FEMS Pathogens and Disease, 73, 2015, ftv065

doi: 10.1093/femspd/ftv065
Advance Access Publication Date: 2 September 2015
Research Article

RESEARCH ARTICLE

Pathogenic potential of *Escherichia coli* clinical strains from orthopedic implant infections towards human osteoblastic cells

Lise Crémet[1,2], Alexis Broquet[1], Bénédicte Brulin[3], Cédric Jacqueline[1], Sandie Dauvergne[1], Régis Brion[3], Karim Asehnoune[1,2,3,4], Stéphane Corvec[1,2], Dominique Heymann[3] and Nathalie Caroff[1,*]

[1]UPRES EA3826, Laboratory of Clinical and Experimental Therapeutics of Infections., Medicine Faculty, University of Nantes, 1, rue G. Veil, F-44000 Nantes, France, [2]Department of Bacteriology-Hygiene, Nantes University Hospital, F-44000 Nantes, France, [3]INSERM, UMR 957, Pathophysiology of Bone Resorption Laboratory and Therapy of Primary Bone Tumors, Medicine Faculty, University of Nantes, 1, rue G. Veil, F-44000 Nantes, France and [4]Intensive Care Unit, Anesthesia and Critical Care Department, Nantes University Hospital, F-44000 Nantes, France

*Corresponding author: UPRES EA3826, Laboratory of Clinical and Experimental Therapeutics of Infections., Medicine Faculty, University of Nantes, 1, rue G. Veil, F-44000 Nantes, France. Tel: +33-2-53-48-41-66; Fax: +33-2-53-48-43-59; E-mail: nathalie.caroff@univ-nantes.fr
One sentence summary: This study describes the interaction of *Escherichia coli* clinical strains with an osteoblastic cell line, showing no internalization unlike *Staphylococcus aureus*, but a strong cytotoxicity of the Hly-A producing strains.
Editor: Patrik M. Bavoil

ABSTRACT

Escherichia coli is one of the first causes of Gram-negative orthopedic implant infections (OII), but little is known about the pathogenicity of this species in such infections that are increasing due to the ageing of the population. We report how this pathogen interacts with human osteoblastic MG-63 cells *in vitro*, by comparing 20 OII *E. coli* strains to two *Staphylococcus aureus* and two *Pseudomonas aeruginosa* strains. LDH release assay revealed that 6/20 (30%) OII *E. coli* induced MG-63 cell lysis whereas none of the four control strains was cytotoxic after 4 h of coculture. This high cytotoxicity was associated with hemolytic properties and linked to *hlyA* gene expression. We further showed by gentamicin protection assay and confocal microscopy that the non-cytotoxic *E. coli* were not able to invade MG-63 cells unlike *S. aureus* strains (internalization rate <0.01% for the non-cytotoxic *E. coli* versus 8.88 ± 2.31% and 4.60 ± 0.42% for both *S. aureus*). The non-cytotoxic *E. coli* also demonstrated low adherence rates (<7%), the most adherent *E. coli* eliciting higher IL-6 and TNF-α mRNA expression in the osteoblastic cells. Either highly cytotoxic or slightly invasive OII *E. coli* do not show the same infection strategies as *S. aureus* towards osteoblasts.

Keywords: *E. coli*; human osteoblasts; cytotoxicity; internalization; alpha-hemolysin; TNF-α

Received: 29 May 2015; **Accepted:** 7 August 2015
© FEMS 2015. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

INTRODUCTION

Despite serious technological advances in orthopedic surgery and improvement in clinical preventive practice, orthopedic implant infections (OII) continue to be a problem, with an incidence increasing with the boom of prosthetic joint implantations (Kurtz *et al.* 2012; Lima *et al.* 2013). Approximately 6–23% of all OII are caused by Gram-negative bacilli, and *Escherichia coli* is the most frequently isolated microorganism in these cases (Zmistowski *et al.* 2011; Peel *et al.* 2012; Rodriguez-Pardo *et al.* 2014).

Progress has been made over the past decade in the understanding of the physiopathogenesis of staphylococcal OII. Based on research in this field, bacterial adherence and biofilm formation on the implant surface represent major factors involved in the pathogenesis process, providing protection against antimicrobial therapy and host immune responses (Montanaro *et al.* 2011; Ribeiro, Monteiro and Ferraz 2012; Molina-Manso *et al.* 2013). Regarding bone infection, *Staphylococcus aureus* has been shown to invade osteoblasts, persist intracellularly and induce the secretion of key proinflammatory mediators and potent stimulators of osteoclastogenesis and bone resorption (Alexander *et al.* 2003; Marriott 2004; Marriot *et al.* 2005; Somayaji *et al.* 2008; Wright and Nair 2010; Ning *et al.* 2011; Hamza *et al.* 2013). Several virulence determinants, such as specific microbial surface components recognizing adhesive matrix molecules with in particular the fibronectin-binding proteins (FnBPs), the surface-bound protein A (SpA) or a class of pore-forming toxins named the phenol-soluble modulins (PSMs), have been demonstrated to play a role in *S. aureus* invasiveness and bone destruction (Wright and Nair 2010; Cassat *et al.* 2013; Claro *et al.* 2013; Rasigade *et al.* 2013).

Escherichia coli is known as a harmless commensal of the intestinal flora, but through virulence and fitness genes gain, evolves as a highly diverse and adapted pathogen (Köhler and Dobrindt 2011; Croxen *et al.* 2013). Very few data currently exist on the pathophysiological mechanisms involved in *E. coli* OII. As previously demonstrated by our group (Crémet *et al.* 2012), most of *E. coli* strains recovered from peri-implant tissues show a high virulence potential, but no molecular pathogenic signature distinguishes these strains from other extraintestinal pathogenic *E. coli* (ExPEC) like uropathogenic strains (UPEC). Furthermore, we showed that only a small number of OII *E. coli* forms strong biofilms on inert surfaces in experimental conditions (Crémet *et al.* 2012).

Little is known about the interaction of *E. coli* with bone. Major insights into osteoblasts and osteoclasts responses to *E. coli* infection come from studies of bone cells stimulated with the bacterial cell-wall component LPS (lipopolysaccharide) (Suda *et al.* 2004; Inada *et al.* 2006; Ochi *et al.* 2010; Yan *et al.* 2010; Gao *et al.* 2013; Guo *et al.* 2014; Nakao *et al.* 2014). Thus, in the present study, we used a well-characterized panel of 20 clinical OII *E. coli* strains to investigate whether this species can infect and survive within a human osteoblastic cell line and whether this infection elicits the secretion of proinflammatory mediators and promotes bone destruction (Crémet *et al.* 2012).

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 20 clinical strains of *E. coli* (Ec1 to Ec20) involved in hip (14 strains) or knee (6 strains) OII were selected for this study. All OII *E. coli* were obtained from cultures of intraoperative tissue specimens of 20 patients, who displayed typical clinical signs of OII with acute presentation, and fulfilled diagnostic criteria for OII (Crémet *et al.* 2012). In five cases (Ec1, Ec3, Ec5, Ec14, Ec17), blood cultures were positive with the same *E. coli*, and in four patients, *E. coli* isolates were recovered from polymicrobial infections (Ec4, Ec8, Ec10, Ec12). Only one isolate per patient was included. The genetic relatedness of the 20 OII *E. coli* was studied by MLST analysis according to the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli). The strains were also investigated for the most common O-serotypes of UPEC and 20 established or putative virulence factors, by PCR (Table 1) (Li *et al.* 2010; Crémet *et al.* 2012). For comparison, we used the well-characterized osteomyelitis strain of *S. aureus* ATCC 49230 (Sa49230), the *Pseudomonas aeruginosa* laboratory reference strain PaO1 and two clinical strains from our collection: one *S. aureus* (Saclin) involved in infection of a total hip prosthesis and one *P. aeruginosa* (Paclin) recovered from an infected locking compression plate used to treat a tibia–fibula fracture. Two isogenic variants of the *E. coli* strain A0 34/86 [ZKLR$^+$ (HlyA$^+$) and Zhly$^-$ (Δ*hlyA* mutant)] were also introduced as controls in some experiments (Table 1) (Sheshko *et al.* 2006).

