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Original article

Restoring catalase activity in *Staphylococcus aureus* subsp. *anaerobius* leads to loss of pathogenicity for lambs

Ricardo DE LA FUENTE[†*], Rosa M. DÍEZ[†], Gustavo DOMÍNGUEZ-BERNAL, José A. ORDEN, Susana MARTÍNEZ-PULGARÍN

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

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Abstract – *Staphylococcus aureus* subsp. *anaerobius*, a microaerophilic and catalase-negative bacterium, is the etiological agent of abscess disease, a specific chronic condition of sheep and goats, which is characterized by formation of necrotic lesions that are located typically in superficial lymph nodes. We constructed an isogenic mutant of *S. aureus* subsp. *anaerobius* (RDKA84) that carried a repaired and functional catalase gene from *S. aureus* ATCC 12600, to investigate whether the lack of catalase in *S. aureus* subsp. *anaerobius* plays a role in its physiological and pathogenic characteristics. The catalase activity had no apparent influence on the in vitro growth characteristics of RDKA84, which, like the wild-type, did not grow on aerobically incubated agar plates. Restoration of catalase activity in RDKA84 substantially increased resistance to H_2O_2 when analyzed in a death assay. The intracellular survival rates of the catalase-positive mutant RDKA84 in polymorphonuclear neutrophils (PMN) isolated from adult sheep were significantly higher than those of the wild-type, while no differences were found with PMN isolated from lambs. RDKA84 showed significantly lower survival rates in murine macrophages (J774A.1 cells) than the wild-type strains did, whereas, in bovine mammary epithelial cells (MAC-T), no differences in intracellular survival were observed. Interestingly, the virulence for lambs, the natural host for abscess disease, of the catalase-positive mutant RDKA84 was reduced dramatically in comparison with wild-type *S. aureus* subsp. *anaerobius* in two experimental models of infection.

***S. aureus* subsp. *anaerobius* / pathogenesis / catalase / abscess disease / sheep**

1. INTRODUCTION

Staphylococcus aureus subsp. *anaerobius* was identified originally by de la Fuente and Suárez [8] as a respiratory deficient variant of *S. aureus* because of its lack of aerobic growth, catalase activity and cytochromes, and was classified subsequently as a new subspecies of *S. aureus* [9]. *S. aureus* subsp. *anaerobius* is related very closely to *S. aureus*, and therefore, both bacteria share the ability to produce extracellular toxins and enzymes [8–10] that have been related traditionally to staphylococcal pathogenicity. However, the pathogenic abilities of both bacteria are very different. Thus, *S. aureus* subsp. *anaerobius* is the etiological agent of abscess disease, which is a specific lymphadenitis of sheep and goats. It is characterized by abscesses in the superficial lymph nodes and affects mainly young animals up to 5–6 months of age [8]. *S. aureus* is a major pathogen that is responsible for a wide range of acute and chronic infections in humans and animals.

[†] These authors contributed equally to this work.
[*] Corresponding author: rifuente@vet.ucm.es

Together with the lack of growth under aerobic conditions, one of the main phenotypic differences between *S. aureus* and *S. aureus* subsp. *anaerobius* is the lack of catalase activity in the latter [9]. Sanz et al. [25] have demonstrated that the catalase deficiency in *S. aureus* subsp. *anaerobius* is associated with mutations within the structural gene. More specifically, a deletion located at 1 338 bp from the initiation codon, which is responsible for the premature translation termination, causes the loss of the final 50 amino acids from the C terminus and a point replacement in residue 317, which affects the heme-binding site.

Catalase is an enzyme that is involved in oxidative stress resistance, and converts H_2O_2 generated during cellular metabolism to water and molecular oxygen. As a result, catalase has been proposed as a potential virulence factor in many bacterial pathogens [3, 7, 19], because its activity might protect them from the reactive oxygen species (ROS) generated by eukaryotic cells, mainly polymorphonuclear neutrophils (PMN) and other inflammatory cells during phagocytosis. In *S. aureus*, catalase has been implicated as a virulence determinant [17, 20]. However, recent studies with *S. aureus katA* mutants have revealed no differences in virulence with the corresponding wild-type strains in different murine models of infection [6, 15, 21].

The aim of the present study was to construct an isogenic mutant of *S. aureus* subsp. *anaerobius* that carried a repaired and functional catalase gene, in order to investigate the influence of catalase activity on the physiological, biochemical and pathogenic characteristics of the mutant in comparison with those of the wild-type.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table I. The *S. aureus* subsp. *anaerobius* strain MVF-84 (CECT 7640), a clinical isolate from a 4-month-old lamb affected by abscess disease, and its catalase-positive mutant RDKA84 were grown routinely at 37 °C in brain heart infusion (BHI) broth under static conditions. Solid media such as BHI agar were incubated microaerophilically (candle jar system). Media were supplemented when appropriate with erythromycin (5 μg/mL for plasmid pLUG277).

