ACINETOBACTER QUORUM SENSING CONTRIBUTES TO INFLAMMATION-INDUCED INHIBITION OF ORTHOPAEDIC IMPLANT OSSEOINTEGRATION

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Abstract

Implant infection impairs osseointegration of orthopaedic implants by inducing inflammation. Acinetobacter spp. are increasingly prevalent multi-drug resistant bacteria that can cause osteomyelitis. Acinetobacter spp. can also cause inflammation and thereby inhibit osseointegration in mice. The purpose of the present study was to investigate the role of quorum sensing in this context. Therefore, wild-type bacteria were compared with an isogenic abal mutant defective in quorum sensing in a murine osseointegration model. The abal quorum-sensing mutant affected significantly less osseointegration and interleukin (IL) 1β levels, without detectably altering other pro-inflammatory cytokines. Wild-type bacteria had fewer effects on IL1 receptor (IL1R)−/− mice. These results indicated that quorum sensing in Acinetobacter spp. contributed to IL1β induction and the resultant inhibition of osseointegration in mice. Moreover, targeting the Gram-negative acyl-homoserine lactone quorum sensing may be particularly effective for patients with Acinetobacter spp. infections.

Keywords: Acinetobacter, implant infection, osseointegration, osteolysis, quorum sensing.

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Introduction

The Gram-negative *Acinetobacter calcoaceticus-A. baumannii* complex is a relatively common cause of osteomyelitis and delayed healing of orthopaedic injuries (Davis et al., 2005; Johnson et al., 2007; Yun et al., 2008). For example, *Acinetobacter* spp. were identified in 50-70 % of osteomyelitis cases in American soldiers wounded in Afghanistan or Iraq (Davis et al., 2005; Johnson et al., 2007; Yun et al., 2008). Those osteomyelitis cases required multiple surgical debridements of necrotic bone and led to delayed fracture healing. Extended courses of antibiotics were administered and were likely responsible for the low frequency of recurrent infection with *Acinetobacter* spp. (Davis et al., 2005; Johnson et al., 2007; Yun et al., 2008). *Acinetobacter* spp. can persist in healthcare environments (Weber et al., 2010) and also frequently acquires multi-drug resistance, further complicating clinical outcomes (Davis et al., 2005; Fily et al., 2019; Munoz-Price and Weinstein, 2008; Perez et al., 2011; Tan and Moenster, 2019; Weber et al., 2010). Consistent with inflammatory responses induced by *Acinetobacter* spp. in soft tissues and the bloodstream (Doi et al., 2015; Dou et al., 2017; Feng et al., 2014; Lin et al., 2012; Mortensen and Skaar, 2012), *Acinetobacter* spp. also cause inflammation and thereby inhibit osteointegration in mice (Choe et al., 2022).

Bacterial implant infection is a devastating complication for orthopaedic patients that induces inflammation and osteolysis and thereby inhibits osteogenesis and osseointegration, causing loosening of previously well-fixed implants (Campoccia et al., 2006). A difficulty in treatment of implant infection is associated with formation of a biofilm. Bacterial biofilms reduce clearance of implant infections by antibiotics and the host immune system (Arnold et al., 2014; Costerton et al., 2007; Lazar et al., 2021). Bacterial density within biofilms is controlled by quorum sensing mediated by autoinducers that regulate bacterial gene expression (Bhargava et al., 2010). Quorum sensing in *Acinetobacter* spp. also regulates virulence, motility, antimicrobial tolerance and modulation of the host immune system (Bhuiyan et al., 2016; Clemmer et al., 2011; Dou et al., 2017; Glucksam-Galnoy et al., 2013; Sun et al., 2021; Tang et al., 2020). Similar to most other Gram-negative bacteria, the primary quorum sensing mediators in *Acinetobacter* spp. are acyl-homoserine lactones, produced by an autoinducer synthase encoded by the *abal* gene (Anbazhagan et al., 2012; Bhargava et al., 2010; Gonzalez et al., 2009; Niu et al., 2008). The receptor for the acyl-homoserine lactones in *Acinetobacter* spp. is encoded by *abaR* (Bhargava et al., 2010). Recent RNA-sequencing analysis showed that the *abal/abaR* quorum-sensing system can regulate expression of numerous genes in *Acinetobacter* spp., including genes that are important for virulence, biofilm formation, antibiotic resistance, energy metabolism, degradation of branched-chain amino acids and lipid metabolism (Sun et al., 2021).

