Simultaneous lack of catalase and beta-toxin in *Staphylococcus aureus* leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models

Susana Martínez-Pulgarín,[†] Gustavo Domínguez-Bernal, José A. Orden and Ricardo de la Fuente

Correspondence

Ricardo de la Fuente
rifuente@vet.ucm.es

Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid[‡], Avda. Puerta de Hierro s/n, 28040 Madrid, Spain

Staphylococcus aureus produces a variety of virulence factors that allow it to cause a wide range of infections in humans and animals. In the latter, *S. aureus* is a leading cause of intramammary infections. The contribution of catalase (KatA), an enzyme implicated in oxidative stress resistance, and beta-toxin (Hlb), a haemolysin, to the pathogenesis of *S. aureus* is poorly characterized. To investigate the role of these enzymes as potential virulence factors in *S. aureus*, we examined the intracellular survival of $\Delta katA$, Δhlb and $\Delta katA \Delta hlb$ mutants in murine macrophages (J774A.1) and bovine mammary epithelial cells (MAC-T), and their virulence in different murine and ovine models. Catalase was not required for the survival of *S. aureus* within either J774A.1 or MAC-T cells. However, it was necessary for the intracellular proliferation of the bacterium after invasion of MAC-T cells. In addition, catalase was not needed for the full virulence of *S. aureus* in mice. Deletion of the *hlb* gene had no effect on the intracellular survival of *S. aureus* in J774A.1 cells but did cause a slight increase in survival in MAC-T cells. Furthermore, like catalase, beta-toxin was not required for complete virulence of *S. aureus* in murine models. Unexpectedly, the $\Delta katA \Delta hlb$ mutant showed a notably increased persistence in both cell lines, and was significantly less virulent for mice than were the wild-type strain and single mutants. Most interestingly, it was also markedly attenuated in intramammary and subcutaneous infections in ewes and lambs.

Received 28 October 2008
Revised 20 February 2009
Accepted 26 February 2009

INTRODUCTION

Staphylococcus aureus is a ubiquitous micro-organism that is responsible for a wide range of both acute and chronic infections in humans and animals. In animals, *S. aureus* is frequently associated with intramammary infections in lactating females, which cause substantial economic losses in dairy production (Sutra & Poutrel, 1994). The ability of *S. aureus* to cause disease has been attributed to its ability to produce a variety of surface-expressed (protein A, fibrinogen-, fibronectin- and collagen-binding proteins) and secreted (haemolysins, enterotoxins, proteases, lipase and coagulases) virulence factors that allow it to colonize

and multiply in tissues (Dinges *et al.*, 2000; Kerro Dego *et al.*, 2002). Furthermore, although *S. aureus* is not traditionally considered to be an intracellular pathogen, numerous studies have clearly demonstrated that *S. aureus* can internalize and survive in a wide variety of mammalian cells (Bayles *et al.*, 1998; Menzies & Kourteva, 1998; Wesson *et al.*, 1998), including phagocytic cells (Brouillette *et al.*, 2003; Hébert *et al.*, 2000).

Catalase (KatA) is an enzyme involved in oxidative stress resistance, and converts hydrogen peroxide generated during cellular metabolism to water and molecular oxygen. As a result, catalase has been proposed to be a potential virulence factor in many bacterial pathogens, because its activity might protect them from the reactive oxygen species (ROS) generated by eukaryotic cells. Thus, this enzyme has been demonstrated to be an essential factor for the intracellular survival of bacteria such as *Mycobacterium tuberculosis* (Manca *et al.*, 1999), *Campylobacter jejuni* (Day *et al.*, 2000) and *Helicobacter pylori* (Basu *et al.*, 2004). In *S. aureus*, the role of catalase in virulence remains unclear;

[†]Present address: Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Crta. de La Coruña km. 7.5, 28040 Madrid, Spain.

[‡]The University Complutense of Madrid (Spain) has filed a patent on the $\Delta katA \Delta hlb$ strain as a vaccine against ruminant mastitis.

Abbreviations: CAMP, Christie–Atkins–Munch–Petersen; CI, competitive index; SCCs, somatic cell counts.

while some researchers have observed a correlation between virulence and catalase activity (Kanafani & Martin, 1985; Mandell, 1975), others have not found any evidence of this (Cosgrove *et al.*, 2007; Horsburgh *et al.*, 2001).

Beta-toxin (Hlb) is one of the haemolysins produced by *S. aureus*, and works as a sphingomyelinase C, degrading sphingomyelin in the outer layer of eukaryotic cell membranes. This toxin is also known for producing 'hot-cold' haemolysis on sheep blood agar plates. It is characterized by incomplete haemolysis at 37 °C, followed by complete haemolysis during incubation at 4 °C. It has been proposed that the cytolytic activity of beta-toxin results from induction of changes in the physical properties of the cell membrane, and induction of apoptosis by generation of ceramide, a potent secondary messenger that arises from sphingomyelin hydrolysis (Bayles *et al.*, 1998; Hannun & Obeid, 1995). A high percentage (75–97 %) of *S. aureus* strains isolated from bovine mastitis produce beta-toxin, whereas only 10–15 % of human isolates express this factor (Aarestrup *et al.*, 1999; Larsen *et al.*, 2002). To date, the role of beta-toxin in *S. aureus* pathogenesis is little known. However, some studies have suggested that this toxin is important in intramammary infection. Purified toxin has been shown to cause moderate inflammatory changes in mouse and rabbit mammary glands (Calvinho *et al.*, 1993; Ward *et al.*, 1979) and to be cytotoxic for bovine mammary epithelial cells (Cifrian *et al.*, 1996). In addition, non-beta-toxin-producing mutants have been found to be less virulent than wild-type strains in a mouse model of intramammary infection (Bramley *et al.*, 1989) but not in non-mammary models (Nilsson *et al.*, 1999; O'Callaghan *et al.*, 1997).

The aim of this work was to investigate the role of catalase and beta-toxin as potential virulence factors in *S. aureus*. We constructed $\Delta katA$, Δhlb and $\Delta katA \Delta hlb$ mutants and compared their ability to survive in macrophages and epithelial cells, as well as their virulence in different murine and ovine models, with those of the wild-type strain.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were routinely grown at 37 °C in brain heart infusion (BHI) broth with shaking or on BHI agar plates. For protoplast regeneration, DM3 medium was used (Götz *et al.*, 1981). When appropriate, erythromycin (5 µg ml⁻¹) was incorporated. Columbia agar with 5 % sheep blood was utilized in order to observe haemolytic activity. *Escherichia coli* strains were cultured in Luria-Bertani medium, with ampicillin (50 µg ml⁻¹) and erythromycin (300 µg ml⁻¹) added as required. The *S. aureus* strain 2386 was isolated from a cow with acute mastitis. This strain was chosen for our studies due to its potentially high intramammary pathogenicity, which was later confirmed in experimental infections in ewes. The 2386 strain belongs to spa type t543 and sequence type ST479.