2.2. DNA manipulation and transformation

Total DNA from *S. aureus* and *S. aureus* subsp. *anaerobius* was extracted by the cetyltrimethylammonium bromide method after pretreatment of bacteria with lysostaphin (30 μg/mL; Sigma-Aldrich, Tres Cantos, Madrid, Spain) at 37 °C for 1 h in Tris/EDTA/sucrose [2]. Plasmid DNA isolation was performed using the Plasmid Purification Kit (Qiagen, Las Matas, Madrid, Spain). Plasmids were transformed into *S. aureus* by protoplast transformation [12]. Protoplast transformation was not possible in *S. aureus* subsp. *anaerobius*, therefore, plasmids were introduced in this bacterium by electroporation in 0.2-mm cuvettes (Bio-Rad, Alcobendas, Madrid, Spain) at 2.5 kV, 25 μF and 100 Ω, using a Bio-Rad Gene Pulser, as described previously [1]. Transformation of *Escherichia coli* was performed by standard procedures [2]. 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 (Cerdanyola del Vallés, Barcelona, Spain). PCR were carried out with Amplitaq Gold polymerase (Applied Biosystems, Alcobendas, Madrid, Spain) as recommended by the manufacturer. DNA sequencing was carried out on double-stranded plasmid DNA templates as described previously [25]. Oligonucleotide primers were purchased from Isogen Bioscience BV (As Maarssen, Netherlands).

2.3. Construction of catalase-positive mutants of *S. aureus* subsp. *anaerobius*

Total DNA from *S. aureus* ATCC 12600 was digested with *Hin*dIII and *Sac*I to obtain a 2-kb fragment that contained the *katA* gene. This DNA fragment was cloned initially in pBT2, an *E. coli–Staphylococcus* shuttle vector. The DNA fragment was excised from pBT2 using *Eco*RI and *Pvu*II and ligated into pCR2.1 which had been digested previously with *Eco*RI and *Eco*RV. Finally, the DNA fragment that contained the *katA* gene was cut out from pCR2.1 by *Spe*I and *Xba*I, and cloned into the *Xba*I site of the pE194 thermosensitive vector to generate pLUG277 (Tab. I). This recombinogenic plasmid was introduced into *S. aureus* RN4220 by protoplast transformation, and later transformed into *S. aureus*

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

Strain or plasmid	Description	Reference or source
Strains		
S. aureus subsp. *anaerobius*		
MVF-84 (CECT 7640)	Clinical isolate from a 4-month-old lamb affected by abscess disease	Our laboratory
RDKA84	Catalase-positive mutant of MVF-84	This study
S. aureus		
ATCC 12600	Type strain	
RN4220	Restriction-deficient mutant of *S. aureus* 8325-4	[18]
MN-42	Clinical isolate from ovine gangrenous mastitis	Our laboratory
MN-45	Clinical isolate from ovine gangrenous mastitis	Our laboratory
MN-73	Clinical isolate from ovine gangrenous mastitis	Our laboratory
DGA-1	Clinical isolate from acute bovine mastitis	Our laboratory
E. coli		
DH5α	Cloning host strain	Our laboratory
TOP10	Cloning host strain	Invitrogen
Plasmids		
pBT2	*E. coli–Staphylococcus amp cat* shuttle vector	[4]
pCR2.1	T-vector for cloning of PCR products	Invitrogen
pE194	Temperature-sensitive *erm* vector for allelic exchange in *S. aureus*	[14]
pLUG277	Recombinogenic plasmid used in the construction of the catalase-positive mutant of MVF-84 (pE194 inserted with a 2 kb *Hind*III-*Sac*I fragment containing the *katA* gene)	This study
Primers		
Cat1	TATAAATTGTGGAGGGATGAT	[25]
Cat2	TCATAAACTGCTCAACTACGC	[25]

subsp. *anaerobius* MVF-84 by electroporation. MVF-84 transformants were screened for bubble-positive phenotypes by the H_2O_2 assay. In order to replace the catalase gene of *S. aureus* subsp. *anaerobius* (*katB*) with the *katA* gene, transformants were cultured in BHI with erythromycin (5 μg/mL) at 32 °C for 48 h. Cultures were diluted into fresh medium and grown at 40 °C (non-permissive for plasmid replication) for 24 h, to induce plasmid integration into the chromosome by homologous recombination. Double crossover and plasmid loss was achieved by regularly subculturing at 37 °C in BHI without erythromycin, and plating appropriate dilutions on BHI agar. Catalase-positive mutants were selected for bubble-positive phenotypes by the H_2O_2 assay, and they were tested for sensitivity to erythromycin. The catalase gene was amplified by PCR using oligonucleotide primers Cat1 and Cat2 (Tab. I), and sequenced to verify the replacement of the native *katB* sequence with the sequence of *katA*.