Before infection of the osteoblast cultures, the strains were grown overnight at 37°C in 10 mL of Luria-Bertani (LB) broth, harvested by centrifugation for 10 min at 800 *g* and washed once in 5 mL of phosphate-buffered saline (PBS). The pellets were resuspended in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Belgium). Bacterial suspensions were adjusted with a nephelometer to obtain 1×10^8 CFU mL^{-1} and then diluted in DMEM.

Human osteoblast cultures

The human osteoblast-like osteosarcoma cell line MG-63 purchased from ATCC was cultured in a 5% CO_2 atmosphere at 37°C in DMEM supplemented with 5% of fetal bovine serum (Hyclone Perbio, France). One day before infection, the cells were seeded at 10^5 cells/wells in 24-well culture plates to obtain confluent monolayers.

Lactate dehydrogenase (LDH) release assays

Cytotoxicity was determined by quantifying LDH release into MG-63 cell culture supernatants, after 2 or 4 h of infection with the different strains at a multiplicity of infection (MOI) of 10:1. The enzymatic activity of LDH was measured with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), as described by the manufacturer. Cells mortality was calculated relative to that of uninfected cells (set at 0%), and cells lysed with 1% Triton X-100 (positive control, 100%).

Hemolysis assays

The hemolytic activity of the strains was quantified in defibrinated horse blood (bioMérieux, Marcy l'Etoile, France) diluted to 10% (v/v) in PBS with Ca^{2+} and Mg^{2+}. For these experiments, 200 μL of each bacterial suspension at a concentration of 10^8 CFU mL^{-1} in PBS with Ca^{2+} and Mg^{2+} was added to 200 μL of the erythrocytes suspension. Following 2, 6, or 24 h of incubation at 37°C, the unlysed erythrocytes were pelleted by centrifugation at 1500 × *g* for 2 min, and the optical density (OD) of 150 μL of the cell-free supernatants was measured at 450 nm. In each assay, a negative control (0% lysis) and a positive control (100% lysis) were obtained by addition of 200 μL of PBS with Ca^{2+} and Mg^{2+} or 1% Triton X-100 in place of the bacterial suspension,

Table 1. Virulence profiles of the 20 OII *E. coli* and 2 *E. coli* control strains studied.

Isolate[a]	Site of infection	Phylogenetic group	MLST	Serotype	Virulence factors[b]: *fimH*, *papC*, *papGII*, *papGIII*, *sfa/foc*, *hra*, *tsh*, *iucC*, *hlyA*, *traT*, *sat*, *cnf1*, *usp*, *cdt*, *vat*, *ibeA*, *ompT*, *neuB*, *csgA* and *pgaA*	Reference
Ec1	Knee	B2	ST95	O2	*fimH*, *papC*, *papGII*, *iucC*, *traT*, *usp*, *vat*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec2	Knee	B2	ST80	O75	*fimH*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *vat*, *ibeA*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec3	Hip	B2	ST537	O75	*fimH*, *papC*, *papGIII*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *cdt*, *vat*, *ibeA*, *ompT*, *csgA* and *pgaA*	This study
Ec4	Knee	B2	ST126	-	*fimH*, *papC*, *papGIII*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *cdt*, *vat*, *ibeA*, *ompT*, *csgA* and *pgaA*	This study
Ec5	Hip	B2	ST141	O2	*fimH*, *papC*, *papGIII*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *vat*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec6	Hip	B2	ST404	O75	*fimH*, *papGII*, *iucC*, *sat*, *usp*, *vat*, *ompT*, *csgA* and *pgaA*	This study
Ec7	Hip	B2	ST73	-	*fimH*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *vat*, *ompT*, *csgA* and *pgaA*	This study
Ec8	Hip	B2	ST73	O6	*fimH*, *papC*, *papGII*, *sfa/foc*, *iucC*, **hlyA**, *sat*, *usp*, *vat*, *ompT*, *csgA* and *pgaA*	This study
Ec9	Hip	B2	ST550	O75	*fimH*, *sfa/foc*, *iucC*, *sat*, *usp*, *vat*, *ompT*, *csgA* and *pgaA*	This study
Ec10	Hip	B2	ST95	O1	*fimH*, *papC*, *papGII*, *iucC*, *traT*, *usp*, *vat*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec11	Knee	B2	ST141	O2	*fimH*, *papC*, *papGIII*, *sfa/foc*, *hra*, **hlyA**, ***cnf1***, *usp*, *vat*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec12	Hip	B2	ST1618	O4	*fimH*, *sfa/foc*, *usp*, *cdt*, *vat*, *ompT*, *csgA* and *pgaA*	This study
Ec13	Hip	B2	ST95	O1	*fimH*, *papC*, *papGII*, *iucC*, *traT*, *usp*, *vat*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec14	Hip	D	ST354	O1	*fimH*, *iucC*, *traT*, *usp*, *ibeA* and *csgA*	This study
Ec15	Knee	D	ST405	O2	*fimH*, *papC*, *hra*, *iucC*, **hlyA**, *traT*, *sat*, *ompT*, *csgA* and *pgaA*	This study
Ec16	Hip	D	ST420	–	*fimH*, *usp*, *vat*, *ibeA*, *ompT*, *neuB*, *csgA* and *pgaA*	This study
Ec17	Hip	A	ST361	–	*fimH*, *traT*, *csgA* and *pgaA*	This study
Ec18	Knee	A	ST88	O8	*fimH*, *papC*, *hra*, *iucC*, *csgA* and *pgaA*	This study
Ec19	Hip	A	ST10	–	*fimH*, *papC*, *hra*, *iucC*, *traT*, *ompT*, *csgA* and *pgaA*	This study
Ec20	Hip	B1	ST1167	O21	*fimH*, *iucC*, *ompT*, *csgA* and *pgaA*	This study
ZKLR[±]	–	B2	–	O83	*fimH*, *papC*, *papGII*, *papGIII*, *sfa/foc*, *hra*, *tsh*, **hlyA**, ***cnf1***, *usp*, *vat*, *ibeA*, *ompT*, *csgA* and *pgaA*	(Sheshko *et al.* 2006)
Zhly[-]	–	B2	–	O83	*fimH*, *papC*, *papGII*, *papGIII*, *sfa/foc*, *hra*, *tsh*, ***cnf1***, *usp*, *vat*, *ibeA*, *ompT*, *csgA* and *pgaA*	(Sheshko *et al.* 2006)

[a] *E. coli* strains that induced osteoblasts lysis *in vitro* are underlined.
[b] Virulence factors significantly associated with the osteoblasts lysis potential are shown in bold type.

Table 2. Primer sequences used in RT-PCR analysis.