The present study used a previously described murine model to assess effects of implant infection on osseointegration (Choe et al., 2015; 2022) in wild-type mice or mice null for IL1R. A. *nosocomialis* strain M2 was used, which was previously known as A. *baumannii* strain M2 and was originally isolated from a hip infection (Carruthers et al., 2013). To address the role of quorum sensing, a strain M2 mutant was used that lacks quorum sensing and has modestly reduced biofilm formation due to a transposon insertion (abal::EZTn5<kan>) into *abal* (Niu et al., 2008). Findings revealed that *Acinetobacter* spp. quorum sensing contributes to inflammation and impaired osseointegration in mice.

Material and Methods

Preparation of implants with adherent bacteria

Titanium alloy screw-shaped implants (Ti-6Al-4V, 3.2 mm length, 1.0 mm diameter, Antrin Miniature Specialties Inc, Fallbrook, CA, USA) were rigorously cleaned following five cycles of alternating treatments in alkali ethanol (0.1 mol/L NaOH and 95 % ethanol at 32 °C) and 25 % nitric acid (Bonsignore et al., 2011). Wild-type *A. nosocomialis* strain M2 was compared with *abal* isogenic mutant (Niu et al., 2008) to determine effects of quorum sensing. 1 d before each implant surgery, a single colony of wild-type or *abal* mutant *A. nosocomialis* strain M2 was inoculated into 5 mL of MHB medium (Fisher Scientific) and incubated at 37 °C overnight in a bacterial shaker. Overnight suspensions were diluted 100-fold in MHB medium and incubated at 37 °C until early log phase was reached (A600/0.1 cm light path = 0.05; Nanodrop 1000; Fisher Scientific). Those low-concentration bacterial suspensions (1·3  × 10⁸ CFUs/mL) were centrifuged (1,500 × g, 5 min) and resuspended in 1/30 volume of MHB broth to obtain high concentration suspensions (3·9  × 10¹⁰ CFUs/mL). Rigorously cleaned implants were incubated with high concentration bacterial suspensions for 24 h at 37 °C with gentle shaking to obtain the *A. nosocomialis* strain M2 dose previously found to routinely provide chronic localised implant infections without any signs of systemic sepsis (Choe et al., 2022). Implants with
adherent bacteria were rinsed 3-times in PBS (Pro200H, Pro Scientific, Oxford, CT, USA) (pH 7.4) and immediately implanted into mice as described below. Additional implants were simultaneously prepared to measure the adherent CFUs following sonication in PBS with 0.3 % Tween-80 for 10 min (50 W, 43,000 Hz) and vortexing for 5 min (Bernthal et al., 2010; Pribaz et al., 2011). Adherent wild-type and abaI (Niu et al., 2008) isogenic mutant A. nosocomialis CFUs were $0.3-1 \times 10^7$ CFUs/implant, without any statistical difference among different bacterial groups.

Animal surgery
Wild-type C57BL/6J and IL1R−/− mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and MAFIA mice (Burnett et al., 2004; Chinnery et al., 2009) were a gift from Dr Eric Pearlman (CWRU Department of Ophthalmology, Cleveland, OH, USA). All experiments with IL1R−/− mice included wild-type control mice matched for genetic background (C57BL/6J), age (6-8 weeks old) and sex. Mice were maintained in the CWRU Animal Resource Center and all procedures were approved by the CWRU Institutional Animal Care and Use Committee. Mice were randomised among groups, anaesthetised and treated with analgesics (0.5 mg/kg local marcare and 1.0 mg/kg systemic slow-release buprenorphine) according to a previously established protocol (Choe et al., 2015; 2022). Briefly, a unicortical pilot hole was made manually (0.75 mm pilot hole drill, KLS Martin, Jacksonville, FL, USA) at the anterior medial aspect of the femoral diaphysis and the implants were manually screwed into the pilot hole. The femur fractured during implantation in one of the 169 mice used for the study. That mouse was euthanised immediately and excluded from the analysis. The remaining 168 mice tolerated the surgery well, could ambulate immediately and were included in the study.

FLI
In MAFIA mice, a monocyte/macrophage-specific c-fms promoter drives expression of both enhanced green fluorescent protein and a modified version of fas that can induce apoptosis in response to the small molecule inducer AP20187 (Burnett et al., 2004; Chinnery et al., 2009). Since FLI signals are severely attenuated by overlying tissues, the femora, implants and surrounding soft tissues were exposed for ex vivo imaging by dissection and opening the soft tissue. FLI signals were defined by automatic spectral segmentation and quantified in automatically selected ROIs encompassing femora and surrounding soft tissues using a Maestro Imaging System (Perkin Elmer) in the CWRU Center for Imaging Research.