DNA manipulations and transformation. Chromosomal DNA from *S. aureus* was extracted using a GFX Genomic Blood DNA

Purification kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol, except for a first lysis step with lysostaphin (50–100 µg ml⁻¹, Sigma) at 37 °C for 30 min. Plasmid DNA isolation was performed using a Plasmid Purification kit (Qiagen), adding pre-incubation with lysostaphin for *S. aureus*, as described above. Plasmids were transformed into *S. aureus* by protoplast transformation (Götz *et al.*, 1981). Transformation of *E. coli* was performed by standard procedures (Ausubel *et al.*, 1992). DNA fragments were isolated with the Qiaquick PCR Purification kit (Qiagen) and the Qiaquick Gel Extraction kit (Qiagen). Restriction enzymes were supplied by Amersham Pharmacia Biotech.

PCRs were carried out with AmpliTaq Gold polymerase (Applied Biosystems), as recommended by the manufacturer. Oligonucleotide primers used in this study (Table 1) were designed using the *S. aureus* COL genome sequence (TIGR Microbial Database) and purchased from Isogen Bioscience.

Construction of $\Delta katA$, Δhlb and $\Delta katA \Delta hlb$ mutants. *S. aureus* strain 2386, a clinical isolate from bovine mastitis, was used for construction of the mutants. In order to make a $\Delta katA$ mutant, oligonucleotide primer pairs KAT1/KATR1 and KATR2/KAT2 (Table 1) were used to PCR-amplify two 0.5 kb DNA fragments that flanked the 5' and 3' ends of *katA*, respectively, from chromosomal DNA of *S. aureus* 2386. Primers KATR1 and KATR2 carried complementary sequences (underlined in Table 1) that were used to fuse the two fragments by recombinant PCR (Vallejo *et al.*, 1994). The resulting chimeric PCR product was inserted into the pCR2.1 vector, excised using *SpeI* and *XbaI*, and cloned into the *XbaI* site of the pE194ts thermosensitive vector to generate pERkat (Table 1). To construct a Δhlb mutant, two 1 kb DNA fragments that flanked the 5' and 3' ends of *hlb* were amplified using oligonucleotide primer pairs HLB1/HLBR1 and HLB2/HLBR2 (Table 1), fused and cloned into pE194ts as described above, which yielded pERhlb (Table 1). These recombinogenic plasmids were introduced into *S. aureus* 2386 by protoplast transformation (Götz *et al.*, 1981), and transformants were selected by incubation at 32 °C on DM3 agar plates that contained 5 µg erythromycin ml⁻¹.

For gene deletion, transformants were cultured in BHI broth with erythromycin (5 µg ml⁻¹) at 32 °C overnight. Then, cultures were diluted into fresh medium and grown at 43 °C (non-permissive for plasmid replication) for 6 h to induce plasmid integration into the chromosome by homologous recombination. Double crossover and plasmid loss were achieved by regular subculturing at 37 °C in BHI broth without erythromycin and plating appropriate dilutions on BHI agar. The $\Delta katA$ mutant was selected for bubble-negative phenotype by the hydrogen peroxide assay (Fig. 1). The Δhlb mutant was selected for lack of the outer halo of incomplete haemolysis on sheep blood agar (Fig. 1). Both mutants were tested for sensitivity to erythromycin and, by PCR, for the presence of the deletion using oligonucleotide primer pairs KAT1/KAT2 and HLB1/HLB2.

To obtain the $\Delta katA \Delta hlb$ double mutant, pERkat was introduced into the Δhlb mutant, using the same protocol as described above.

Complementation of the mutants. To complement the *katA* mutation, a 1.6 kb DNA fragment that contained the *katA* ORF was amplified using the oligonucleotide primer pairs CAT1/CAT2 (Table 1). The amplification product was cloned into pCR2.1, excised by digestion with *EcoRI*, and then subcloned into the pHpS9 shuttle vector to generate pHkat (Table 1). Likewise, to complement the *hlb* mutation, a 3 kb DNA fragment that contained the *hlb* ORF was amplified using the oligonucleotide primer pairs HLB1/HLB2 (Table 1), and directly cloned into the *SmaI* site of pHpS9 to obtain pHhlb (Table 1). Finally, to complement the double mutant, pHkathlb (Table 1) was constructed by cloning the *katA*-containing DNA fragment into pHhlb. Plasmids were introduced into the

Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Description or sequence	Reference or source
Strains		
<i>S. aureus</i>		
2386	Clinical isolate (bovine mastitis)	Laboratory stock
2386 Δ <i>katA</i>	<i>katA</i> deletion mutant derived from 2386	This study
2386 Δ <i>katA</i> (pHkat)	Δ <i>katA</i> mutant derived from 2386 complemented with pHkat	This study
2386 Δ <i>hlyB</i>	<i>hlyB</i> deletion mutant derived from 2386	This study
2386 Δ <i>hlyB</i> (pHhlyB)	Δ <i>hlyB</i> mutant derived from 2386 complemented with pHhlyB	This study
2386 Δ <i>katA</i> Δ <i>hlyB</i>	<i>katA/hlyB</i> deletion mutant derived from 2386	This study
2386 Δ <i>katA</i> Δ <i>hlyB</i> (pHkathlyB)	Δ <i>katA</i> Δ <i>hlyB</i> mutant derived from 2386 complemented with pHkathlyB	This study
<i>E. coli</i>		
DH5 α	Cloning host strain	Laboratory stock
TOP10	Cloning host strain	Invitrogen
<i>R. equi</i>		
MAD	Cholesterol oxidase-hyperproducing strain	Ripio <i>et al.</i> (1995)
Plasmids		
pCR2.1	T-vector for cloning of PCR products	Invitrogen
pE194ts	Temperature-sensitive <i>erm</i> vector for allelic exchange in <i>S. aureus</i>	Horinouchi & Weisblum (1982)
pHpS9	<i>E. coli</i> – <i>Bacillus</i> <i>erm</i> shuttle vector	Haima <i>et al.</i> (1990)
pERkat	Recombinogenic plasmid used in the construction of the Δ <i>katA</i> and Δ <i>katA</i> Δ <i>hlyB</i> mutants (pE194ts with a PCR fragment insert containing 0.5 kb flanking sequences to 5' and 3' ends of <i>katA</i>)	This study
pERhlyB	Recombinogenic plasmid used in the construction of the Δ <i>hlyB</i> mutant (pE194ts with a PCR fragment insert containing 1 kb flanking sequences to 5' and 3' ends of <i>hlyB</i>)	This study
pHkat	1.6 kb CAT1/CAT2 fragment containing the <i>katA</i> gene inserted into pHpS9	This study
pHhlyB	3 kb HLB1/HLB2 fragment containing the <i>hlyB</i> gene inserted into pHpS9	This study
pHkathlyB	1.6 and 3 kb fragments containing <i>katA</i> and <i>hlyB</i> genes, respectively, inserted into pHpS9	This study
Primers		
KAT1	ATGACCACAATGCCCAATACA	This study
KATR1*	<u>ATCAAATTATGACATAGTCATCCCTCCAC</u>	This study
KATR2*	<u>GACTATGTCATAAAATTTGATATGTAGTTTC</u>	This study
KAT2	TTCCGCTTTGAAATTTTAAAT	This study
HLB1	AACTTTCTTTGTTCCATTTT	This study
HLBR1*	<u>AGCACTATTTCAACATCATTATCACTCCTT</u>	This study
HLBR2*	<u>AATGATGGTGAAATAGTGCTGAACCTAACTA</u>	This study
HLB2	TTATTCAGAGACGATTAGTTA	This study
CAT1	AGTCTCCCATCTATTTAAAT	This study
CAT2	TCATAAAGTCTCAACTACGC	Sanz <i>et al.</i> (2000)

*Complementary sequences between KATR1 and KATR2 and between HLBR1 and HLBR2 are underlined. Sequences for target recognition in these primers are indicated by bold type.

mutants by protoplast transformation, and transformants were selected by incubation at 37 °C on DM3 agar plates supplemented with 5 μ g erythromycin ml⁻¹. Transformants were also checked for wild-type phenotype restoration.