Primer	Nucleotide sequence (5′-3′)
gapA-F	ACGAAGTTGGTGTTGACGTTG
gapA-R	ATAACCACTTTCTTCGCACCA
hlyA-F	AGCAGGACAAAGCACGAAA
hlyA-R	GTAATCGCCGTGCCATTCTT
cnf1-F	ATGAAGCCCCGGTTGAAGTA
cnf1-R	TCCCCGTCCTTTAAGCACAT
β-actin-F	CCCAGCCATGTACGTTGCTA
β-actin-R	AGGGCATACCCCTCGTAGATG
h-IL-6-F	TCCACAAGCGCCTTCGGTCCAG
h-IL-6-R	CTCAGGGCTGAGATGCCGTCG
h-TNF-α-F	CAGCCTCTTCTCCTTCCTGAT
h-TNF-α-R	GCCAGAGGGCTGATTAGAGA

respectively. The percentage of hemolysis was calculated as follows: (OD_{450nm} supernatants – OD_{450nm} negative control)/(OD_{450nm} positive control – OD_{450nm} negative control) × 100.

Measurement of *hlyA* and *cnf1* mRNA expression

The expression of the *hlyA* and *cnf1* genes was analyzed in a relative quantification assay by real-time PCR, using the *E. coli gapA* housekeeping gene as a reference. The strains were grown overnight in LB broth, then adjusted to a density of 0.5 MacFarland units and diluted one-tenth in 10 mL of LB broth. The diluted suspensions were cultured for an additional 3 h in a 37°C water bath, and the total RNA from 1 mL of each suspension was then extracted using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instructions. The RNA yield was assessed by UV absorbance, and 100 ng of total RNA was reverse transcribed and amplified using a One Step SYBR® PrimeScript RT-PCR Kit II (TaKaRa) with primers listed in Table 2.

Analysis of the chromosomal region upstream of the *hlyC* gene

Primer pairs previously developed from sequences of PAI I (81f/r) and PAI II (72f/r) from the UPEC strain 536 were used for amplification and sequencing of the region upstream of the *hlyC* gene (Burgos and Beutin 2010). Primers ops-hly-f 5′ ACATGAGCAAAACGGATGGG 3′and ops-hly-r 5′ ATCCCAGCACGCAAAAGAAG 3′ were used for amplification and sequencing of the *ops* element (operon polarity suppressor) upstream of the *hlyCABD* genes.

Internalization and adherence assays

For bacterial internalization experiments, the MG-63 monolayers were infected during 2 h (MOI of 10:1). The number of bacteria present in the inocula was verified by plating dilutions on Tryptic Soy Agar (TSA) plates. After 2 h of infection, the MG-63 cells were washed twice with PBS and incubated for further 4 h in 1 mL of DMEM containing either 100 μg mL^{-1} gentamicin for cells infected with the *S. aureus* or *E. coli* strains or 1 mg mL^{-1} gentamicin for cells infected with the *P. aeruginosa* strains, to kill the remaining extracellular bacteria (all strains were susceptible to gentamicin with the VITEK2® system). The MG-63 monolayers were then washed three times and lysed with 0.1% Triton X-100. The lysates were serially diluted, and plated on TSA plates to determine the number of intracellular bacteria.

To measure the number of adherent bacteria, the MG-63 monolayers were infected as for internalization experiments, without the use of gentamicin to kill extracellular bacteria. After washing three times with PBS, MG-63 cells were lysed as described above, and lysates were serially diluted and plated on TSA plates.

Adherence and invasion efficiencies were calculated as the ratio of the number of cell-associated bacteria or cell-internalized bacteria, respectively, and the number of bacteria used to infect MG-63 monolayers.

Bacterial internalization assessment by confocal microscopy

MG-63 cells were seeded onto glass cover slips and infected with three clinical strains from OII (Saclin, Paclin and Ec2), as described above for the internalization assays. Following incubation with gentamicin, the MG-63 cells were washed three times, fixed with 4% paraformaldehyde and then permeabilized and blocked with 0.05% Triton X-100 + 1% bovine serum albumin. FITC-labeled rabbit anti-*E. coli*, *S. aureus* or *P. aeruginosa* antibodies (Thermo Fisher Scientific) were used to visualize the bacteria, along with DRAQ5 far-red fluorescent DNA dye (Eurobio) and Alexa Fluor 546® phalloidin (Life Technologies) for labeling F-actin. Slides were acquired on a Nikon A1R-Si confocal microscope, and pictures were analyzed with the Fiji software.

Measurement of IL-6 and TNF-α mRNA expression by relative quantitative RT-PCR

Following 4 or 6 h of infection (MOI of 100:1), the MG-63 monolayers were washed twice with PBS and RNA was isolated using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instructions. The RNA yield was assessed by UV absorbance, and 2 μg of total RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Life technologies) with random primers (Promega), as recommended by the manufacturers. Real-time amplification of the cDNA was performed using the SYBR® *Premix Ex Taq* (TaKaRa) and primers were listed in Table 2. The expression of the lL-6 and TNF-α mRNAs was normalized against β-actin mRNA and quantified using the comparative $2^{-\Delta CT}$ method.

IL-6 and TNF-α secretion assays

The release of IL-6 and TNF-α in culture supernatants of uninfected and infected MG63 cells (5 × 10^5 cells/wells in 6-well culture plates, MOI of 1:100) was quantified by ELISA, according to the manufacturer's instructions (eBioscience, Paris, France), after 24 h of incubation or 72 h, including a treatment with 100 μg mL^{-1} gentamicin at 24 h post-infection.

Statistical analysis

All assays were performed on three separate occasions. Results were expressed as means ± SD. Fisher's exact test and Kruskal–Wallis test with Dunn's multiple comparison post test were used to calculate *P* values. *P* values less than 0.05 were considered statistically significant.

RESULTS

Some OII *E. coli* strains induce osteoblasts lysis *in vitro*

Viability of the MG-63 cells was assessed following infection with the 20 *E. coli* and 6 control strains. Osteoblasts were not lysed following 2 or 4 h of infection with the *S. aureus* or *P. aeruginosa* control strains. By contrast, 6/20 OII *E. coli* induced

