested, these data suggest that the uptake of *S. lugdunensis* by osteoblasts is similar or even lower than that observed for most *S. epidermidis* strains. The Bonferroni/Dunn test, applied to cross-compare all isolates, showed that differences were not statistically significant.
Internalization of the strains belonging to the other remaining ribogroups was generally as low as that found for *S. epidermidis* and *S. lugdunensis*, despite the more concentrated inocula. The restricted number of isolates per ribogroup and strain diversity did not allow to infer a significant association of highly internalizing strains with a specific ribogroup.

However, in contrast to what observed in *S. lugdunensis*, the Bonferroni/Dunn test did demonstrate some statistically significant difference from the comparison of the 21 strains of *E. faecalis*. Figure 9 illustrates the internalization of the isolates organized per ribogroup, reporting the results of the cross-comparison by statistical analysis.

**Cross-comparison of the different species investigated**

Although tested with high-titer inocula, the clinical isolates of all three opportunistic species investigated in the present study exhibited marginal rates of internalization with respect to *S. aureus*. The bar-graph in Figure 10 reports in a logarithmic scale the mean value ± SD of all isolates of each opportunistic species. It may be noticed that, as well documented in literature, the levels of internalization of *S. aureus* appear important already at a relatively low inoculum size. Conversely, the isolates of the two CNS species and of *E. faecalis* showed an only marginal internalization, 2–3 orders of magnitude lower than that found for *S. aureus*, despite the high MOI utilized.

**DISCUSSION**

The present study addresses the following issues: whether three opportunistic bacterial species, namely *S. epidermidis*,

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**FIGURE 9.** *E. faecalis* cell invasiveness. The bar-graph illustrates the internalization of 21 different *E. faecalis* isolates belonging to seven distinct ribogroups. It may be noticed that some *E. faecalis* strains exhibited a relatively significant number of internalized bacteria (1402 CFU in the case of cra1244/1). Among different ribogroups, cra-116-S-1, cra-118-S-2, and cra-115-S-4 contained the strains exhibiting higher internalization. The other ribogroups showed levels of internalization similar to those of *S. epidermidis* and *S. lugdunensis* in spite of the increased size of inocula. The internalization of the different isolates was crosscompared by Bonferroni/Dunn test (significance level: 5%). Comparisons were significant only with *p* values <0.0002. Legend: a significant difference was found with: *(a)* all strains except for cra2168, cra2174, cra1365, and cra1571; *(b)* all strains except for cra2168, cra2174, cra1365, cra1571, and cra1244; and *(c)* cra1983, cra2397, cra1590, cra1705, cra2175, cra1806, cra1884, cra1970, and cra2341.

**FIGURE 10.** The bar-graph illustrates on a logarithmic scale the internalization of the different opportunistic pathogens investigated with respect to the two *S. aureus* strains. The different size of inocula does not allow a direct comparison of these species. Nonetheless, considering that the scale is logarithmic, it is noticeable as the opportunistic species investigated, in spite of the high inocula, remain orders of magnitude distant from a pathogenic species such as *S. aureus*, invading osteoblastic cells in conspicuous numbers already at relatively low ratios of bacteria/eukaryotic cells.
S. lugdunensis, and E. faecalis, known as new pathogens, are causative of serious implant-related orthopedic infections, are able to penetrate into osteoblastic cells; whether internalization plays a role in the pathogenesis of periprosthetic bone infections caused by the three species; and, consistently, whether intracellularly active antibiotics are really necessary in a prophylactic/therapeutic strategy for thwarting/treating bone peri-implant infections caused by these bacterial species.

Focusing first on S. epidermidis, it should be considered that, besides being a main human opportunistic pathogen, this bacterium is also well-known as an etiologic agent of clinical and subclinical mastitis in dairy cattle. It is presumably for this reason that investigations on S. epidermidis intracellular invasiveness started with a veterinary study. That study showed that S. epidermidis and other coagulase-negative staphylococci, such as Staphylococcus xylosus and Staphylococcus hyicus, are able to internalize into bovine mammary cells in a receptor(s)-mediated process that exploits the host signal transduction and cytoskeleton for inducing an uptake signal. It is only in 2007 that Khalil et al. reported that some S. epidermidis strains could be internalized by human osteoblasts. However, on the contrary of what observed for S. aureus, internalization was not common among S. epidermidis strains. Only three of the six investigated strains were consistently internalized by osteoblasts, respectively, the reference strain NCTC11047 and two clinical strains, namely 19 and HB, both fibrinogen binding. The former, strain 19, had been isolated from a case of peritonitis and the latter from an osteomyelitis. Based on this study, the intracellular invasive properties vary not only depending on the species but also, within the same species, on the strain type. Although reporting slightly lower counts than in the early work by the group of Khalil et al., Valour et al. confirmed the invasiveness potential of the strain NCTC11047 against human bone cells (MG63 and osteoblasts). These pioneering findings warrant further investigations, extended to large collections of clinical isolates, in order to better define the role played by bacterial internalization under different clinical circumstances. This is particularly true for the emerging opportunistic species, where just a few strain types could have acquired/developed virulence traits and exhibit cell invasive characteristics.