2.4. Biochemical and growth characteristics

The biochemical profile of the recombinant mutant RDKA84 in comparison to that of the wild-type strain MVF-84 was evaluated using API 32 STAPH (bioMérieux, Madrid, Spain). Catalase plate assays were performing by plating a drop of 3% H_2O_2 onto the edge of the colonies. A positive reaction was indicated by formation of oxygen bubbles. Growth characteristics were determined by monitoring OD_{600} of BHI cultures of each strain, under static conditions without shaking at 37 °C for

48 h. In order to maintain stable microaerophilic conditions, independent cultures were used for each measurement time. Colony morphology on BHI and sheep blood agar plates was also compared.

2.5. H_2O_2 sensitivity assays

Cultures of MVF-84, RDKA84 and four *S. aureus* strains isolated from clinical cases of bovine acute mastitis (strain DGA-1) or ovine gangrenous mastitis (strains MN-42, MN-45 and MN-73) were grown in BHI under static conditions until the stationary phase (OD_{600} 1.4). Aliquots of 100 µL were then added to the tubes that contained 0.9-mL volumes of different concentrations (0.4, 10, 20, 30, 40 and 50 mM) of H_2O_2, and allowed to sit at 37 °C for 30 min. Tenfold dilutions were then spotted onto BHI plates to determine the number of viable bacteria. The sensitivity to H_2O_2 was also analyzed by the disk diffusion method using BHI agar and 6-mm non-impregnated disks (bioMérieux) spotted with 30 µL of H_2O_2 (5 mM, 50 mM, 500 mM and 1 M).

2.6. Intracellular survival in ovine PMN

Peripheral blood PMN were obtained from healthy adult sheep and lambs (3 months old). Jugular blood was collected in 10-mL venoject tubes that contained sodium heparin (150 IU/mL). The isolation of PMN was performed as described previously [16]. The final suspension was adjusted to a concentration of 4×10^6 cells/mL. The purity of the PMN suspension, as assessed by staining with May–Grunwald/Giemsa, was greater than 95%, and its viability, determined by trypan-blue exclusion, was more than 95%. Bacteria were cultured during 48 h in BHI medium at 37 °C under microaerophilic conditions, and washed twice with Hanks balanced salt solution (HBSS), resuspended in HBSS–gelatin (HBSS containing 0.1% gelatin), and adjusted to the desired concentration of 2×10^7 CFU/mL. The intracellular survival assay was performed as described previously [27], with slight modifications. In brief, 45 µL of bacterial suspension was placed into each well of four sterile 96-well flat-bottomed microtitration plates. Each plate represented a different time point in the experiment. Samples were taken at 0, 30, 60 and 90 min after phagocytosis. Bacteria were opsonized for 10 min at 37 °C with 5 µL of pooled ovine sera (diluted 1:2) or HBSS (negative control). Forty-five microliters of the PMN suspension was added to each well, which gave a bacterium/PMN ratio of 5:1, and plates were reincubated for 15 min to allow phagocytosis. Five microliters of lysostaphin (100 µg/mL) were added to the wells, and the plates were incubated once more for 20 min in order to lyse any extracellular bacteria. At the different sampling times, 8 µL trypsin (25 mg/mL) were added to inactivate the lysostaphin in the wells of the appropriate plate. Immediately, 100 µL were transferred from the wells of the plate at time zero into a 1.5-mL tube and incubated for 5 min, in order to allow inactivation to occur. One hundred microliters of chilled (4 °C), sterile distilled water was added to the tubes, which were placed in ice for 5 min. Later, these tubes were agitated vigorously to lyse the PMN and release surviving intracellular staphylococci. The other plates were incubated with lysostaphin and samples were taken after 30, 60 and 90 min. At these times, samples were treated as mentioned above. Tenfold dilutions were spotted onto BHI plates in 100-µL portions. After 72 h incubation at 37 °C under microaerophilic conditions, the colonies were counted and the percentage of intracellular survivors was calculated, taking the number of bacteria phagocytosed, point zero, as 100%.

2.7. Intracellular survival in J774A.1 and MAC-T cell lines

The ability of the RDKA84 mutant and its parental strain MVF-84 to survive inside J774A.1, a murine macrophage cell line (ATCC TIB-67), and MAC-T, an established bovine mammary epithelial cell line, was determined as described previously [21].