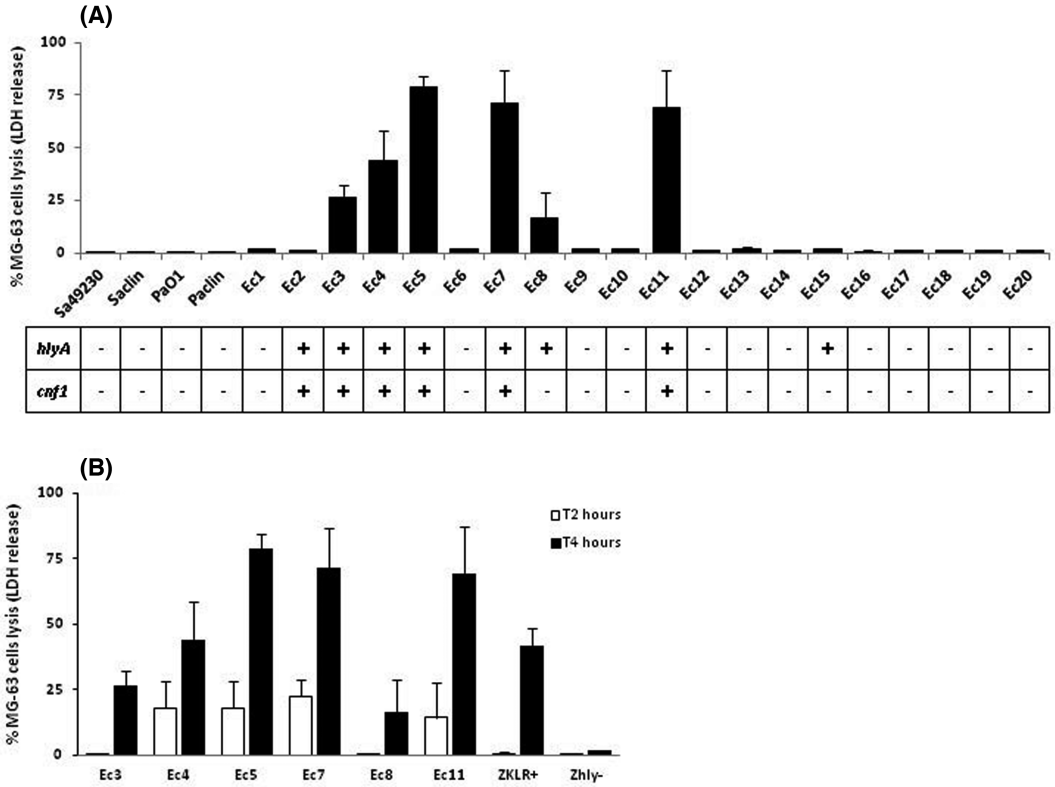

Figure 1. LDH release assays. *In vitro* lytic effects on MG-63 cells of the 20 OII *E. coli* and six control strains. (**A**) Percentage of MG-63 cells lysis after 4 h of infection (MOI of 10:1), and correlation with the detection of two virulence genes (*hlyA* and *cnf1*), by PCR. (**B**) Levels of mortality obtained after 2 and 4 h of infection (MOI of 10:1) for the six cytotoxic *E. coli* strains of our panel, and both *E. coli* control strains: ZKLR$^+$, positive for the *hlyA* gene, and Zhly$^-$, the Δ*hlyA* mutant of ZKLR$^+$.

cytotoxicity following 4 h (Fig. 1A), and cell lysis could already be observed at 2 h for four strains (Fig. 1B). No cytotoxic effect was detected when the strains were heat inactivated at 100°C for 10 min before infection.

Cytotoxicity, hemolytic activity and alpha-hemolysin (HlyA) expression are linked

Investigation of the virulence profiles of the 20 *E. coli* strains showed that the cytolytic potential was significantly associated with two virulence genes (*hlyA* and *cnf1*, $P = 0.0007$ and 0.0022, respectively; Fisher's exact test) (Table 1, Fig. 1A). The six strains that induced cytotoxicity after 4 h of infection were positive for *hlyA*, and experiments with both *E. coli* ZKLR$^+$ (*hlyA*+) and Zhly$^-$ (ZKLR$^+$ Δ*hlyA* mutant) confirmed the link between the *hlyA* gene and MG-63 cells lysis (Fig. 1B). Since two *hlyA*-positive strains (Ec2 and Ec15) did not induce lysis (Fig. 1A), we analyzed the hemolytic activity of the eight *hlyA*-positive strains of our panel and studied the *hlyA* gene expression in these strains (Fig. 2), to characterize the influence of alpha-hemolysin production on MG-63 cells lysis. In accordance with the results shown above, two *hlyA*-positive strains (Ec2 and Ec15) were non-hemolytic. The six cytotoxic *E. coli* strains and the ZKLR$^+$ control, but not the Zhly$^-$ control, caused hemolysis with some differences, the maximum effect being obtained after 6 h of incubation with erythrocytes (Fig. 2A). The observed differences were correlated with the levels of *hlyA* gene expression (Fig. 2B), and with the lytic effects on MG-63 cells (some variations observed for Ec3 being probably due to differences in response between the two cell types). Genetic analysis of the regions located upstream of the *hlyCABD* genes revealed that the eight *hly* operons were encoded on pathogenicity islands (PAI) II, and that the sequences of the respective *ops* elements, involved in transcriptional activation of the *hlyCABD* genes, were identical. The *cnf1* gene was less strongly expressed than the *hlyA* gene, and while both genes were weakly expressed in the non-cytotoxic Ec2 strain, results obtained with the Zhly$^-$ *E. coli* confirmed that this virulence factor was not responsible for the MG-63 cells lysis (Figs 1B and 2C).

OII *E. coli* strains show fairly inefficient internalization in osteoblasts

Evaluation of bacterial internalization was performed with the *S. aureus* and *P. aeruginosa* control strains and the 16 *E. coli* strains that did not induce cytotoxicity following 2 h of infection. All *E. coli* strains were less invasive than the control strains towards osteoblasts (mean percentage of internalized bacteria <0.01% for the 16 *E. coli* strains versus 8.88 ± 2.31% and 4.60 ± 0.42% for Saclin and Sa49230, respectively, or 0.57 ± 0.17% and 0.04 ± 0.03% for Paclin and PaO1, respectively). Determination of bacterial subcellular localization by confocal microscopy confirmed the intracellular presence of *S. aureus* or *P. aeruginosa* and nearly no internalization for *E. coli* (Fig. 3).

OII *E. coli* strains show differences in adherence to osteoblasts

The adherence assays revealed differences among the 16 non-cytotoxic strains of *E. coli* (Fig. 4). Only two strains (Ec6 and Ec12) showed an adherence rate higher than 4%, and eight showed

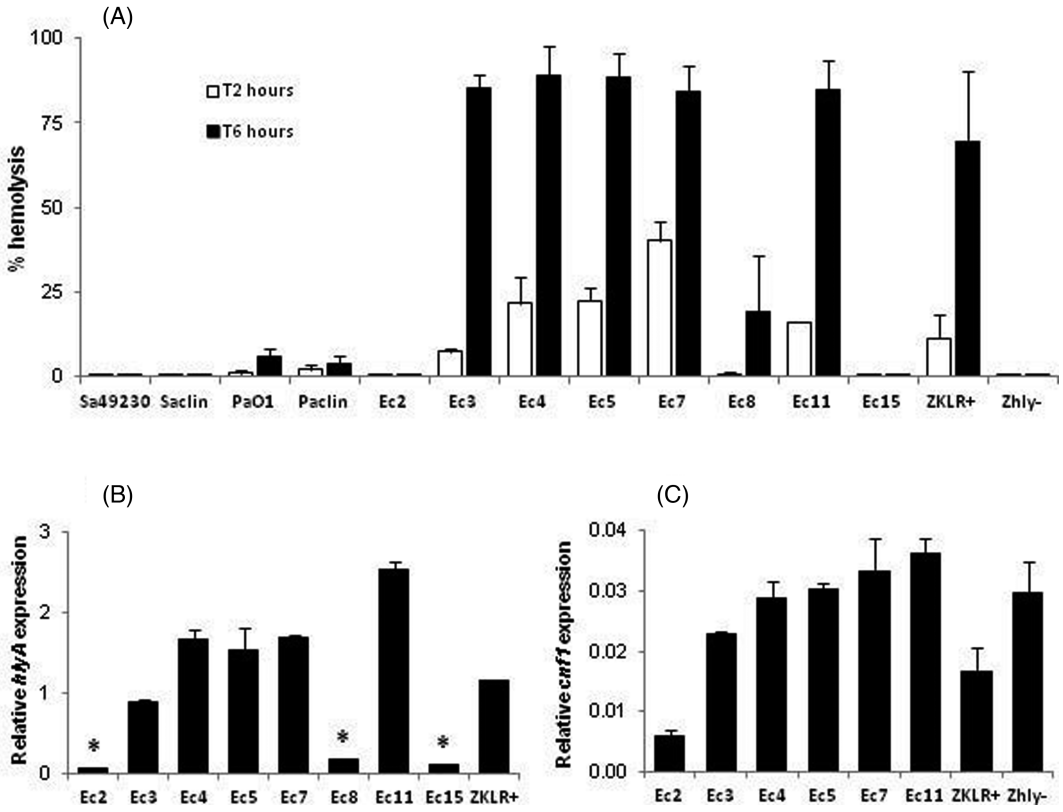

Figure 2. Analysis of the hemolytic potential of *hlyA*-positive strains of *E. coli*. (**A**) Hemolytic activity of the eight *hlyA*-positive *E. coli* of our panel, and the six control strains of our study, after 2 and 6 h of incubation with horse erythrocytes. The 12 *hlyA*-negative *E. coli* of our panel did not induce hemolysis and are not shown. (**B**) Relative expression of the *hlyA* gene in the eight *hlyA*-positive strains, and the ZKLR$^+$ *E. coli* control strain. * $P < 0.01$ versus Ec3, Ec4, Ec5, Ec7, Ec11 or ZKLR$^+$ (Kruskal–Wallis test followed by a Dunn's post test). (**C**) Relative expression of the *cnf1* gene in the six *cnf1*-positive strains, and both ZKLR$^+$ and Zhly$^-$ *E. coli* control strain.

an adherence rate lower than 1%. No significant association was found between specific virulence genes and the ability of the strains to adhere to MG-63 cells *in vitro*.