Histomorphometry
Dissected femora were prepared for histomorphology assessment as described previously (Bonsignore et al., 2011). Briefly, femora were fixed in formalin for

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**Fig. 1.** abaI mutation reduced the effect of *Acinetobacter* on osseointegration. (a-c) Biomechanical and (d-e) histomorphometric measures of osseointegration and CFUs on (f) implants and (g) in surrounding femora of control groups without bacteria and of isogenic *A. nosocomialis* strain M2 wild-type and abaI mutant groups were measured in C57BL/6J mice. Solid horizontal bars show means for parametric analysis (*p < 0.05). Dashed bars show medians for non-parametric analysis (#p < 0.05).
24 h and dehydrated in 70 % ethanol. Undecalcified ground cross-sections (100 μm) were stained with Sanderson’s Rapid Bone Stain (Surgipath Medical Industries, Richmond, IL, USA). Bone-to-implant contact and peri-implant bone were measured using ImageJ analysis software (National Institutes of Health). The percentage of direct bone-to-implant contact was calculated in a ROI extending from the periosteal surface of the cortex to the tip of the last implant thread. The percentage of peri-implant bone was calculated in a ROI between the implant threads. The bottom edge of the implant was excluded from all calculations (Bonsignore et al., 2011).

Biomechanical testing
Pull-out testing was performed immediately after euthanasia as previously described (Bonsignore et al., 2011), except a Test Resources 100R Series Single Column Frame (Test Resources, Shakopee, MN, USA) with a 100R Controller was used. Briefly, femora were placed under wire loops embedded in poly-methyl methacrylate and the implant was gripped by a custom-designed jig, which was then attached to the Test Resources Frame. Force was measured through a 4.5 kg capacity load cell and testing was performed at a displacement rate of 1 mm/min. Ultimate force, average stiffness and work to failure were determined from load versus displacement curves according to ASTM standards, F543-07. To reduce pre-loading variability, calculations of work began when force equalled 0.3 N.

CFU counting
CFUs on implants and in surrounding femora were quantified after pull-out testing (Choe et al., 2015; 2022). Implants were sonicated for 10 min (50 W, 43,000 Hz) in PBS with 0.3 % Tween-80 followed by vortexing for 5 min (Bernthal et al., 2010; Pribaz et al., 2011). Femora were homogenised in PBS (Bernthal et al., 2010). CFUs in sonicates and homogenates were counted on MHB broth agar plates.

Evaluation of pro-inflammatory cytokines and chemokine
Femur homogenates were centrifuged (9,000 ×g, 10 min) and supernatants were stored at −20 °C. The concentrations of TNFα, IL1α, IL1β, IL6, RANKL and CCL2 were measured using ELISA DuoSet mini-kits (catalogue numbers DY410, DY400, DY401, DY406, DY462 and DY479, R&D Systems). Biomechanical testing, CFU counting and cytokine measurements were all done on the same mice.

Statistical analysis
All statistical analyses were performed using Prism 7 software (GraphPad Software). Statistical significance was determined by Student’s t-test or one-way ANOVA followed by Bonferroni post-hoc test in experiments with multiple groups. Non-parametric Mann-Whitney tests or Kruskal-Wallis analysis of variance followed by Student-Newman-Keuls post-hoc tests were applied to data sets that were not normally distributed or were not of equal variance. Tests were reported as significant for *p* < 0.05.

Results

Effect of Acinetobacter on osseointegration
No signs of systemic infection were observed in any mice. Osseointegration increased in groups without bacteria between 7 and 14 d post-implantation (circles in Fig. 1a-c, Fig. 2a). In contrast, all three biomechanical (diamonds in Fig. 1a-c) and both histomorphometric (diamonds in Fig. 1d,e, Fig. 2b)
Effect of abaI-deficiency on osseointegration in Acinetobacter infection

To determine mechanisms responsible for effects of A. nosocomialis strain M2, an abaI-deficient isogenic mutant was used that lacks quorum sensing (Niu et al., 2008). The abaI quorum-sensing mutant (upward triangles in Fig. 1a-e, Fig. 2c) had less effect than the wild-type strain. For example, the wild-type and abaI mutant strains were significantly different regarding all three biomechanical measures of osseointegration at day 14 (Fig. 1a-c) and with regard to both histomorphometric measures at day 7 (Fig. 1d,e). Importantly, effects of the abaI mutation were not due to different bacterial growth since neither mutant altered the number of bacteria on implants or in surrounding bones (Fig. 1f,g).