2.8. LD_{50} determination in mice

LD_{50} was calculated using the method of Reed and Muench [22]. Bacteria were cultured in BHI medium at 37 °C under static conditions for 48 h, centrifuged at 10 000 *g* for 10 min, washed twice with sterile PBS (pH 7.0), and suspended in the same buffer. Groups of five female Swiss mice, 4 weeks old, were inoculated intraperitoneally with 10-fold serial dilutions of bacterial suspension (dose range from 10^5 to 10^{10} CFU/mL). The precise inoculum was confirmed by serial dilution and counting on BHI agar plates. Inoculated mice were monitored for death every 24 h for 14 days.

2.9. Experimental infections in lambs

Sixteen lambs of the Manchego breed were used. Animals in each experimental group were housed together in one pen and fed a mixture of Lucerne chaff and concentrate pellets ad libitum.

2.9.1. Infection of superficial skin incisions

Ten 2-month-old lambs were used in this experiment. Three of the animals, which served as positive controls for development of abscess disease, were inoculated with the wild-type strain MVF-84. The remaining seven lambs were infected with the catalase-positive mutant RDKA84. The experimental infections were carried out as described previously [23]. Briefly, superficial incisions (3–5) of about 1 cm each were made with a scalpel in the skin of the left side of the lower lip and the left forelimb above the carpal joint. Incisions were rubbed for about 30 s with a swab impregnated in a BHI culture of MVF-84 or RDKA84, which contained about 3.5×10^7 CFU/mL.

2.9.2. Subcutaneous infections

Two groups of three 10-week-old lambs were used. Lambs were inoculated simultaneously by the subcutaneous route in the right armpit, just behind the elbow joint, with the wild-type strain MVF-84, and in the left armpit with the catalase-positive mutant RDKA84. In the first group, low doses of bacteria (5×10^3 CFU of MVF-84 and 2.4×10^4 CFU of RDKA84) were used, while in the second group, lambs were inoculated with high doses (1.3×10^7 CFU of MVF-84 and 2.6×10^7 CFU of RDKA84).

Rectal temperatures were recorded once a day from day 3 before inoculation till the experiments ended. The infected animals were clinically examined daily during the first week following inoculation, and every 3 days afterwards, paying special attention to the local reactions at the inoculation sites and to any swelling of the lymph nodes draining the experimental areas. When there were abscesses in the inoculation site and the abscesses were fistulated, a specimen was collected for bacterial culture. Lambs were sacrificed 6 (subcutaneously infected) or 8 (infection of superficial skin incision method) weeks after inoculation and subjected to full necropsy. At necropsy, the regional lymph nodes draining the inoculated sites were dissected in situ for evidence of abscess formation.

2.10. 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 Animal Experimentation of the Complutense University of Madrid.

2.11. Statistical analysis

A two-tailed Student's *t*-test was used to determine statistically significant differences. Differences were described as statistically significant when p was < 0.05.

3. RESULTS

3.1. Construction and characterization of *S. aureus* subsp. *anaerobius* catalase-positive mutant RDKA84

Plasmid pLUG277 was transformed into MVF-84 by electroporation, and transformants were incubated at different temperatures to induce homologue recombination and plasmid curing. Finally, a catalase positive and Em^s mutant was selected and designated as RDKA84. The nucleotide sequence of the *kat* gene from mutant RDKA84 was identical to that of the *katA* gene from *S. aureus* ATCC 12600. Restoration of catalase activity had no apparent influence on in vitro growth of *S. aureus* subsp. *anaerobius*, since the growth curves of the MVF-84 parental strain and the RDKA84 mutant were similar (data not shown). Moreover, like the wild-type, the catalase-positive mutant did not grow on aerobically incubated agar plates. No differences were seen between the wild-type and the catalase-positive mutant in the colony morphology on BHI or sheep blood agar incubated under microaerophilic conditions. Furthermore, compared with the wild-type strain, the RDKA84 mutant did not show any difference in biochemical features analyzed by API 32 STAPH.

3.2. Sensitivity to H_2O_2

The sensitivity to H_2O_2 was first analyzed using a death assay. In this assay, the catalase-positive mutant RDKA84 was clearly more resistant to H_2O_2 than its parental strain MVF-84. Indeed, the survival of MVF-84 diminished progressively and sharply as the concentration of H_2O_2 increased from 10 mM (Fig. 1), while the survival level of the