Adherence of OII *E. coli* strains to osteoblasts elicits weak induction of IL-6 and TNF-α responses

We examined the effects of the *S. aureus* and *P. aeruginosa* control strains and six *E. coli* strains on IL-6 and TNF-α mRNAs expression in MG-63 cells. Both most adherent *E. coli* (Ec6 and Ec12), and four other *E. coli* strains [including three (Ec2, Ec9 and Ec10) that showed adherence rates < 1%] were studied. As shown in Fig. 5, the strain Sa49230 induced much higher level of IL-6 mRNA than any other strain, 6 h post-infection, and was the only strain to induce IL-6 secretion 24 and 72 h post-infection (54.08 ± 26.73 and 131.71 ± 43.25 pg mL^{-1}, respectively). Both most adherent Ec6 and Ec12 strains induced significantly higher levels of IL-6 mRNA than the less adherent Ec2 and Ec9 strains. Similarly, induction of TNF-α mRNA expression was correlated with the adherence rates of the *E. coli* strains. Thus, the levels of TNF-α mRNA expressed after 6 h by the Ec6 or Ec12-infected MG-63 cells were comparable to those of the Saclin, PaO1, or Paclin-infected cells, but were significantly higher than those of the Ec2, Ec9 or Ec10-infected cells. TNF-α was not detected in supernatants of the infected cells 24 or 72 h post-infection, even for the strain Sa49230, which was the most efficient to induce TNF-α mRNA expression.

DISCUSSION

Several subgroups of ExPEC through production of large arrays of virulence factors have been shown to adhere to and cross different epithelial barriers (e.g. intestine, bladder), triggering severe tissue damages and proinflammatory responses (Croxen *et al.* 2013; Ulett *et al.* 2013). In this investigation, we wanted to determine if such virulence mechanisms are developed by OII *E. coli* towards human osteoblasts. For this purpose, we used a panel of 20 clinical strains of *E. coli* from OII. These strains belonged to different sequence types and serotypes, most of them presenting the characteristics of UPEC (serotypes and PAI-encoded virulence genes of UPEC).

The osteoblasts, derived from mesenchymal bone marrow precursors, control the bone remodeling process by synthesizing the components of bone matrix, and by modulating the activity of the bone-resorbing osteoclasts. These cells have also been shown to produce different soluble inflammatory mediators [IL-6, IL-1, TNF-α, receptor activator of NF-κB ligand (RANKL)], following *S. aureus* infection (Marriott 2004; Somayaji *et al.* 2008; Wright and Nair 2010; Ning *et al.* 2011; Claro *et al.* 2013). Thus, the bone destruction induced by *S. aureus* exposure results mainly from the inflammatory reaction elicited by the infection, even if some *S. aureus* lineages that overexpress the newly studied family of PSM peptides share cytolytic properties following invasion of osteoblasts (Rasigade *et al.* 2013). The present study provides pieces of evidence that 30% of the *E. coli* strains of our panel are also strongly cytotoxic towards osteoblasts. Interestingly, we observed that most of these cytotoxic strains at an MOI as low as

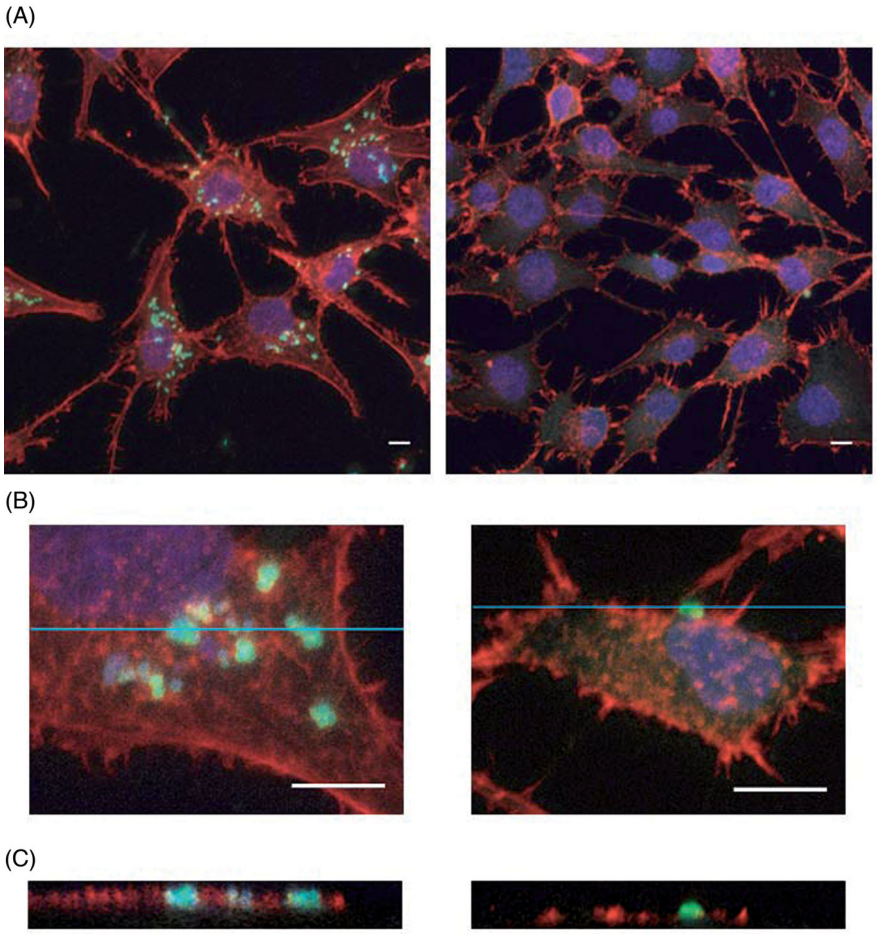

Figure 3. Bacterial internalization assessment by confocal microscopy. Immunofluorescence stainings of F-actin (red, phalloidin), nuclei (blue, DRAQ5) and bacteria (green, polyclonal anti *S. aureus* or *E. coli*), performed after 2 h of coculture of MG-63 cells with the Saclin strain or with the OII *E. coli* strain Ec2 (MOI of 10:1) and 4 h of incubation with 100 μg mL^{-1} gentamicin. (**A**) The image on the left shows several *S. aureus* cells associated with the MG-63 cells, whereas very few *E. coli* cells were detected (image on the right). (**B**) Cropped images of the stacks analyzed in A confirm the presence of aggregates of *S. aureus* cells (image on the left) and a single *E. coli* cell (image on the right) in close contact with the MG-63 cells. (**C**) Orthogonal views derived from the blue lines of the crops analyzed in B show the intracellular location of the *S. aureus* cells (image on the left, green signals framed with red), whereas the *E. coli* cell was extracellular (image on the right, no red frame around the green signal). Scale bars: 10 μm.