Biochemical and growth characteristics. In order to test the biochemical profile of the Δ *katA*, Δ *hlyB* and Δ *katA* Δ *hlyB* mutants in comparison with those of the wild-type strain, API 32 STAPH and API 50 CH systems (bioMérieux) were used. The API 32 STAPH system is based on 32 miniature biochemical tests and is used for the identification of staphylococci to species level. The API 50 CH system contains 50 biochemical tests for the study of the carbohydrate

metabolism of micro-organisms. Growth characteristics were determined by monitoring the OD₆₀₀ of BHI cultures of each mutant and wild-type strain at 37 °C with shaking at 175 r.p.m. for 24 h. Optical density measurements were made in a Bio-Rad SmartSpec3000 spectrophotometer with 1.5 ml cuvettes.

Intracellular survival assays. J774A.1, a murine macrophage cell line (ATCC TIB-67), and MAC-T, an established bovine mammary epithelial cell line (Huynh *et al.*, 1991), were used for experiments. J774A.1 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Pen/Strep/Fungizone mix (Biowhittaker). MAC-T cells were cultured in

Fig. 1. Phenotypic changes caused by deletion of *katA* and *hlb* genes. (a) $\Delta katA$ mutants showed a bubble-negative phenotype in contact with hydrogen peroxide. (b) Δhlb mutants were characterized by absence of the outer halo of incomplete haemolysis on sheep blood agar. (c) Spontaneous and cooperative CAMP-like haemolysis with *R. equi* (vertical streak indicated by an arrowhead) on sheep blood agar. Δhlb mutants did not produce a shovel-shaped CAMP-like reaction. WT, wild-type strain.

Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FBS, 5 μg insulin ml^{-1} , 1 μg hydrocortisone ml^{-1} and 1% Pen/Strep/Fungizone mix. Prior to each experiment, cells were seeded at 3×10^4 cells per well in 24-well tissue-culture plates and grown for 48 h at 37 °C with 5% CO_2 . Approximately 16 h before the assay, growth medium was replaced with 1 ml invasion medium (growth medium without antibiotics). Prior to infection, medium was removed and cells were given 0.5 ml fresh invasion medium. Wells were inoculated with 5×10^5 c.f.u. (J774A.1 assay) or 2.5×10^6 c.f.u. (MAC-T assay) of washed bacteria from an overnight culture suspended in 0.5 ml invasion medium. Infected cell monolayers were centrifuged for 5 min at 450 g at room temperature, and incubated for 1 h at 37 °C with 5% CO_2 . Medium was then removed, cell monolayers were washed twice with sterile PBS, and 1 ml invasion medium that contained 100 μg gentamicin ml^{-1} to kill extracellular bacteria was added to each well. After incubation of co-cultures at 37 °C with 5% CO_2 had continued for 1 (internalized bacteria), 4, 8 and 24 h in the presence of gentamicin, the supernatants were removed and discarded. Cell monolayers were then washed twice with sterile PBS and lysed with 0.2% Triton X-100 in sterile PBS. Cell lysates were serially diluted 10-fold and plated in duplicate on BHI agar plates to quantify viable intracellular bacteria. The number of intracellular bacteria at each time point was expressed as a percentage of the number of internalized bacteria.

LD₅₀ determination in mice. LD₅₀ was calculated by employing the method of Reed & Muench (1938). Groups of five female Swiss mice, 4 weeks old, were inoculated intraperitoneally with 10-fold serial

dilutions of bacterial suspension (doses ranged from 1×10^6 to 1×10^{11} c.f.u. ml^{-1}) in 0.4 ml sterile PBS. The precise inoculum was confirmed by serial dilution and counting on BHI agar plates. Inoculated mice were monitored for death every 24 h for 7 days.

Competitive index (CI) determination in a murine skin abscess model. CI was measured as described previously (Benton *et al.*, 2004) in an established murine abscess model of infection (Horsburgh *et al.*, 2001), with the following modifications. Mutant and wild-type strains were separately grown to stationary phase in BHI broth, harvested, washed and resuspended in sterile PBS. Then, each single mutant was mixed with the wild-type strain at a concentration of 4×10^8 c.f.u. ml^{-1} (1:1 ratio, 8×10^8 c.f.u. ml^{-1} total), and the double mutant was mixed with the wild-type strain in a 10:1 ratio at concentrations of 8×10^9 and 8×10^8 c.f.u. ml^{-1} (8.8×10^9 c.f.u. ml^{-1} total), respectively. To determine the exact input ratio, serial dilutions of these mixtures were plated on BHI agar or Columbia agar with 5% sheep blood, to distinguish between mutant and wild-type strains by the hydrogen peroxide assay or the haemolytic phenotype, respectively. Groups of three female 6–8-week-old BALB/c mice were inoculated subcutaneously on the back with 0.2 ml mutant/wild-type mixtures. After 7 days, mice were euthanized with CO_2 , abscesses were aseptically opened, and the contents were homogenized in 1 ml sterile ice-cold PBS. Output ratios were determined by plating serial dilutions of these homogenates, as described above, to calculate input ratio. The CI for each mutant was calculated as the ratio between the mutant and the wild-type strain in the output (abscess content) divided by the ratio in the input (initial inoculum).

Experimental intramammary infections in ewes. The experimental infection was induced as described previously (Cucarella *et al.*, 2002). At 20 to 25 days after parturition, a group of six healthy lactating Manchego ewes was inoculated with 5×10^2 c.f.u. of the wild-type strain, and three groups of three ewes were inoculated with different doses of the $\Delta katA \Delta hlb$ double mutant (1.5×10^3 , 1.5×10^4 and 1.7×10^5 c.f.u.) in 1 ml sterile PBS. Ewes were separated from their lambs 2 h before the inoculation to guarantee the presence of milk (as a natural lubricant) in the teat duct at the time of inoculation. After the teats were disinfected with 70% ethanol, the bacterial inoculum was introduced into both glands through a 21-gauge cannula. Following inoculation, because suckling can favour the removal of the bacteria, lambs were separated from their mothers for 3 h to increase the bacterial colonization of the mammary gland. Milk samples for bacteriological analysis and somatic cell counts (SCCs) were obtained prior to inoculation, once a day for the first week after inoculation, every 2 days and every 3 days in the second and third weeks, respectively, and at 28 days. SCCs were analysed at the Centro de Selección y Reproducción Animal-Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario CENSYRA-IMIDRA Dairy Control Laboratory (Madrid, Spain). For bacteriological analysis, serial dilutions of milk samples were placed on Baird-Parker agar and Columbia agar with 5% sheep blood. Rectal temperature was regularly recorded from day 2 before inoculation to 10 days post-infection. A detailed clinical examination of the mammary gland was carried out during the same time interval as milk sampling.