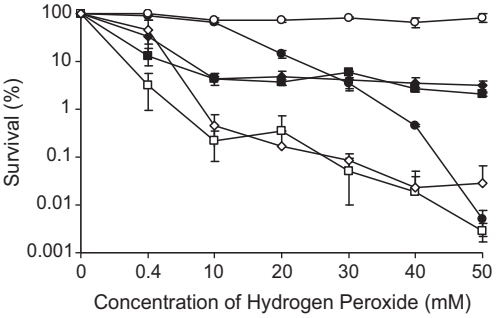

Figure 1. H_2O_2 sensitivity of MVF-84 (wild-type *S. aureus* subsp. *anaerobius*) (●), RDKA84 (catalase-positive mutant of MVF-84) (○) and four strains of *S. aureus* isolated from clinical cases of mastitis (MN-42 (■), MN-45 (□), MN-73 (♦) and DGA-1 (◇)). These strains were exposed to various concentrations of H_2O_2. Each point represents the mean (± S.D.) of three experiments. Each experiment was performed in triplicate. Error bars indicate confidence level (95%); *p* values were determined using the Student's *t*-test.

RDKA84 mutant remained high at all H_2O_2 concentrations (Fig. 1). In the disk diffusion method, however, there was no difference in the sensitivity to H_2O_2 between the RDKA84 and MVF-84 strains. At the two lowest H_2O_2 concentrations tested in the death assay (0.4 and 10 mM), both the MVF-84 wild-type and RDKA84 mutant proved to be significantly more resistant than the four *S. aureus* strains used for comparison (DGA-1, MN-42, MN-45 and MN-73) (Fig. 1). MVF-84 and RDKA84 strains were also more resistant than the four *S. aureus* strains in the disk diffusion assay, when disks were spotted with 30 µL 50 mM H_2O_2 (no zone of inhibition versus 15 mm). However, MVF-84 and RDKA84 were more sensitive than the four *S. aureus* strains when disks were soaked with 30 µL 500 mM and 1 M H_2O_2 (zone of inhibition 35 mm versus 27 mm with 500 mM, and 44 mm versus 33 mm with 1 M). Moreover, in the death assay, the RDKA84 mutant showed significantly higher survival than the *S. aureus* strains at all the H_2O_2 concentrations tested (Fig. 1).

3.3. Intracellular survival of RDKA84 mutant and its parental strain

To investigate whether restoration of catalase activity had any effect on intracellular survival of *S. aureus* subsp. *anaerobius*, we examined the ability of the RDKA84 mutant and its parental strain MVF-84 to survive inside ovine PMN, murine macrophage-like cell line J774A.1, and bovine mammary epithelial cell line MAC-T.

3.3.1. Assay with PMN from ewes and lambs

The intracellular survival rate of the catalase-positive mutant RDKA84 in PMN isolated from adult sheep was significantly higher than that of the wild-type at 60 ($p < 0.05$) and 90 ($p < 0.001$) min (Fig. 2A). In the assay with PMN isolated from lambs, unlike that seen with those from adult sheep, no differences in the intracellular survival between the two bacteria were observed at any of the three times (Fig. 2B). When the intracellular survival of *S. aureus* subsp. *anaerobius* MVF-84 in PMN isolated from adult sheep was compared with that in PMN from lambs, no differences were seen at 30 and 60 min. However, at 90 min, the rate of survival of the MVF-84 strain (13.53 ± 2.0%) in the PMN from lambs was significantly higher than that observed in the PMN from adult sheep (7.39 ± 0.84%) ($p < 0.001$). No differences were found between the intracellular survival of the catalase-positive mutant RDKA84 in PMN from adult sheep and lambs at 30 and 60 min. However, at 90 min, on the contrary to that observed with the wild-type strain, the survival rate of the mutant in the PMN from lambs was significantly lower (20.12 ± 3.06%) than that in the PMN from adult sheep (31.43 ± 1.51%) ($p < 0.001$).

3.3.2. J774A.1 and MAC-T assays

Intracellular viability of the RDKA84 mutant and its parental strain in J774A.1 and MAC-T cells was determined over a period of 24 h. The RDKA84 mutant showed a significantly lower survival rate in J774A.1 cells ($p < 0.05$)