10:1 reduced osteoblasts cells viability by approximately 20% after 2 h of infection, whereas both *S. aureus* strains of our study were non-cytotoxic. Other authors reported cytolytic effects for *S. aureus* infection of the same MG-63 cell line, at higher MOI (100:1) and after 24 h of infection (Rasigade *et al.* 2013).

In our study, two virulence factors, namely an alpha-hemolysin (HlyA) and a toxin named cytotoxic necrotizing factor (CNF1), were significantly associated with the cytolytic activity on MG63 cells. This cytolytic activity was also confirmed on human mesenchymal stem cells (derived from bone marrow) that are precursors of osteoblasts (data not shown). HlyA is an extracellular pore-forming cytolysin from the RTX (repeats-in-toxin) family, while the protein CNF1 belongs to a family of factors activating intracellular Rho GTPases (Landraud *et al.* 2003; Burgos and Beutin 2010; Garcia *et al.* 2013; Ulett *et al.* 2013). Both virulence factors are known to induce apoptosis or necrosis/lysis, depending on concentration, in several cell types, including macrophages, epithelial cells and neutrophils (Burgos and Beutin 2010; Garcia *et al.* 2013; Ulett *et al.* 2013). In UPEC -producing CNF1, the *cnf1* gene has been shown to be coupled to the hemolysin operon (*hlyCABD*) in the PAI II (Landraud *et al.* 2003). Transcription of both *hlyCABD* operon and *cnf1* gene is enhanced by the regulator RfaH which binds on the *ops* DNA site located downstream from the *hlyCABD* promoter, and suppresses transcription polarity (Leeds and Welch 1997; Landraud *et al.* 2003). In the present study, all cytotoxic *E. coli* strains exhibited a hemolytic activity linked to the expression of the *hlyA* gene. We observed that non-hemolytic strains were not cytotoxic, regardless of whether or not they carried a *cnf1* gene. We found, in fact, that the *cnf1* gene was less expressed than the *hlyA* gene, and that its expression was well correlated with that of *hlyA* gene. Accordingly, all *hlyCABD* operons were found encoded on PAIs II, with an *ops* element upstream of the *hlyC* gene. Several polymorphisms were found in the upstream sequences of the *hlyC* genes, but no nucleotide deletions and/or substitutions was detected in the *ops* elements of the strains that weakly expressed the *hlyA* gene. Thus, while no explanation was found for the low *hlyA* expression in some *E. coli*, our results highlight the cytolytic effect of a functioning hemolysin on osteoblasts, and are in accordance with those of Island *et al.* (1998), who found that CNF1 does not affect the cytotoxicity of hemolytic isolates towards human bladder cells *in vitro*. In fact, the Zhly$^-$ control (ZKLR$^+$ Δ*hlyA* mutant) that expressed *cnf1* did not induce a cytolytic effect in our experimental conditions.

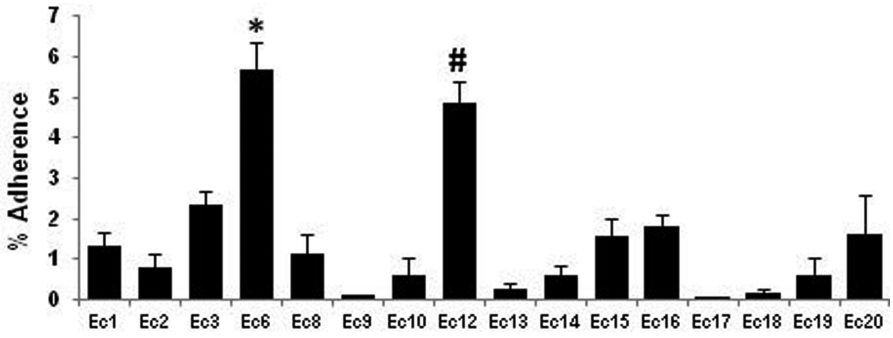

Figure 4. Adherence efficiencies of the OII *E. coli* strains. Percentage of adherent bacteria after 2 h of coculture of MG-63 cells with the 16 non-cytotoxic *E. coli* strains (MOI of 10:1). The four *E. coli* strains (Ec4, Ec5, Ec7 and Ec11) that induced osteoblasts lysis after 2 h of infection were not tested. The Ec6 and Ec12 strains were tested statistically with the Kruskal–Wallis test followed by a Dunn's post test. *, $P < 0.05$ versus Ec2, Ec9, Ec10, Ec13, Ec14, Ec17, Ec18 or Ec19; #, $P < 0.05$ versus Ec9, Ec10, Ec13, Ec14, Ec17, Ec18 or Ec19.

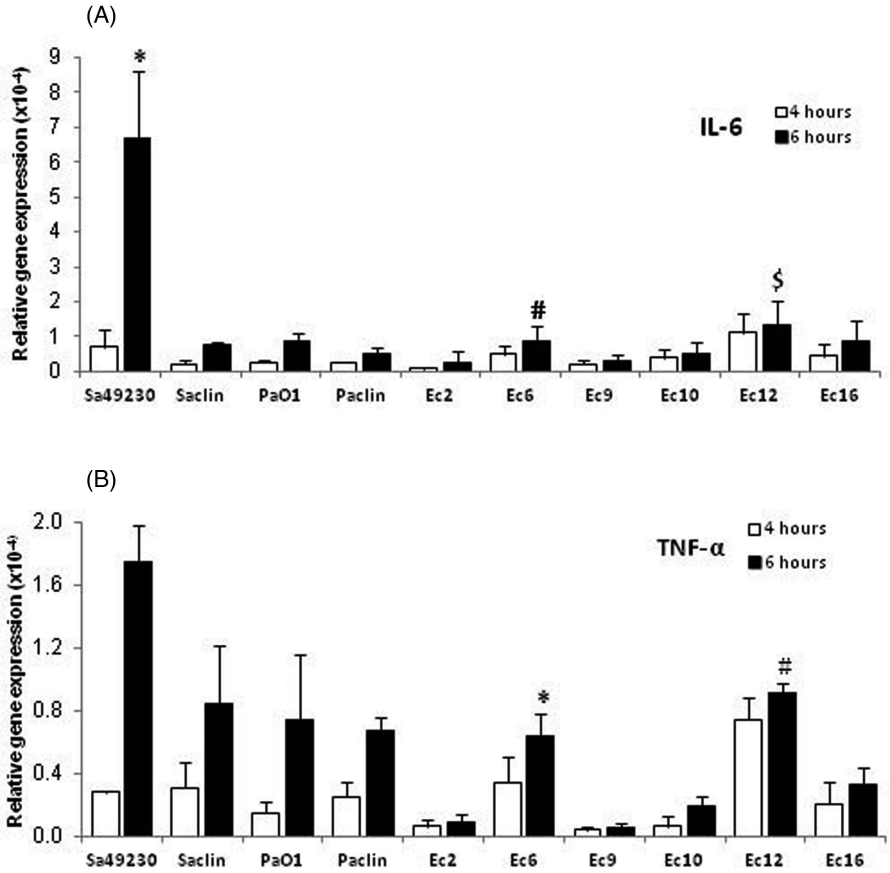

Figure 5. Relative expression of IL-6 and TNF-α mRNAs in MG-63 cells. The cells were infected with the four control strains or six selected OII *E. coli* strains, for 4 or 6 h (MOI of 100:1), before total RNA extraction. Results are expressed as relative gene expression after normalization using the β-actin housekeeping gene. (**A**) IL-6 mRNA expression. The Sa49230, Ec6 and Ec12 strains were tested statistically with the Kruskal–Wallis test followed by a Dunn's post test. *, $P < 0.05$ versus Saclin, PaO1, Paclin, Ec2, Ec6, Ec9, Ec10 or Ec16 (6 h of infection); #, $P < 0.05$ versus Sa49230, Ec2 or Ec9 (6 h of infection); $, $P < 0.05$ versus Paclin, Ec2, Ec9 or Ec10 (6 h of infection). (**B**) TNF-α mRNA expression. The Ec6 and Ec12 strains were tested statistically with the Kruskal–Wallis test followed by a Dunn's post test. *, $P < 0.05$ versus Sa49230, Ec2, Ec9 or Ec10 (6 h of infection); #, $P < 0.05$ versus Ec2, Ec9, Ec10 or Ec16 (6 h of infection).