Aiming at screening numerous strains, it was thought to rely on an experimental design offering the option to contemporarily assay diverse bacterial isolates on the same plate together with appropriate controls. Thus, we adapted the assay for intracellular invasiveness to microtiter plates. Although reduced in scale, this new approach was found to be sensitive and to allow the detection of extremely low levels of bacterial intracellular invasiveness.

In the present study, a considerable number of finely characterized and typed clinical strains from orthopedic implant infections were investigated. Our data indicate that for the clinical isolate exhibiting the highest internalization potential, S. epidermidis cra1231, only 0.002% of the inoculum was internalized. Definitively, most S. epidermidis clinical isolates from orthopedic infections do not appear to possess a consistent osteoblast intracellular invasiveness. It has to be emphasized as the broad variety of epidemic ribgroups of S. epidermidis that was considered in this study strengthens the value of this conclusion. It remains to be verified whether endothelial cells, anyway present in vascularized tissues, could represent also for this species, as well as for S. aureus, a significant reservoir of viable bacteria in bone tissue. Indeed S. aureus is characterized by a high potential of virulence in terms of intracellular invasiveness toward both endothelial and bone cells, these endowments providing a plausible explanation for the relapses following medical treatments of implant-related osteomyelitis.

S. epidermidis internalization appears very critical in various veterinary and clinical conditions, in view of the high internalization levels of S. epidermidis in bovine epithelial and endothelial cells and in human embryonic kidney cells.

When the most internalized S. epidermidis cra1231 was investigated, the optimal MOI concentration appeared to be greater than that previously reported in literature for the NCTC11047 reference strain. Apart from the differences related to the in vitro model adopted, it is conceivable that different curves of MOI versus internalized bacteria can be found depending on the mechanism/s of invasion of the strain under study. Differently from S. aureus, which requires a very light inoculum for reaching a high internalization rate, S. epidermidis cell invasiveness remains low and marginal for most strains even with highest inocula. This finding indicates that the active mechanisms of invasion exhibited by S. aureus are either absent or, if any, much less efficient in S. epidermidis.

As far as the other two opportunistic species S. lugdunensis and E. faecalis are concerned, this study is the first attempting to ascertain if orthopedic clinical isolates are consistently capable of entering human osteoblasts. The very low level of internalization observed for the clinical isolates of S. lugdunensis and E. faecalis (for S. lugdunensis, as for S. epidermidis, of the order of 1 internalized bacterium every 100 eukaryotic cells; for E. faecalis, up to four bacteria every 100 eukaryotic cells achieved with very large inocula) was a consistent finding (Fig. 10).

The minimal levels of endocytosis observed with some strains of the three bacterial species could simply depend on a nonspecific passive internalization by osteoblast cells. And indeed, even internalization of inanimate microparticles is known to occur in nonphagocytic cells. In presence of extremely low levels of internalization, as in the case of the strains of the opportunistic species here investigated, it could be inappropriate to talk of invasiveness, as the phenomenon could possibly take place even with inanimate particles and does not imply specific and active mechanisms. Under such circumstances, internalization or, more appropriately, uptake takes place without the specific signal transduction mechanisms supposedly implicated in the infection pathogenesis. Up to now, this point has not been exhaustively addressed in literature and, thus, unjustified.
properties of invasiveness are sometimes attributed to bacterial isolates that are incapable to actively internalize. Uptake of nonviable solid microspheres consisting of plastics (e.g., polystyrene), metals (e.g., titanium) or other materials is known for a variety of nonprofessional phagocytes and host cell types. Likely, properties of repulsion/adhesion to eukaryotic cell surfaces could determine variations of cell uptake among microspheres of similar sizes. It would be therefore relevant to compare to which extent the uptake of bacterial strains differs from that of inert material microparticles. In this regard, it should be remarked that studies on *Escherichia coli* invasiveness toward vegetable cells demonstrated that there was no difference between the internalization of fluorescent microspheres and that of the bacterium.