Experimental subcutaneous infection in lambs. Groups of two 8–10-week-old Manchego lambs were simultaneously inoculated by the subcutaneous route in the right armpit, just behind the elbow joint, with the wild-type strain, and in the left armpit with the same dose of the $\Delta katA \Delta hlb$ double mutant. Doses of 1.5×10^5 , 1.1×10^7 and 1.2×10^8 c.f.u. in 0.2 ml sterile PBS were used. Infected animals were clinically examined for the 3 weeks following inoculation, paying special attention to the local reactions at the inoculation sites and to any inflammation of the lymph nodes that drained the experimental

area. When an abscess appeared at the inoculation site, a sample was obtained for bacteriological analysis.

Ethical considerations. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the Ethical Committee for the Animal Experimentation, CEA, of the University Complutense of Madrid.

Statistical analysis. Data were analysed by a two-tailed Student's *t* test for unpaired data to determine statistically significant differences. Differences were considered statistically significant when *P* was <0.05 in all cases. Analyses were made with the help of GraphPad InStat software (version 3.05).

RESULTS

Construction and characterization of *S. aureus* *katA* and *hlyB* mutants

In order to determine the potential role of catalase and beta-toxin in *S. aureus* pathogenesis we obtained single and double mutants by deleting the *katA* and *hlyB* genes in the bovine clinical isolate *S. aureus* 2386. Deletion of the *katA* gene caused a bubble-negative phenotype in contact with hydrogen peroxide (Fig. 1a). The $\Delta hlyB$ mutation was characterized by a lack of the outer halo of incomplete haemolysis (Fig. 1b) and a negative shovel-shaped cooperative Christie–Atkins–Munch–Petersen (CAMP)-like haemolysis in the presence of *Rhodococcus equi* (Fig. 1c) on sheep blood agar. Compared with the wild-type strain, none of the three mutants showed any difference in biochemical features or utilization of a panel of 50 carbohydrates analysed by API 32 STAPH and API 50 CH, respectively. In addition, deletion of the *katA* and/or *hlyB* gene had no apparent influence on *in vitro* growth of *S. aureus*, since the growth curves of the wild-type and mutant strains were similar (data not shown). Therefore,

any differences in the behaviour of the mutants could not be attributed to differences in housekeeping gene activities or growth capability. Finally, to confirm that the deletion of the *katA* or *hlyB* genes was responsible for the observed phenotypes, a genetic complementation study was performed. As expected, complementation of the mutants with plasmids that carried the *katA* and/or *hlyB* genes restored the wild-type phenotype (data not shown).

Intracellular survival of the $\Delta katA$, $\Delta hlyB$ and $\Delta katA \Delta hlyB$ mutants

To investigate whether catalase and beta-toxin are involved in intracellular survival of *S. aureus*, we examined the ability of the mutants, complemented mutants and wild-type strain to survive inside two different cell types. We specifically used the murine macrophage-like cell line J774A.1 and the bovine mammary epithelial cell line MAC-T.

(i) J774A.1 assay. These cells retain the ability to display a respiratory burst and are quite permissive towards a variety of intracellular pathogens (Day *et al.*, 2000; Seral *et al.*, 2003). Intracellular viability of the mutants and the wild-type strain in J774A.1 cells was determined over a period of 24 h. All strains exhibited a decrease in the number of viable bacteria after phagocytosis (Fig. 2a). Intracellular survival of the $\Delta katA$ and $\Delta hlyB$ mutants was not substantially different to that of the wild-type strain. However, the $\Delta katA \Delta hlyB$ mutant showed significantly higher survival rates than the wild-type strain and the single mutants ($P < 0.01$ at 4 and 8 h; $P < 0.05$ at 24 h). All complemented mutants had a behaviour similar to that of the wild-type strain (Fig. 2a).

(ii) MAC-T assay. MAC-T cells have been used in numerous studies to characterize the ability of *S. aureus*

Fig. 2. Intracellular survival of wild-type strain (●), $\Delta katA$ (■), $\Delta hlyB$ (◆) and $\Delta katA \Delta hlyB$ (▲) mutants, and $\Delta katA$ (pHkat) (□), $\Delta hlyB$ (pHhlyB) (◇) and $\Delta katA \Delta hlyB$ (pHkat hlyB) (△) complemented mutants in J774A.1 (a) and MAC-T (b) cell lines. Numbers of viable intracellular bacteria at 4, 8 and 24 h are expressed as a percentage of internalized bacteria and represent the mean $\pm$ SEM of at least three independent experiments.

to invade and survive within eukaryotic cells (Bayles *et al.*, 1998; Qazi *et al.*, 2001; Wesson *et al.*, 1998). Wild-type *S. aureus* 2386 was internalized by and proliferated inside MAC-T cells (Fig. 2b). Thus, the number of viable intracellular bacteria at 4 h was ~1.7-fold higher than the number of internalized bacteria. After this initial increase in titre, the viability of the wild-type strain gradually declined, and reached about 7.3% of that of the internalized bacteria at 24 h. Unlike the wild-type strain, the $\Delta katA$ mutant did not exhibit an early increase in the number of viable bacteria, and viability diminished after internalization (Fig. 2b). However, despite this apparent lack of intracellular multiplication, the survival rates of the $\Delta katA$ mutant at 8 and 24 h were similar to those of the wild-type strain. With respect to the Δhlb mutant, it initially had similar kinetics to those of the wild-type strain (Fig. 2b). The Δhlb mutant grew intracellularly during the first 4 h, and then progressively declined, but it showed a significantly higher intracellular survival at 8 and 24 h ($P=0.0043$ and $P=0.0206$, respectively) than the wild-type strain. Finally, the $\Delta katA \Delta hlb$ mutant also proliferated intracellularly, but its growth was delayed in comparison with that of the wild-type strain (Fig. 2b). Thus, the number of intracellular bacteria increased almost twofold within the first 8 h. Together with the delayed intracellular proliferation, the double mutant showed a significantly greater persistence level in MAC-T cells compared with the wild-type strain ($P=0.0001$ at 8 h; $P=0.0207$ at 24 h). In all cases, complementation with plasmids that carried the deleted gene(s) returned to the mutants the characteristics observed in the wild-type strain (Fig. 2b).

Virulence of the mutants in murine models.