Detailed analyses of the interaction between *S. aureus* and bone revealed that the *S. aureus* surface-associated proteins FnBPs and SpA mediate attachment of the bacterium to fibronectin and tumor necrosis factor receptor-1 (TNFR-1) on osteoblasts, and leads to bacterial uptake via an integrin $\alpha5\beta1$-mediated mechanism (Claro *et al.* 2013). Consistent with this model, both *S. aureus* strains of our study showed the highest internalization capacity (approximately 5 and 9% of internalized bacteria). The percentage of internalized *P. aeruginosa* was comparatively 10-fold to 100-fold lower, and all strains of *E. coli* presented an extremely low internalization rate (<0.01%). The poor uptake of *E. coli* could be reasonably attributed to the low ability of the strains to adhere to osteoblasts, since the adherence rates of our OII *E. coli* were not higher than 7%, half of the strains showing an adherence rate lower than 1%. Interestingly, these results are very similar to those from Fiedler *et al.* (2013)

that recently investigated the adherence and internalization in adipose-derived mesenchymal stem cells of one *E. coli* obtained from wound infection (i.e. 1.4% of adherent bacteria and 0.008% of internalized bacteria). As far as we know, there is not any animal model showing histopathological evidence of *E. coli* adherence to osteoblasts during an experimental infection. Nonetheless, *E. coli* is well known to express various adhesins, including several FnBPs. The amyloid fibers curli, involved in biofilm formation, are the most important and were previously identified in all OII *E. coli* studied (Barnhart and Chapman 2006; Henderson *et al.* 2011; Crémet *et al.* 2012). Approximately 60% (12/20) of our *E. coli* strains demonstrated a strong affinity for the curli-binding dye, congo red (data not shown), including both most adherent strains, Ec6 and Ec12.

Finally, a major component of the outer membrane of *E. coli*, namely the LPS, has been proposed to be a potent stimulator of bone destruction in several bone inflammatory diseases (Ochi *et al.* 2010; Yan *et al.* 2010). Thus, following binding to Toll-like receptor 4 (TLR4) on osteoblasts, LPS promotes synthesis of proinflammatory mediators, among which IL-6, TNF-α, RANKL and PGE2 inhibit osteoblast differentiation, promote osteoblast apoptosis and stimulate osteoclastogenesis directly or indirectly (Suda *et al.* 2004; Inada *et al.* 2006; Ochi *et al.* 2010; Yan *et al.* 2010; Gao *et al.* 2013; Guo *et al.* 2014). However, LPS is a surrogate marker of Gram-negative bacteria, which do not sufficiently reflect whole bacteria and only partly mimics osteoblasts infection, with the risk of non-physiological stimuli. There are very few experimental models of osteoblasts infection by Gram-negative organisms, the existing studies being essentially focused on the intracellular pathogens *Salmonella* and *Brucella* (Alexander *et al.* 2001; Marriott 2004; Delpino, Fossati and Baldi 2009; Scian *et al.* 2011). Furthermore, these studies are often based on small numbers of clinical strains, even in the case of *S. aureus* infections, where the osteomyelitis clinical strain ATCC 49230 has been the most studied (Alexander *et al.* 2001, 2003; Marriott *et al.* 2005; Somayaji *et al.* 2008; Ning *et al.* 2011).

In the present study, in accordance with the current knowledge on osteoblasts responses to *S. aureus*, we found that infection with the strain Sa49230 elicited the secretion of IL-6 from MG-63 cells (Ning *et al.* 2011). This secretion seemed to be strain dependent, since stimulation of osteoblasts with the other *S. aureus* elicited a lower IL-6 response and did not result in IL-6 secretion. Moreover, we show that infection with both *S. aureus* induced TNF-α mRNA expression, but did not induce TNF-α release from MG-63 cells. These results are consistent with previous data that either showed an endogenous cell-surface TNF-α expression of MG-63 cells or established that *S. aureus*- or *Brucella*-infected osteoblasts do not secrete detectable levels of TNF-α *in vitro* (Bu *et al.* 2003; Delpino, Fossati and Baldi 2009; Scian *et al.* 2011).

Differences in IL-6 or TNF-α mRNA expression were observed between the *E. coli* strains, and adherence to osteoblasts seemed to contribute to the induction of IL-6 or TNF-α mRNA expression. Our findings indicate that the most adherent OII *E. coli* strains elicit IL-6 or TNF-α responses, which are equivalent to those elicited by *S. aureus* or *P. aeruginosa* strains recovered in the same clinical context. It would be now interesting to extend those findings to other proinflammatory mediators and to study the molecular mechanisms mediating osteoblasts immune response to infection by these different bacterial species. Furthermore, even though the low adherence seemed correlated to a poor inflammatory response, further experiments could be helpful to rule out a subversion of immune response by the bacteria, as previously described in ExPEC isolates (Cirl *et al.* 2008).

In conclusion, our study is the first to investigate the effects of 20 clinical strains of *E. coli* from OII on human osteoblastic cells. Even if we did not find that OII *E. coli* shared common virulence properties towards osteoblasts, our data provide evidence that the HlyA-producing strains, known to be highly virulent, are associated with cytolytic phenotypes. The others are poorly internalized into osteoblasts, the most adherent of them eliciting IL-6 or TNF-α responses in the same range as those induced by some strains of *S. aureus* or *P. aeruginosa* from OII.

ACKNOWLEDGEMENTS

We thank Dr J. Holubova and Pr P. Sebo (Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Prague, Czech Republic) for kindly providing the ZKLR$^+$ and Zhly$^-$ *E. coli* control strains used in this study.

FUNDING

This work was supported by the French 'Ministère de l'Enseignement Supérieur et de la Recherche'.

Conflict of interest. None declared.

REFERENCES

Alexander EH, Bento JL, Hughes FM, Jr, *et al. Staphylococcus aureus* and *Salmonella enterica* serovar Dublin induce tumor necrosis factor-related apoptosis-inducing ligand expression by normal mouse and human osteoblasts. *Infect Immun* 2001;**69**:1581–6.

Alexander EH, Rivera FA, Marriott I, *et al. Staphylococcus aureus*-induced tumor necrosis factor-related apoptosis-inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts. *BMC Microbiol* 2003;**3**:5.

Barnhart MM, Chapman MR. Curli biogenesis and function. *Annu Rev Microbiol* 2006;**60**:131–47.

Bu R, Borysenko CW, Li Y, *et al.* Expression and function of TNF-family proteins and receptors in human osteoblasts. *Bone* 2003;**33**:760–70.

Burgos Y, Beutin L. Common origin of plasmid encoded alpha-hemolysin genes in *Escherichia coli*. *BMC Microbiol* 2010; **10**:193.

Cassat JE, Hammer ND, Campbell JP, *et al.* A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host Microbe* 2013;**13**:759–72.

Cirl C, Wieser A, Yadav M, *et al.* Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. *Nat Med* 2008;**14**: 399–406

Claro T, Widaa A, McDonnell C, *et al. Staphylococcus aureus* protein A binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection. *Microbiology* 2013;**159**: 147–54.

Crémet L, Corvec S, Bémer P, *et al.* Orthopædic-implant infections by *Escherichia coli*: molecular and phenotypic analysis of the causative strains. *J Infect* 2012;**64**:169–75.