Differently from the majority of *S. epidermidis* strains, NCTC11047 and a few other *S. epidermidis* strains that have been reported to exhibit internalization properties toward human osteoblasts, could have acquired the possibility to interact with osteoblasts by inducing actively endocytosis through more efficient cell-targeted mechanisms.

Based on the present observations, *S. lugdunensis* and *E. faecalis* orthopedic isolates show very low intracellular invasiveness, similar to that of the many *S. epidermidis* orthopedic clinical strains of the investigated collection. We hypothesize that the opportunistic species examined could rely their survival to host immune defenses and medical treatments on the ability to form biofilm on colonized implant surfaces. In this connection, it is interesting to underline that all *S. lugdunensis* and *E. faecalis* and nearly 50% of *S. epidermidis* strains of our collection have been recognized as biofilm forming. These findings do not anyway exclude that, in other clinical infections involving different tissues, the same bacterial species can be endowed with or can express invasins able to target host cells. Hussain et al. have very recently assayed the biofilm formation by the *S. lugdunensis* clinical strain SI253 and explored its intracellular invasiveness in epithelial, endothelial and fibroblast cells, demonstrating that internalization abilities were associated to the presence of the autolysin Atl. The autolysin Atl could therefore play for *S. lugdunensis* a role analogue to that played by the autolysins Atl in *S. aureus* and AtlE in *S. epidermidis*, which were found to mediate adhesion and internalization into human endothelial cells through the interaction with the Hsc70 receptor. The involvement of molecular mechanisms in bacterial internalization is also underlined by the finding that *S. aureus* Cowan internalization is completely inhibited by cytochlasin D, an inhibitor of F-actin polymerization, while *S. lugdunensis* internalization is just slightly affected. Furthermore, the internalization of *S. lugdunensis* clinical strains to bladder carcinoma and endothelial cells was found not to be influenced by the presence/absence of the fibrinogen-binding protein Fbl, but rather associated to the adhesiveness of the respective strains to solid-phase fibronectin. Thus, even within the same *Staphylococcus* genus, there is evidence that the different species have evolved distinct invasins, implicating different molecular mechanisms of interaction and different specificities toward eukaryotic cell types. Even within the same species, different strains may exhibit a different invasive capacity. Given the critical importance of intracellular invasion for the related risk of infection relapse, especially in orthopedics, it is of great importance to gain a comprehensive knowledge of the infection pathogenesis in different scenarios and to achieve sound indications for an effective antibiotic chemotherapy.

**CONCLUSIONS**

The approach of the microtiter plate method to investigate bacterial internalization in eukaryotic cells has been found of appropriate sensitivity to accurately detect internalization even in case of bacterial species characterized by very low rate of intracellular uptake.

For the *S. epidermidis* strains of our collection, isolated from orthopedic implant-associated infections, we have observed that internalization in osteoblasts is not as relevant as in *S. aureus*. Future efforts should be made to study the genome of those few *S. epidermidis* strains that have been reported in literature to escape to this rule and exhibit in human osteoblasts an efficient internalization, equivalent to that of *S. aureus*.

Moreover, results have also demonstrated, for the first time, that *S. lugdunensis* and *E. faecalis* are incompetent to penetrate osteoblasts. Thus, based on current knowledge, *S. aureus* therapies for osteomyelitis and implant infections should comprehend the use of antibiotics permeable to host cells and active intracellularly. Vice versa, our results suggest that the use of intracellularly active antibiotics should not be generally necessary for infections caused by *S. epidermidis, S. lugdunensis* or *E. faecalis*, the first being in absolute, straight after *S. aureus*, the major etiologic agent of orthopedic implant infections, the other two emerging opportunistic pathogens causative of serious orthopedic (and cardiovascular) implant-related infections.

Finally, enhancing microparticle internalization by nonphagocytic cells is of great interest for a vast number of potential applications in the biomedical field, these ranging from the creation of biosensor systems, the development of intracellular drug delivery systems, to therapeutic implants. The knowledge of bacterial molecular mechanisms capable of inducing efficient and cell type-selective uptake of microparticles is expected to have in the future relevant implications for innovative approaches in the biomaterials field.

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