(i) LD₅₀ determinations. To analyse whether *katA* or *hlb* has a role in the virulence of *S. aureus*, LD₅₀ assays in mice were performed. Mice were infected intraperitoneally. As shown in Table 2, the mean LD₅₀ for the $\Delta katA$ mutant was lower than that of the wild-type, but this difference was not statistically significant. Likewise, no significant differences in virulence were observed between the Δhlb mutant and the wild-type strain in this model. In contrast, the double

$\Delta katA \Delta hlb$ mutant was five times less virulent than the wild-type strain in this model and the differences observed were statistically significant ($P=0.0199$). We also observed a statistically significant reduction in the virulence of the double mutant by comparison with both the $\Delta katA$ ($P=0.0074$) and the Δhlb ($P=0.0374$) mutants. Furthermore, complementation restored the virulence of the double mutant (Table 2).

(ii) CI determination in a murine skin abscess model. CI is a sensitive measure of the relative degree of virulence attenuation of a particular mutant in mixed infections with the wild-type strain (Benton *et al.*, 2004). To provide more data about the virulence of the mutants, CI was determined *in vivo*, using an established mouse subcutaneous model of infection (Horsburgh *et al.*, 2001). As with the results of the LD₅₀ assay, the $\Delta katA$ and Δhlb mutants were not attenuated in this model (Table 3). Only the $\Delta katA \Delta hlb$ mutant was out-competed by the wild-type strain, consistent with significant attenuation of the mutant compared with the wild-type strain (Table 3). Furthermore, since CI values lower than 0.4 are considered to represent defects in virulence (Ramos-Morales *et al.*, 2003), the double mutant was severely attenuated in this model (CI=0.036).

Virulence of the $\Delta katA \Delta hlb$ mutant in ovine models

In order to further characterize the virulence potential of the $\Delta katA \Delta hlb$ mutant, we induced intramammary and subcutaneous infections in ewes and lambs.

(i) Experimental intramammary infections in ewes. The wild-type *S. aureus* 2386 strain was highly virulent in this model. Five out of the six ewes inoculated with 500 c.f.u. developed clinical mastitis with different degrees of severity (Table 4). Thus, two ewes developed a gangrenous mastitis in both mammary glands and they were euthanized at 48–72 h post-infection. In another two ewes, the wild-type strain caused severe mastitis in one mammary gland and mild mastitis in the other. One ewe developed mild mastitis in one gland, whereas in the other, mastitis was subclinical, and in the remaining ewe the experimental infection was subclinical in both glands (Table 4).

The clinical signs observed had a direct correlation with infection severity. In ewes with clinical mastitis, mammary glands were swollen, hard and painful, and even cyanotic in cases of gangrenous mastitis. Moreover, these glands produced abnormal milk, with clots and even blood in the most serious cases. Ewes also exhibited an increase in rectal temperature and a decrease in appetite. Elevated rectal temperatures were detected at 24 and 48 h post-infection, and reached a maximum at 24 h (Fig. 3a).

Bacteria were isolated from all the inoculated mammary glands from day 1 post-infection until the end of the experimental period (Fig. 3b). The number of bacteria in

Table 2. LD₅₀ determination in mice

Strain	LD ₅₀ *	P value†
WT	$2.80 \pm 1.35 \times 10^9$	—
$\Delta katA$	$5.68 \pm 0.78 \times 10^8$	0.1735
$\Delta katA$ (pHkat)	$4.26 \pm 0.43 \times 10^8$	0.1531
Δhlb	$3.75 \pm 2.03 \times 10^9$	0.7143
Δhlb (pHhlb)	$1.00 \pm 0.34 \times 10^8$	0.1157
$\Delta katA \Delta hlb$	$1.41 \pm 0.27 \times 10^{10}$	0.0199
$\Delta katA \Delta hlb$ (pHkathlb)	$5.77 \pm 1.80 \times 10^8$	0.1773

*Data represent the mean $\pm$ SEM from three independent experiments.

†LD₅₀ values for mutants or complemented mutants were compared with that for the wild-type (WT) strain.

Table 3. CI in mice

Strains compared	Challenge route	Inoculum ratio	CI*	Attenuation†
$\Delta katA$ versus WT	Subcutaneous	1:1	1.411 ± 0.154	No
Δhlb versus WT	Subcutaneous	1:1	0.837 ± 0.178	No
$\Delta katA \Delta hlb$ versus WT	Subcutaneous	10:1‡	0.036 ± 0.004	Yes

*CI is the ratio between the mutant and the WT *S. aureus* in the output (bacteria recovered from abscess content homogenates, 7 days after infection) divided by their ratio in the input (initial inoculum). Data represent the mean $\pm$ SEM obtained from a minimum of five mice from three independent experiments.

†Mutants are considered to be attenuated when CI < 0.4 (Ramos-Morales *et al.*, 2003).

‡Inoculum ratio was increased because the 1:1 ratio did not allow recovery of mutant bacteria from the lesion.

milk was in general greater in the mammary glands with clinical mastitis. Mean values were very high ($\sim 10^8$ c.f.u. ml⁻¹) and relatively constant over the 28 days of the experiment. Furthermore, the SCCs increased significantly in response to infection with the wild-type strain and remained elevated until the end of the experiment (Fig. 3c).

Unlike the wild-type strain, the $\Delta katA \Delta hlb$ mutant did not cause any clinical mastitis in the inoculated udders, not even with the highest dose used, almost 350 times greater than that of the wild-type strain (Table 4). Moreover, there were no apparent differences in the responses of the animals to the different doses of mutant used. Apart from slight swelling at 24 h post-infection in eight out of the 18 mammary glands inoculated, and a very slight increase in rectal temperature, no other clinical alterations were observed. The highest temperature (39.8 ± 0.20 °C) was detected at 48 h post-infection (Fig. 3a), and it was significantly lower than that reached (40.82 ± 0.34 °C) at 24 h post-infection in the ewes infected with the wild-type strain ($P=0.0173$).

Like the wild-type strain, the $\Delta katA \Delta hlb$ mutant infected all the inoculated mammary glands and it was shed in milk throughout the experiment (Fig. 3b). However, overall bacterial counts in milk were significantly lower than those found with the wild-type strain ($P<0.05$). SCCs also

increased greatly after infection with the double mutant and remained high over the entire period of the experiment (Fig. 3c). SCCs were generally lower than those reached in the mammary glands infected with the wild-type strain; however, the differences were not statistically significant.

(ii) Experimental subcutaneous infection in lambs. Three groups of two lambs were used for the assay. Animals were simultaneously inoculated in the right armpit with the wild-type strain, and in the left one with the same dose of the $\Delta katA \Delta hlb$ mutant (Table 5). Lambs infected with the lowest dose (1.5×10^5 c.f.u.) did not show clinical alterations at the inoculation sites or in the regional lymph nodes. The lambs inoculated with 1.1×10^7 and 1.2×10^8 c.f.u. of both strains showed a similar response to the experimental infections. Thus, no local alterations were detected at the left inoculation site or in the left superficial cervical lymph node in these lambs (Table 5). In contrast, the wild-type strain caused a clear local inflammatory response at 24–48 h post-infection, which led to abscess development in all lambs. Abscesses reached a diameter of ~ 2 cm at 4–6 days post-infection, then gradually regressed, and were practically resolved at 20 days post-infection. Moreover, the wild-type strain also caused enlargement of the right superficial cervical lymph node in these animals.