Croxen MA, Law RJ, Scholz R, *et al.* Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin Microbiol Rev* 2013;**26**:822–80.

Delpino MV, Fossati CA, Baldi PC. Proinflammatory response of human osteoblastic cell lines and osteoblast-monocyte interaction upon infection with *Brucella* spp. *Infect Immun* 2009;**77**:984–95.

Fiedler T, Salamon A, Adam S, *et al.* Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells. *Exp Cell Res* 2013;**18**:2883–92.

Gao A, Kantarci A, Herrera BS, *et al.* A critical role for suppressors of cytokine signaling 3 in regulating LPS-induced transcriptional activation of matrix metalloproteinase-13 in osteoblasts. *Peer J* 2013;**1**:e51.

Garcia TA, Ventura CL, Smith MA, *et al.* Cytotoxic necrotizing factor 1 and hemolysin from uropathogenic *Escherichia coli* elicit different host responses in the murine bladder. *Infect Immun* 2013;**81**:99–109.

Guo C, Yuan L, Wang JG, *et al.* Lipopolysaccharide (LPS) induces the apoptosis and inhibits osteoblast differentiation through JNK pathway in MC3T3-E1 cells. *Inflammation* 2014;**37**:621–31.

Hamza T, Dietz M, Pham D, *et al.* Intra-cellular *Staphylococcus aureus* alone causes infection *in vivo*. *Eur Cell Mater* 2013;**25**: 341–50.

Henderson B, Nair S, Pallas J, *et al.* Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. *FEMS Microbiol Rev* 2011;**35**:147–200.

Inada M, Matsumoto C, Uematsu S, *et al.* Membrane-bound prostaglandin E synthase-1-mediated prostaglandin E2 production by osteoblast plays a critical role in lipopolysaccharide-induced bone loss associated with inflammation. *J Immunol* 2006;**177**:1879–85.

Island MD, Cui X, Foxman B, *et al.* Cytotoxicity of hemolytic, cytotoxic necrotizing factor 1-positive and -negative *Escherichia coli* to human T24 bladder cells. *Infect Immun* 1998;**66**:3384–9.

Köhler CD, Dobrindt U. What defines extraintestinal pathogenic *Escherichia coli*? *Int J Med Microbiol* 2011;**301**:642–7.

Kurtz SM, Lau E, Watson H, *et al.* Economic burden of periprosthetic joint infection in the United States. *J Arthroplasty* 2012;**27**:61–5e1.

Landraud L, Gibert M, Popoff MR, *et al.* Expression of *cnf1* by *Escherichia coli* J96 involves a large upstream DNA region including the *hlyCABD* operon, and is regulated by the RfaH protein. *Mol Microbiol* 2003;**47**:1653–67.

Leeds JA, Welch RA. Enhancing transcription through the *Escherichia coli* hemolysin operon, *hlyCABD*: RfaH and upstream JUMPStart DNA sequences function together via a postinitiation mechanism. *J Bacteriol* 1997;**179**:3519–27.

Li D, Liu B, Chen M, *et al.* A multiplex PCR method to detect 14 *Escherichia coli* serogroups associated with urinary tract infections. *J Microbiol Meth* 2010;**82**:71–7.

Lima AL, Oliveira PR, Carvalho VC, *et al.* Periprosthetic joint infections. *Interdiscip Perspect Infect Dis* 2013;**2013**:542796–802.

Marriott I. Osteoblast responses to bacterial pathogens. *Immunol Res* 2004;**30**:291–308.

Marriott I, Gray DL, Rati DM, *et al.* Osteoblasts produce monocyte chemoattractant protein-1 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. *Bone* 2005;**37**:504–12.

Molina-Manso D, del Prado G, Ortiz-Pérez A, *et al.* *In vitro* susceptibility to antibiotics of staphylococci in biofilms isolated from orthopædic infections. *Int J Antimicrob Ag* 2013;**41**:521–3.

Montanaro L, Speziale P, Campoccia D, *et al.* Scenery of Staphylococcus implant infections in orthopedics. *Future Microbiol* 2011;**6**:1329–49.

Nakao J, Fujii Y, Kusuyama J, *et al.* Low-intensity pulsed ultrasound (LIPUS) inhibits LPS-induced inflammatory responses of osteoblasts through TLR4-MyD88 dissociation. *Bone* 2014;**58**:17–25.

Ning R, Zhang X, Guo X, *et al.* *Staphylococcus aureus* regulates secretion of interleukin-6 and monocyte chemoattractant protein-1 through activation of nuclear factor kappaB signaling pathway in human osteoblasts. *Braz J Infect Dis* 2011;**15**:189–94.

Ochi H, Hara Y, Tagawa M, *et al.* The roles of TNFR1 in lipopolysaccharide-induced bone loss: dual effects of TNFR1 on bone metabolism via osteoclastogenesis and osteoblast survival. *J Orthop Res* 2010;**28**:657–63.

Peel TN, Cheng AC, Buising KL, *et al.* Microbiological ætiology, epidemiology, and clinical profile of prosthetic joint infections: are current antibiotic prophylaxis guidelines effective? *Antimicrob Agents Ch* 2012;**56**:2386–91.

Rasigade JP, Trouillet-Assant S, Ferry T, *et al.* PSMs of hypervirulent *Staphylococcus aureus* act as intracellular toxins that kill infected osteoblasts. *PLoS One* 2013;**8**:e63176.

Ribeiro M, Monteiro FJ, Ferraz MP. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. *Biomatter* 2012;**2**:176–94.

Rodriguez-Pardo D, Pigrau C, Lora-Tamayo J, *et al.* Gram-negative prosthetic joint infection: outcome of a debridement, antibiotics, and implant retention approach. A large multicenter study. *Clin Microbiol Infect* 2014;**20**:O911–9.

Scian R, Barrionuevo P, Giambartolomei GH, *et al.* Granulocyte-macrophage colony-stimulating factor- and tumor necrosis factor alpha-mediated matrix metalloproteinase production by human osteoblasts and monocytes after infection with *Brucella abortus*. *Infect Immun* 2011;**79**:192–202.

Sheshko V, Hejnova J, Rehakova Z, *et al.* HlyA knock out yields a safer *Escherichia coli* A0 34/86 variant with unaffected colonization capacity in piglets. *FEMS Immunol Med Micr* 2006;**48**:257–66.

Somayaji SN, Ritchie S, Sahraei M, *et al.* *Staphylococcus aureus* induces expression of receptor activator of NF-kappaB ligand and prostaglandin E2 in infected murine osteoblasts. *Infect Immun* 2008;**76**:5120–6.

Suda K, Udagawa N, Sato N, *et al.* Suppression of osteoprotegerin expression by prostaglandin E2 is crucially involved in lipopolysaccharide-induced osteoclast formation. *J Immunol* 2004;**172**:2504–10.

Ulett GC, Totsika M, Schaale K, *et al.* Uropathogenic *Escherichia coli* virulence and innate immune responses during urinary tract infection. *Curr Opin Microbiol* 2013;**16**:100–7.

Wright JA, Nair SP. Interaction of staphylococci with bone. *Int J Med Microbiol* 2010;**300**:193–204.

Yan C, Cao J, Wu M, *et al.* Suppressor of cytokine signaling 3 inhibits LPS-induced IL-6 expression in osteoblasts by suppressing CCAAT/enhancer-binding protein β activity. *J Biol Chem* 2010;**285**:37227–39.

Zmistowski B, Fedorka CJ, Sheehan E, *et al.* Prosthetic joint infection caused by Gram-negative organisms. *J Arthroplasty* 2011;**26**(6 Suppl):104–8.