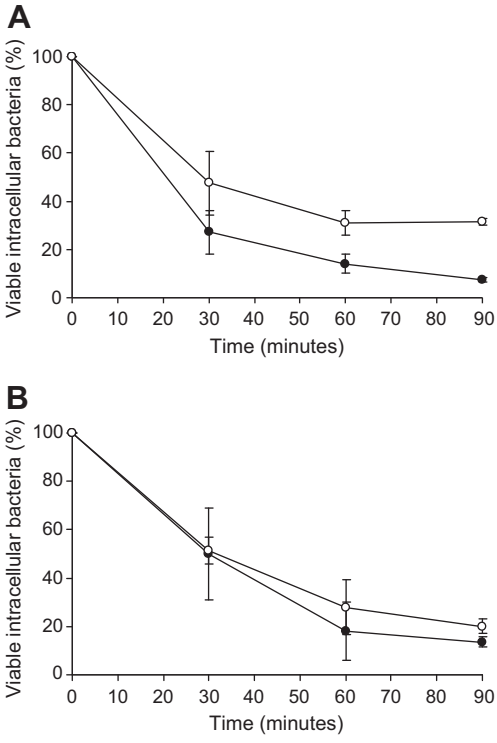

Figure 2. Intracellular killing of *S. aureus* subsp. *anaerobius* strains MVF-84 (●) and RDKA84 (○) by PMN isolated from ewes (A) and lambs (B). Survival of MVF-84 and RDKA84 was determined using dilutional plate counts. Numbers of surviving bacteria are represented as the percentage of the original number of phagocytosed bacteria. Each point represents the mean (± S.D.) of three experiments. Each experiment was performed in triplicate. Error bars indicate confidence level (95%); *p* values were determined using the Student's *t*-test.

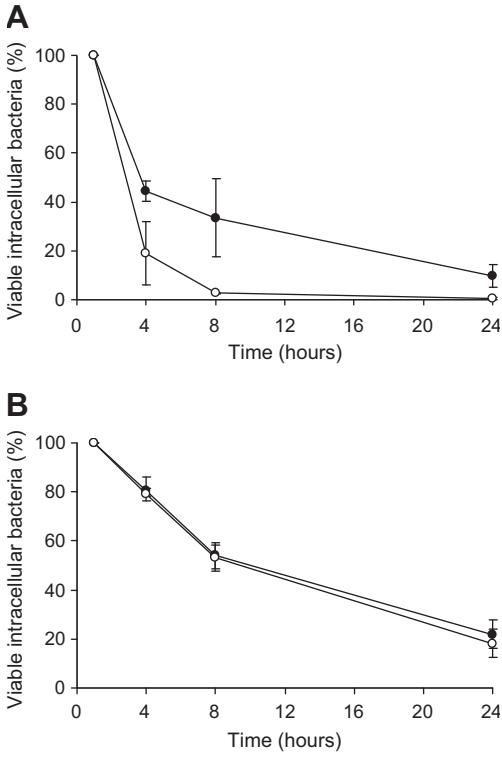

Figure 3. Intracellular survival of *S. aureus* subsp. *anaerobius* strains MVF-84 (●) and RDKA84 (○) in murine macrophage-like cell line J774A.1 (A) and bovine mammary epithelial cell line MAC-T (B). Survival of MVF-84 and RDKA84 was determined using dilutional plate counts. Data of viable intracellular bacteria at 4, 8 and 24 h are expressed as a percentage of internalized bacteria and represent the mean ± S.D. of three independent experiments running in triplicate. Error bars indicate confidence level (95%); *p* values were determined using the Student's *t*-test.

than the wild-type strain did, at all three time points (Fig. 3A). In MAC-T cells, however, no differences in the intracellular survival between the parental and mutant strains were observed at any of the time points (Fig. 3B).

3.4. LD_{50} determinations in mice

The mean LD_{50} for the RDKA84 mutant was higher ($2.9 \pm 1.22 \times 10^8$) than that of the wild-type ($5.5 \pm 1.31 \times 10^7$) but this difference was not statistically significant.

3.5. Virulence of RDKA84 mutant for lambs

In order to characterize the virulence potential of the RDKA84 mutant for lambs, two infection models were used.

3.5.1. Infection of superficial skin incisions

In accordance with previous studies with *S. aureus* subsp. *anaerobius* [23], abscess

Table II. Results of the experimental subcutaneous infection in lambs with *S. aureus* subsp. *anaerobius* MVF-84 and its catalase-positive mutant RDKA84.

Experimental group	Strain inoculated	Doses (CFU)	Side inoculated	Lamb	Abscess at the inoculation point	Abscess at necropsy in the superficial cervical lymph node
A	MVF-84	5×10^3	Right	A1	No	No
				A2	Yes	Yes
				A3	Yes	No
	RDKA84	2.4×10^4	Left	A1	No	No
				A2	No	No
				A3	No	No
B	MVF-84	1.3×10^7	Right	B1	Yes	Yes
				B2	Yes	Yes
				B3	Yes	No
	RDKA84	2.6×10^7	Left	B1	No	No
				B2	No	No
				B3	No	No

disease was induced successfully by infecting superficial skin incisions with the wild-type strain MVF-84. The three inoculated lambs developed abscesses in some of the lymph nodes that drained the infected sites, which resembled those seen in the natural disease. The development of the pathological process in the three lambs was similar; thus, all the skin incisions healed by the end of the first week and no clinical alterations were detected during this week. A small increase in rectal temperature (0.3–0.4 °C) was recorded from days 6–7 until days 9–10 post-infection. From days 7–8 post-infection, the regional (mandibular and superficial cervical) lymph nodes on the left site were palpably enlarged in comparison to the contralateral normal nodes. The size of the affected lymph nodes increased gradually and abscesses in lymph nodes, especially in mandibular nodes, became evident at 3 weeks post-infection. At necropsy, abscesses were detected in the left mandibular lymph nodes of the three lambs and in the left superficial cervical lymph nodes of two lambs. These results contrast with those obtained with the catalase-positive mutant RDKA84. The seven lambs infected with the mutant were free of any clinical alteration throughout the experiment without exception, and no abscesses were detected at necropsy in any of the regional lymph nodes.