Table 4. Experimental intramammary infection in ewes

Group (n)*	Strain	Dose (c.f.u.)	Number of mammary glands infected	Severity of intramammary infection†			
				Subclinical	Clinical		
					Mild	Severe	Gangrenous
A (6)	WT	5×10^2	12	3	3	2	4
B (3)	$\Delta katA \Delta hlb$	1.5×10^3	6	6	–	–	–
C (3)	$\Delta katA \Delta hlb$	1.5×10^4	6	6	–	–	–
D (3)	$\Delta katA \Delta hlb$	1.7×10^5	6	6	–	–	–

*n refers to the number of healthy lactating Manchego ewes inoculated in each group.

†Animals were clinically examined for 4 weeks.

Fig. 3. Rectal temperature (a), bacterial shedding in milk (b) and SCCs in milk (c) in ewes infected with wild-type *S. aureus* (●) and the $\Delta katA \Delta hlb$ mutant (○). Data represent mean $\pm$ SEM. Mean values for the $\Delta katA \Delta hlb$ mutant were calculated from the data for all infected ewes, regardless of the dose used.

DISCUSSION

The purpose of this study was to investigate the potential role of catalase and beta-toxin in the intracellular survival, and consequently virulence, of *S. aureus*. With this aim,

single and double mutants of *katA* and *hlyB* genes were created by gene deletion.

S. aureus is not generally considered to be a major intracellular pathogen. However, it is well documented that *S. aureus* adheres to, easily invades and survives within non-professional (Bayles *et al.*, 1998; Menzies & Kourteva, 1998; Wesson *et al.*, 1998) and professional phagocytes (Brouillette *et al.*, 2003; Hébert *et al.*, 2000). Factors influencing the internalization of *S. aureus* have frequently been investigated (Alexander & Hudson, 2001), but little is known about those that are necessary for its intracellular survival.

Phagocytosis by polymorphonuclear neutrophils (PMNs) and macrophages is considered to be the first and main defence mechanism of the host against *S. aureus* invasion. Bactericidal mechanisms of phagocytic cells include the production of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals (Cabiscol *et al.*, 2000). However, the generation of ROS is not an exclusive prerogative of professional phagocytes, and it has also been described in non-phagocytic cells (Meier, 2001). Therefore, enzymes implicated in oxidative stress resistance could play an important role in the survival of *S. aureus* inside both phagocytic and non-phagocytic cells. However, in our experiments, catalase did not contribute to the survival of *S. aureus* within J774A.1 macrophages. This indicates either that the production of ROS by these cells, which retain the ability to display a respiratory burst, is not involved in the killing of *S. aureus* or that the $\Delta katA$ mutant compensates for the lack of catalase activity by the expression of other oxidative stress-resistance factors such as alkyl hydroperoxide reductase, which has been demonstrated to have compensatory roles with catalase in cellular physiology (Cosgrove *et al.*, 2007). Catalase also did not significantly participate in the long-term survival of *S. aureus* in MAC-T cells, although it was required for the intracellular proliferation of the bacterium during the initial hours after cell invasion. This finding reveals differences between J774A.1 and MAC-T cells in the bactericidal mechanisms against *S. aureus*, at least in the early stage of intracellular infection.

Current knowledge of the role of bacterial sphingomyelinases in intracellular pathogenesis remains limited. In *Listeria ivanovii*, a sphingomyelinase similar to beta-toxin, SmcL, seems to promote intracellular proliferation of bacteria in bovine epithelial cells by mediating disruption of the phagocytic vacuole and the release of bacteria into the cytosol (González-Zorn *et al.*, 1999). Earlier work has demonstrated that intracellular *S. aureus* escapes from endosomes and then multiplies within the cytoplasm in MAC-T cells (Bayles *et al.*, 1998; Qazi *et al.*, 2001; Shompole *et al.*, 2003). Moreover, staphylococcal haemolysins are involved in endosome rupture and subsequent intracellular replication of *S. aureus* (Shompole *et al.*, 2003). In our study, the lack of beta-toxin did not modify the proliferation of *S. aureus* in MAC-T cells during the

Table 5. Experimental subcutaneous infection in lambs

Group*	Dose (c.f.u.)	Strain	Superficial cervical lymph node†	Abscess in the inoculation site†
A	1.5×10^5	WT	Normal	No
		$\Delta katA \Delta hlb$	Normal	No
B	1.1×10^7	WT	Increased	Yes
		$\Delta katA \Delta hlb$	Normal	No
C	1.2×10^8	WT	Increased	Yes
		$\Delta katA \Delta hlb$	Normal	No

*Groups of two 8–10-week-old Manchego lambs were simultaneously inoculated by the subcutaneous route in the right armpit with the WT *S. aureus* and in the left armpit with the same dose of the $\Delta katA \Delta hlb$ mutant.

†Animals were clinically examined for 3 weeks. Clinical responses were similar in both lambs of each group.

first 4 h post-infection. Therefore, it is possible that the remaining haemolysins were sufficient to allow the release of *S. aureus* from the endosome in these cells. Nevertheless, the absence of beta-toxin, which had no effect on intracellular survival of *S. aureus* in murine J774A.1 macrophages, caused an increase in bacterial persistence in bovine MAC-T epithelial cells at 8 and 24 h post-infection. This divergence might have been caused by differences in the sphingomyelin content of these cells. Thus, a correlation has been observed between membrane sphingomyelin content and the susceptibility of erythrocytes to beta-toxin; therefore, only erythrocytes from ruminants, which have the highest content of all mammals, are highly susceptible (Titball, 1993). The increased persistence of the Δhlb mutant in MAC-T cells in comparison with the wild-type strain could have been a result of lower cytolytic activity, which protected the mutant from the gentamicin present in the extracellular medium. Our results are in agreement with previous studies in which a lack of alpha-toxin (Vann & Proctor, 1988) or a decreased production of haemolysins (von Eiff *et al.*, 1997; Wesson *et al.*, 1998) in *S. aureus* were associated with an increase in its intracellular survival.

Interestingly, the behaviour of the $\Delta katA \Delta hlb$ mutant in the intracellular survival assays was quite different from that of the single mutants. Thus, the simultaneous lack of catalase and beta-toxin caused a notable increase in the survival of *S. aureus* within J774A.1 macrophages at all the three time points examined, and within MAC-T cells at 8 and 24 h post-infection. Moreover, in MAC-T cells, a delay in the proliferation of the double mutant in comparison with the wild-type strain and the Δhlb mutant was observed. The increased survival of the double mutant could initially be attributed to a higher resistance to the bactericidal activity of the cells, and this would imply increased virulence of the double mutant. However, this interpretation, which could be considered when the intracellular assays are analysed in isolation, clearly contrasts with the results obtained in all the experimental infection models used, which undoubtedly showed that the combined mutation leads to a dramatic reduction in

virulence. Therefore, the *in vitro* increased intracellular persistence of the double mutant could be a consequence of the diminished cytotoxicity of the bacterium, which would have delayed death of the infected cells. The mechanism by which catalase and beta-toxin synergistically contribute to intracellular pathogenesis of *S. aureus* in J774A.1 and MAC-T cells is not evident. Further studies on the possible interaction between these two proteins during the intracellular infection of *S. aureus* are needed to determine this mechanism and to establish how the increased intracellular persistence is connected with the attenuation in animal models. Nevertheless, the results of the experimental infections allow us to hypothesize that *in vivo* the simultaneous lack of catalase and beta-toxin results in a limited capacity of the mutant to proliferate and spread.