Difference in induction of cytokines between wild-type Acinetobacter and mutants

Although A. nosocomialis strain M2 infection led to increased macrophage recruitment to the site of infection, the abaI mutation did not alter macrophage recruitment (Fig. 3a). CCL2, TNFα, IL6, IL1α and IL1β were measured in femora surrounding implants as examples of local inflammatory cytokines. CCL2 and TNFα were not induced at either day 7 or day 14 following implantation (Fig. 3b,c). IL6 and IL1α were increased equivalently by wild-type A. nosocomialis strain M2 and the abaI mutant strain (Fig. 3d-f). In contrast, the IL1β level closely tracked with impaired osseointegration as the abaI mutant (upward triangles) had significantly less effect on IL1β at day 7 (Fig. 3f), on bone to implant contact at day 7 (Fig. 1d) and on all three biomechanical measures of osseointegration at day 14 (Fig. 1a-c).

Effect of IL1R on cytokine expression and impaired osseointegration in implant infection with Acinetobacter

To test the functional role of IL1β, effects of A. nosocomialis strain M2 in wild-type and IL1R−/− mice were compared (Glaccum et al., 1997). Consistent with an important role for IL1β, wild-type A. nosocomialis strain M2 had less effect on ultimate force in IL1R−/− mice (diamonds in Fig. 4a) and there was a trend towards less effect on work to failure (p = 0.07, diamonds in Fig. 4b), while stiffness was not affected by IL1R deletion (diamonds Fig. 4c). Similarly, levels of CCL2, IL6 and RANKL were reduced in IL1R−/− mice at day 14 (diamonds in Fig. 4d-f). Importantly, the IL1R deletion did not alter osseointegration or

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**Fig. 3.** abaI mutation reduced the effects of Acinetobacter on IL1β production. (a) Macrophage recruitment was measured in MAFIA mice. Levels of (b) CCL2, (c) TNFα, (d) IL6, (e) IL1α and (f) IL1β in control groups without bacteria as well as in isogenic A. nosocomialis strain M2 wild-type and abaI mutant groups were measured in C57BL/6j mice. Assay ranges were (b) 58.6 pg-7.5 ng/femur for CCL2, (c) 15.6 pg-2 ng/femur for TNFα, (d) 15.6 pg-2 ng/femur for IL6, (e) 16.4 pg-2.1 ng/femur for IL1α, and (f) 78 pg-10 ng/femur for IL1β. Solid horizontal bars show means for parametric analysis (*p < 0.05). Dashed bars show medians for non-parametric analysis (#p < 0.05). N:S: no significant difference.
cytokine production in the absence of bacteria or in the presence of the abaI mutant strain (circles and upward triangles in Fig. 4a-f). The effects of ILR1 deletion were not due to differential bacterial growth since deletion did not affect the number of wild-type bacteria or the abaI mutant on implants or in surrounding bones (Fig. 4g,h).

Discussion

The present study showed that the quorum-sensing system of Acinetobacter spp. infections on implants contributed to macrophage recruitment, production of inflammatory cytokines, osteolysis and impaired osseointegration. These effects are likely due, in part, to regulation by quorum sensing of genes that are important for virulence (Sun et al., 2021; Tang et al., 2020). Also, the effects of Acinetobacter spp. quorum sensing were due, in part, to increased induction of IL1β. Those results were consistent with findings that IL1β contributes to inflammatory osteolysis induced by Staphylococcus aureus in mice (Bernthal et al., 2011; Putnam et al., 2019; Wang et al., 2020) and that single nucleotide polymorphisms in IL1β and IL1R associate with human osteomyelitis (Alves De Souza et al., 2017; Osman et al., 2016). A limitation of the study was that it was not determined whether the impaired osseointegration was due to reduced osteogenesis, increased osteolysis or both (Choe et al., 2022). The study was also restricted to 14 d following bacterial inoculation and it is, therefore, unknown whether the infection, inflammation or impaired osseointegration would resolve at later time points. Also, it was not determined which virulence factors acted downstream of the quorum-sensing system to regulate IL1β production and osseointegration or which mammalian cells were the direct targets of those virulence factors. It is likely that multiple virulence factors contribute as hundreds of gene are regulated by the quorum-sensing system in Acinetobacter spp. (Sun et al., 2021). Virulence factors

![Fig.4. IL-1R mediated the effects of Acinetobacter on osseointegration.](image)