3.5.2. Subcutaneous infections

Lambs were inoculated by the subcutaneous route simultaneously with the wild-type strain MVF-84 (in the right armpit) and the catalase-positive mutant RDKA84 (in the left armpit). The results of the subcutaneous infections are summarized in Table II. In the first experiment, low doses of bacteria (5×10^3 CFU of MVF-84 and 2.4×10^4 CFU of RDKA84) were inoculated in three 10-week-old lambs (group A). In this experimental group, no local reactivity was detected at the injection site on the left side inoculated with the mutant strain, while two of the lambs displayed local abscesses at the right injection site that was inoculated with the parental strain (Tab. II). Development of the local inflammatory reaction in these two lambs was retarded compared to that seen in lambs inoculated with high doses (see below), since the first local alteration, a small indurated nodule, was only palpably detected at 12 and 14 days after inoculation. Afterwards, the evolution of the local lesions was typical, since abscesses increased gradually in size, fistulated spontaneously in the following 15–18 days, and then regressed gradually, although they had not resolved when the animals were sacrificed 6 weeks after infection. At necropsy, besides the local lesions, only one abscess was detected

in the right superficial cervical lymph node of lamb A2 (Tab. II).

In a subsequent experiment, three lambs (group B) were infected subcutaneously with high doses of bacteria (1.3×10^7 CFU of MVF-84 and 2.6×10^7 CFU of RDKA84). All the animals in this group displayed similar clinical signs after infection. Rectal temperature increased by 0.4–0.6 °C during the first 4–5 days post-infection, and then returned to normal. Infection with the MVF-84 strain caused a strong inflammatory reaction at the right inoculation site, detectable from 24 h post-injection, which led to the development of local abscesses by the end of the first week. These local lesions evolved as described above. However, on the left side that was inoculated with a high dose of the catalase-positive mutant, no local reactivity was detected in two of the lambs and only a slight local reaction, characterized by a transient (from day 5 to 8 post-infection) nodular induration that reached a maximal diameter of 9 mm, was seen in the third lamb. At necropsy, abscesses were found in the right superficial cervical lymph nodes of two of the inoculated lambs (Tab. II).

4. DISCUSSION

The purpose of this study was to investigate the influence of catalase deficiency in *S. aureus* subsp. *anaerobius* on its physiological, biochemical and pathogenic properties. We constructed an isogenic mutant from *S. aureus* subsp. *anaerobius* MVF-84, designated RDKA84, which carried the functional *katA* gene from *S. aureus* ATCC 12600.

As expected from previous studies with *katA* mutants of *S. aureus* [6, 21], our results demonstrate that the lack of catalase activity in *S. aureus* subsp. *anaerobius* is not responsible, at least on its own, for the inability of the bacteria to grow aerobically, since restoring the catalase activity had no apparent influence on the in vitro growth characteristics of the bacteria. In addition, compared with the wild-type strain, the catalase activity of the RDKA84 mutant had no effect on the biochemical features analyzed by API 32 STAPH.

Previous studies have shown that catalase has a role in H_2O_2 stress resistance in *S. aureus* [15]. Restoration of catalase activity in the RDKA84 mutant substantially increased the resistance to H_2O_2 at concentrations of $\geq$ 20 mM, when analyzed by death assay. This agrees with the proposal by Cosgrove et al. [6] that catalase is the major component involved in resistance to externally applied H_2O_2, that is, catalase is the primary scavenger of high levels of H_2O_2. At the two lowest H_2O_2 concentrations tested in the death assay (0.4 and 10 mM), the *S. aureus* subsp. *anaerobius* wild-type MVF-84 was significantly more resistant than four *S. aureus* strains isolated from clinical cases of mastitis. This indicates that *S. aureus* subsp. *anaerobius* compensates for the lack of catalase activity by the expression of other H_2O_2 resistance factors, such as alkyl hydroperoxide reductase (AhpC), which has been demonstrated to have a compensatory role for catalase in *S. aureus* [6], and that these other factors are more active in *S. aureus* subsp. *anaerobius* than in *S. aureus*. It has been postulated that catalase is responsible for detoxifying high levels of H_2O_2, whereas AhpC is responsible for the removal of low levels of H_2O_2 [26].