The changes observed in the intracellular behaviour of the mutants may have altered their virulence; therefore, we decided to use two different murine models to investigate this question. Systemic and abscess models allowed us to establish the ability of a bacterium to survive in two distinct host environments. The systemic model involves the adaptation of bacteria to the host environment, survival of the host immune response, dissemination and colonization, and persistence within organs. The abscess model, in contrast, does not require dissemination to organs. Furthermore, during abscess formation, bacterial growth is reduced by influx of neutrophils, as well as oxygen and nutrient limitation.

Catalase has traditionally been implicated as a virulence determinant in *S. aureus* (Kanafani & Martin, 1985; Mandell, 1975). However, recent studies with *S. aureus* *katA* mutants have revealed no differences in virulence from the corresponding wild-type strains in murine models of infection (Cosgrove *et al.*, 2007; Horsburgh *et al.*, 2001). The results of this study supply further experimental evidence that catalase is not required for complete virulence of *S. aureus*, at least not in the murine models used.

The role of beta-toxin in staphylococcal infections is not clear, since divergent results have been reported in various

experimental models with beta-toxin mutants (Bramley *et al.*, 1989; Nilsson *et al.*, 1999; O'Callaghan *et al.*, 1997). In our study, the virulence of the Δhlb mutant was not significantly different from that of the wild-type strain in the two murine models, which agrees with the results of the intracellular survival experiment with the murine cell line J774A.1, which also showed no differences. The finding that beta-toxin does not seem to have any effect on the pathogenesis of *S. aureus* for mice does not allow us to rule out the possibility that this toxin plays an important role in intramammary infections caused by *S. aureus*, especially in ruminants. Beta-toxin is consistently produced by *S. aureus* strains isolated from bovine mastitis; therefore, it has been suggested that it is an active virulence factor in the pathogenesis of bovine *S. aureus* mastitis (Larsen *et al.*, 2002). This hypothesis is supported by the fact discussed above that the lack of beta-toxin caused increased persistence of *S. aureus* in bovine MAC-T cells, possibly due to lower cytolytic activity and a potential relationship with the high membrane content of sphingomyelin in ruminant cells.

Although catalase and beta-toxin per se did not significantly contribute to the virulence of *S. aureus*, the $\Delta katA \Delta hlb$ mutant was clearly attenuated in the two murine models of infection. The *in vivo* results therefore do not support the view that a longer intracellular persistence *in vitro*, even in macrophage-like cells, gives rise to increased virulence. On the contrary, the results of the experimental infections clearly indicate that *in vivo* the double mutant is more susceptible to the host defence mechanisms than the wild-type and single mutants, and correlate well with the interpretation that the double mutant has a notably diminished virulence for cells. They also demonstrate the multifactorial nature of the pathogenesis of *S. aureus*, and hence the necessity to establish how potential virulence factors interact in causing disease.

The results of the experimental infection in mice with the $\Delta katA \Delta hlb$ mutant led us to further investigate the virulence of the double mutant in two different ovine models of infection. Marked attenuation in the virulence of the $\Delta katA \Delta hlb$ mutant was observed in experimental intramammary infection in ewes, based on clinical manifestations. In this model, the wild-type strain was extremely virulent, since 500 c.f.u. caused very severe mastitis in most animals, whereas the double mutant was not able to cause clinical mastitis in the infected ewes, not even in those inoculated with 1.7×10^5 c.f.u. The double mutant, however, caused subclinical mastitis in all the infected udders, as demonstrated by shedding of bacteria in milk and increased SCCs, which means that the $\Delta katA \Delta hlb$ mutant still retained the capacity to invade, colonize and grow in the mammary gland. The attenuation of the double mutant seen in the model of subcutaneous infection in lambs was even more evident. Indeed, the two higher doses of the mutant did not produce any local alteration at the inoculation site, while the corresponding dose of the wild-type caused local inflammation that led to the development of abscesses in all cases.

S. aureus is the most common cause of intramammary infections in ruminants, which result in important economic losses for the dairy industry (Sutra & Poutrel, 1994). The use as live vaccines of attenuated bacteria, such as the $\Delta katA \Delta hlb$ mutant, might be a good strategy to prevent *S. aureus* mastitis.

ACKNOWLEDGEMENTS

This research was supported by grants from the Spanish Ministry of Education and Science (MEC) (AGF98-0743), Universidad Complutense-Comunidad de Madrid (920338) and Laboratorios Hipra, SA (470/2005). We thank Kenneth W. Bayles, University of Nebraska, for the gift of MAC-T cells, A. Vindel (Instituto de Salud Carlos III) for determining the spa and ST type, and R. Sánchez and E. Neves for their technical support.

REFERENCES

- Aarestrup, F. M., Larsen, H. D., Eriksen, N. H., Elsborg, C. S. & Jensen, N. E. (1999). Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. *APMIS* **107**, 425–430.
- Alexander, E. H. & Hudson, M. C. (2001). Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Appl Microbiol Biotechnol* **56**, 361–366.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1992). *Current Protocols in Molecular Biology*. New York: Green Publishing Associates and Wiley-Interscience.
- Basu, M., Czinn, S. J. & Blanchard, T. G. (2004). Absence of catalase reduces long-term survival of *Helicobacter pylori* in macrophage phagosomes. *Helicobacter* **9**, 211–216.
- Bayles, K. W., Wesson, C. A., Liou, L. E., Fox, L. K., Bohach, G. A. & Trumble, W. R. (1998). Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun* **66**, 336–342.
- Benton, B. M., Zhang, J. P., Bond, S., Pope, C., Christian, T., Lee, L., Winterberg, K. M., Schmid, M. B. & Buysse, J. M. (2004). Large-scale identification of genes required for full virulence of *Staphylococcus aureus*. *J Bacteriol* **186**, 8478–8489.
- Bramley, A. J., Patel, A. H., O'Reilly, M., Foster, R. & Foster, T. J. (1989). Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect Immun* **57**, 2489–2494.
- Brouillette, E., Grondin, G., Shkreta, L., Lacasse, P. & Talbot, B. G. (2003). In vivo and in vitro demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. *Microb Pathog* **35**, 159–168.
- Cabiscol, E., Tamarit, J. & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* **3**, 3–8.
- Calvinho, L. F., Donnelly, W. J. & Dodd, K. (1993). Effect of partially purified *Staphylococcus aureus* beta-haemolysin on the mammary gland of the mouse. *Zentralbl Veterinarmed B* **40**, 559–568.
- Cifrian, E., Guidry, A. J., Bramley, A. J., Norcross, N. L., Bastida-Corcuera, F. D. & Marquardt, W. W. (1996). Effect of staphylococcal β toxin on the cytotoxicity, proliferation and adherence of *Staphylococcus aureus* to bovine mammary epithelial cells. *Vet Microbiol* **48**, 187–198.