(a-c) Biomechanical measures of osseointegration, (d-f) levels of CCL2, IL6 and RANKL, (g-h) bacterial burden in control groups without bacteria as well as in isogenic A. nosocomialis strain M2 wild-type and abaI mutant groups were compared in IL1R1+ and their wild-type control mice. Assay ranges were (d) 19.5 pg-2.5 ng/femur for CCL2, (e) 15.6 pg-2 ng/femur for IL6 and (f) 31 pg-4 ng/femur for RANKL. Solid horizontal bars show means for parametric analysis (*p < 0.05). Dashed bars show medians for non-parametric analysis (#p < 0.05).
that might be involved include the acyl-homoserine lactones themselves acting through mammalian T2R receptors, pathogen-associated molecular patterns that activate mammalian pattern recognition receptors and multiple others (Carey and Lee, 2019; Glucksam-Galnoy et al., 2013; Lin et al., 2012; Bhuiyan et al., 2016; Kale et al., 2017; Morris et al., 2019).

*Acinetobacter* spp. quorum sensing, similarly to most other Gram-negative bacteria, is mediated by acyl-homoserine lactones (Anbazhagan et al., 2012; Bhargava et al., 2010; Niu et al., 2008). Novel approaches targeting the Gram-negative quorum-sensing system or the T2R mammalian receptors for acyl-homoserine lactones (Carey and Lee, 2019) may, therefore, be particularly effective for *Acinetobacter* spp. infections (Bhargava et al., 2010; Bjarnsholt and Givskov, 2007; Costerton et al., 2007; Lazar et al., 2021). The potential utility of these quorum-quenching approaches is further enhanced by recent reports that acyl-homoserine lactones induce antibiotic resistance in *Acinetobacter* spp. (Dou et al., 2017). Examples of these approaches include developing antagonistic acyl-homoserine lactones (Stacy et al., 2012), engineering thermostable lactonases that can degrade a broad range of acyl-homoserine lactones (Chow et al., 2014) and repurposing drugs that are FDA-approved for other indications (Seleem et al., 2020). The results regarding quorum sensing in the Gram-negative *Acinetobacter* spp. were reminiscent of the extensive literature reviewed by Urish and Cassat (2020) showing that the peptide-based quorum-sensing systems of Gram-positive bacteria also contribute to inflammatory osteolysis.

Importantly, the observed effects of the gene deletions, either in the *A. nosocomialis* strain M2 or in the mice, were not due to differential bacterial growth since none of them altered the number of bacteria on retrieved implants or in surrounding bone. A limitation of the study was use of a transposon mutant without determining whether the effects were reversed in a complemented strain of bacteria. However, it is unlikely that the *abaI* transposon has a polar effect on expression of downstream genes as *abaI* is the last gene in an operon and there are no genes downstream in the same orientation.

In the present study, *A. nosocomialis* strain M2 caused inflammatory osteolysis around implants in addition to impaired osseointegration. This finding would not have been predicted based on the report that *Acinetobacter* spp. increases osteogenesis in mice without detectably inducing osteolysis (Crane et al., 2009). This discrepancy could be due to testing different amounts (Vidlak and Kielian, 2016) or different strains of *Acinetobacter* spp. For example, it is unknown whether the strain used in the previous report (Crane et al., 2009) possesses a quorum-sensing system.

In conclusion, results showed that novel approaches targeting the quorum-sensing system of Gram-negative bacteria may be particularly effective for *Acinetobacter* spp. infections. The murine model will also be useful for future studies to clarify the mechanism of implant failure due to *Acinetobacter* spp. and to assess novel diagnostic tools or therapeutic agents.

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Contributions are as follow. HC, OA, PR, ZL, RB, EG designed the experiments. HC, BH, KH, EG conducted the experiments. HC, OA, PR, ZL, RB, EG wrote the manuscript.

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**References**


Discussion with Reviewer

Reviewer: In this model, the inoculated bacteria do not establish a long-term infection as evidenced by low or zero CFU at day 14 on the implant. Therefore, any effects of infection are only in the early post-operative phase. Does this suggest that Acinetobacter is not a true bone pathogen but rather induces inflammation in contaminated wounds that may extend to bone, without actually inducing osteomyelitis?

Authors: We agree that the CFUs are lower on day 14 than on day 7, especially on the implants (Fig. 1f). However, Fig. 1g shows substantial CFUs on day 14 in the femora of the groups with either wild-type or abaR mutant bacteria. The study was restricted to
14 d after bacterial inoculation and it is, therefore, unknown whether the infection, inflammation or impaired osseointegration would resolve at later time points. *Acinetobacter* is frequently considered to cause osteomyelitis in human patients (Davis et al., 2005; Fily et al., 2019; Johnson et al., 2007; Tan et al., 2019; Yun et al., 2008).

**Editor's note:** The Scientific Editor responsible for this paper was Fintan Moriarty.