Phagocytosis by PMN 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 or hydroxyl radicals [5, 13]. Therefore, enzymes implicated in oxidative stress resistance may play an important role in the survival of *S. aureus* inside phagocytic cells. Since abscess disease produced by *S. aureus* subsp. *anaerobius* mainly affects young animals up to 5–6 months of age [8], we decided to compare the intracellular survival of the wild-type strain MVF-84 and the catalase-positive mutant RDKA84 in PMN isolated from lambs and adult sheep. Catalase contributed to the survival of RDKA84 mutant in adult sheep PMN (Fig. 2A). This indicates that production of ROS by adult sheep PMN is involved in the killing of *S. aureus* subsp. *anaerobius*, and that catalase in RDKA84 probably neutralizes, at least partially, the bactericidal activity of PMN oxidative stress. However,

catalase did not show any protective effect in the assays performed with lamb PMN, since no differences in the survival levels between the RDKA84 mutant and its parental strain MVF-84 were observed at any of the time points analyzed (Fig. 2B). The difference in the intracellular survival of these two bacteria in PMN from adult and young animals suggests that the bactericidal mechanisms of the PMN from adult sheep and lambs are different, at least against these two bacteria, and that oxidative stress plays a more relevant role in the PMN from adults compared to young animals. This implies a change in the relative importance of oxidative stress in the bactericidal activity of the PMN with animal age. At 90 min after phagocytosis, the wild-type strain MVF-84 was significantly more resistant to intracellular killing by PMN isolated from lambs than adult sheep. Although other factors are no doubt involved in the pathogenesis of natural abscess disease, this result, together with the previous observation that PMN from lambs phagocytosed *S. aureus* subsp. *anaerobius* less efficiently than *S. aureus* [24], explains at least partially the predominant occurrence of this condition in young animals.

While there were no differences in the intracellular survival of the MVF-84 wild-type and RDKA84 mutant in MAC-T cells (Fig. 3B), the catalase-positive mutant was more sensitive to intracellular killing by J774A.1 cells than the wild-type was (Fig. 3A). As suggested previously for *S. aureus* [21], our results revealed differences in the bactericidal mechanism against *S. aureus* subsp. *anaerobius* between J774A.1 and MAC-T cells. The reason why catalase caused a decrease in the survival of the catalase-positive mutant RDKA84 within J774A.1 cells is not evident. In fact, this was unexpected since J774A.1 cells retain the ability to display a respiratory burst [7], and catalase may confer some protection against J774A.1 oxidative stress, similar to that observed in the assays with PMN from adult sheep.

The virulence for mice of the RDKA84 mutant, as determined by LD_{50}, although lower was not significantly different to that of the wild-type strain, which correlates with the results of the intracellular survival experiment with the J774A.1 murine cell line.

The virulence for lambs, the natural host for abscess disease, of the RDKA84 catalase-positive mutant was reduced dramatically in comparison with that of the *S. aureus* subsp. *anaerobius* wild-type strain MVF-84. Thus, in the superficial skin incision model, none of the seven infected lambs showed any clinical manifestation throughout the experiment. This method of infection, although it reliably reproduces abscess disease [23], does not allow one to estimate the degree of attenuation of the mutant, because the number of bacteria that gain entry into the infected host is unknown. To overcome this disadvantage, we decided to infect lambs subcutaneously and inoculate simultaneously with the parental strain and catalase-positive mutant. The subcutaneous route of inoculation is less effective than infecting skin incisions for reproducing abscess disease as seen in the field, however, abscesses at the inoculation sites have been induced consistently in sheep and goats infected subcutaneously [8, 11, 23, 28]. The attenuation of the RDKA84 mutant seen in the model of subcutaneous infection in lambs was even more evident. Indeed, a high dose of 2.6×10^7 of the mutant did not produce any local alteration at the injection point in the three inoculated animals, while a low dose of 5×10^3 of the wild-type caused local inflammation that led to the development of abscesses in two of the three inoculated lambs.

Although no differences were seen in the survival assays with lamb PMN for the RDKA84 mutant and its MVF-84 parental strain, the results of the experimental infections clearly indicate that, in vivo, the mutant is much more susceptible to the host defence mechanisms than the wild-type is. Elucidation of why restoration of catalase activity in *S. aureus* subsp. *anaerobius* leads to a dramatic reduction in virulence for lambs requires further investigation. It may be hypothesized that, from an evolutionary point of view, the loss of functional catalase, possibly together with other, so far unknown, changes in relation to classical *S. aureus*, allows *S. aureus* subsp.

anaerobius to adapt to a specific ecological niche.

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