- Cosgrove, K., Coutts, G., Jonsson, I. M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J. & Foster, S. J. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* **189**, 1025–1035.
- Cucarella, C., Tormo, M. A., Knecht, E., Amorena, B., Lasa, I., Foster, T. J. & Penadés, J. R. (2002). Expression of the biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process. *Infect Immun* **70**, 3180–3186.
- Day, W. A., Jr, Sajceki, J. L., Pitts, T. M. & Joens, L. A. (2000). Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun* **68**, 6337–6345.
- Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* **13**, 16–34.
- González-Zorn, B., Domínguez-Bernal, G., Suárez, M., Ripio, M. T., Vega, Y., Novella, S. & Vázquez-Boland, J. A. (1999). The *smcL* gene of *Listeria ivanovii* encodes a sphingomyelinase C that mediates bacterial escape from the phagocytic vacuole. *Mol Microbiol* **33**, 510–523.
- Götz, F., Ahrne, S. & Lindberg, M. (1981). Plasmid transfer and genetic recombination by protoplast fusion in staphylococci. *J Bacteriol* **145**, 74–81.
- Haima, P., van Sinderen, D., Schotting, H., Bron, S. & Venema, G. (1990). Development of a β -galactosidase α -complementation system for molecular cloning in *Bacillus subtilis*. *Gene* **86**, 63–69.
- Hannun, Y. A. & Obeid, L. M. (1995). Ceramide: an intracellular signal for apoptosis. *Trends Biochem Sci* **20**, 73–77.
- Hébert, A., Sayasith, K., Sénéchal, S., Dubreuil, P. & Lagacé, J. (2000). Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol Lett* **193**, 57–62.
- Horinouchi, S. & Weisblum, B. (1982). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. *J Bacteriol* **150**, 804–814.
- Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E. & Foster, S. J. (2001). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect Immun* **69**, 3744–3754.
- Huynh, H. T., Robitaille, G. & Turner, J. D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an *in vitro* model for bovine lactation. *Exp Cell Res* **197**, 191–199.
- Kanafani, H. & Martin, S. E. (1985). Catalase and superoxide dismutase activities in virulent and nonvirulent *Staphylococcus aureus* isolates. *J Clin Microbiol* **21**, 607–610.
- Kerro Dego, O., van Dijk, J. E. & Nederbragt, H. (2002). Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. *Vet Q* **24**, 181–198.
- Larsen, H. D., Aarestrup, F. M. & Jensen, N. E. (2002). Geographical variation in the presence of genes encoding superantigenic exotoxins and β -hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. *Vet Microbiol* **85**, 61–67.
- Manca, C., Paul, S., Barry, C. E., III, Freedman, V. H. & Kaplan, G. (1999). *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*. *Infect Immun* **67**, 74–79.
- Mandell, G. L. (1975). Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal-leukocyte interaction. *J Clin Invest* **55**, 561–566.
- Meier, B. (2001). Superoxide generation of phagocytes and non-phagocytic cells. *Protoplasma* **217**, 117–124.
- Menzies, B. E. & Kourteva, I. (1998). Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. *Infect Immun* **66**, 5994–5998.
- Nilsson, I. M., Hartford, O., Foster, T. & Tarkowski, A. (1999). Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. *Infect Immun* **67**, 1045–1049.
- O'Callaghan, R. J., Callegan, M. C., Moreau, J. M., Green, L. C., Foster, T. J., Hartford, O. M., Engel, L. S. & Hill, J. M. (1997). Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. *Infect Immun* **65**, 1571–1578.
- Qazi, S. N., Counil, E., Morrissey, J., Rees, C. E., Cockayne, A., Winzer, K., Chan, W. C., Williams, P. & Hill, P. J. (2001). *agr* expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. *Infect Immun* **69**, 7074–7082.
- Ramos-Morales, F., Prieto, A. I., Beuzón, C. R., Holden, D. W. & Casadesús, J. (2003). Role for *Salmonella enterica* enterobacterial common antigen in bile resistance and virulence. *J Bacteriol* **185**, 5328–5332.
- Reed, L. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Am J Hyg* **27**, 493–497.
- Ripio, M. T., Geoffroy, C., Domínguez, G., Alouf, J. E. & Vázquez-Boland, J. A. (1995). The sulphhydryl-activated cytolysin and a sphingomyelinase C are the major membrane-damaging factors involved in cooperative (CAMP-like) haemolysis of *Listeria* spp. *Res Microbiol* **146**, 303–313.
- Sanz, R., Marín, I., Ruiz-Santa-Quiteria, J. A., Orden, J. A., Cid, D., Díez, R. M., Silhadi, K. S., Amils, R. & de la Fuente, R. (2000). Catalase deficiency in *Staphylococcus aureus* subsp. *anaerobius* is associated with natural loss-of-function mutations within the structural gene. *Microbiology* **146**, 465–475.
- Seral, C., Van Bambeke, F. & Tulkens, P. M. (2003). Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. *Antimicrob Agents Chemother* **47**, 2283–2292.
- Shompole, S., Henon, K. T., Liou, L. E., Dziewanowska, K., Bohach, G. A. & Bayles, K. W. (2003). Biphasic intracellular expression of *Staphylococcus aureus* virulence factors and evidence for Agr-mediated diffusion sensing. *Mol Microbiol* **49**, 919–927.
- Sutra, L. & Poutrel, B. (1994). Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J Med Microbiol* **40**, 79–89.
- Titball, R. W. (1993). Bacterial phospholipases C. *Microbiol Rev* **57**, 347–366.
- Vallejo, A. N., Pogulis, R. J. & Pease, L. R. (1994). *In vitro* synthesis of novel genes: mutagenesis and recombination by PCR. *PCR Methods Appl* **4**, S123–S130.
- Vann, J. M. & Proctor, R. A. (1988). Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* α -hemolysin. *Microb Pathog* **4**, 443–453.
- von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G. & Gotz, F. (1997). A site-directed *Staphylococcus aureus* *hemB* mutant is a small-colony variant which persists intracellularly. *J Bacteriol* **179**, 4706–4712.
- Ward, P. D., Adlam, C., McCartney, A. C., Arbuthnott, J. P. & Thorley, C. M. (1979). A histopathological study of the effects of highly purified staphylococcal alpha and beta toxins on the lactating mammary gland and skin of the rabbit. *J Comp Pathol* **89**, 169–177.
- Wesson, C. A., Liou, L. E., Todd, K. M., Bohach, G. A., Trumble, W. R. & Bayles, K. W. (1998). *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis. *Infect Immun* **66**, 5238–5243.

Edited by: J. Lindsay