Catalase deficiency in *Staphylococcus aureus* subsp. *anaerobius* is associated with natural loss-of-function mutations within the structural gene

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Degenerate oligonucleotide primers based on internal peptide sequences obtained by HPLC from purified *Staphylococcus aureus* catalase were used to locate the *S. aureus* and *S. aureus* subsp. *anaerobius* *kat* regions by PCR. Southern hybridization analysis with a probe derived from a 1.1 kb PCR-amplified fragment showed that a single copy of the putative catalase gene was present in the *S. aureus* and *S. aureus* subsp. *anaerobius* chromosome. The nucleotide sequence of *S. aureus* *katA* revealed a 1518 bp open reading frame for a protein with 505 amino acids and a predicted molecular mass of 58347 Da, whereas *S. aureus* subsp. *anaerobius* *katB* is 1368 nt long and encodes a polypeptide of 455 amino acids with a predicted molecular mass of 52584 Da. These catalases are highly homologous to typical monofunctional catalases from prokaryotes. The active-site residues, proximal and distal haem-binding ligands and NADPH-binding residues of the bovine liver catalase-type enzyme were highly conserved in *S. aureus* *KatA*. *Escherichia coli* cells carrying cloned *katA* had a catalase activity approximately 1000 times that of untransformed *E. coli*, but no detectable increase in catalase activity was observed with *E. coli* carrying cloned *katB*. Northern blotting showed the presence of a *kat*-specific transcript in *S. aureus* subsp. *anaerobius*, suggesting that the lack of catalase activity in this bacterium is due to a post-transcriptional alteration. Compared to the nucleotide sequence of *katA*, *katB* showed a single base-pair deletion and six mis-sense mutations, and these alterations were present in three other *S. aureus* subsp. *anaerobius* strains analysed. The deletion, located at 1338 bp from the initiation codon, originates a shift of the nucleotide reading frame and is responsible for the premature translation termination at 1368 bp, generating a *KatB* polypeptide 50 amino acid residues shorter than *KatA*. Moreover, four of the mis-sense mutations present in *katB* lead to non-conservative amino acid replacements, the most significant being that located at residue 317 (Pro in *KatA*→Ser in *KatB*) because the affected amino acid is involved in determining the proximal haem-binding site. Both the main alterations found in *KatB* (the deletion and the substitution in residue 317) seem to contribute to the lack of catalase activity in *S. aureus* subsp. *anaerobius*, as deduced from results obtained with chimeric catalase constructs.

Keywords: catalase, catalase gene, *Staphylococcus aureus* subsp. *anaerobius*

Abbreviation: BLC, bovine liver catalase.

The GenBank accession numbers for the sequences reported in this paper are AJ000472 (*S. aureus* ATCC 12600 *katA*) and AJ000471 (*S. aureus* subsp. *anaerobius* MVF 213 *katB*).

INTRODUCTION

Staphylococcus aureus subsp. *anaerobius* is the aetiological agent of abscess disease, a specific lymphadenitis of sheep and goats which affects mainly young animals up to 5–6 months of age (De la Fuente *et al.*, 1985). The disease is characterized by the presence of abscesses in the superficial lymph nodes (De la Fuente & Suárez, 1985). Experimentally, this bacterium is also pathogenic for ewes by intramammary inoculation, although very high numbers of bacteria (10^8 bacteria ml^{-1}) are required to induce a subclinical or clinical mastitis (De la Fuente *et al.*, 1993). *S. aureus* subsp. *anaerobius* is very closely related to *S. aureus* (De la Fuente *et al.*, 1985, 1987), a major human and animal pathogen. In sheep, *S. aureus* is mainly associated with gangrenous mastitis, dermatitis and pyaemia (Timoney *et al.*, 1988), and ewes are very sensitive to experimental infection with *S. aureus* by the intramammary route: fewer than 100 bacteria are sufficient to produce clinical mastitis (Watson, 1988). Thus, the pathogenic abilities of *S. aureus* and *S. aureus* subsp. *anaerobius* are very different, and this has been attributed to their distinctive biochemical and physiological characteristics (De la Fuente *et al.*, 1993). The main phenotypic differences between *S. aureus* and *S. aureus* subsp. *anaerobius* are the weak or negative aerobic growth and the lack of catalase activity in the latter (De la Fuente *et al.*, 1985). In *S. aureus* a correlation between catalase activity and virulence has been observed (Mandell, 1975; Kanafani & Martin, 1985), suggesting the role of catalase as a defensive mechanism against the oxygen radicals produced by macrophages. *S. aureus* subsp. *anaerobius*, however, shares with *S. aureus* the ability to produce extracellular toxins and enzymes (De la Fuente & Suárez, 1985; De la Fuente *et al.*, 1985), which traditionally have been related to staphylococcal pathogenicity.

Catalase is a haem-containing enzyme involved in dismutation of hydrogen peroxide generated during cellular metabolism to water and molecular oxygen. Most of the catalases characterized can be classified into one of two types based on their enzymological properties: monofunctional or typical catalases, and bifunctional catalase-peroxidases (Loewen, 1992). In many bacteria, both types of catalase are present and each enzyme is encoded by a different gene (e.g. in *Escherichia coli*, *katE* and *katG* code for monofunctional and bifunctional catalases, respectively). Monofunctional catalases have been described as proteins with molecular masses of approximately 220–350 kDa and are normally formed by four identical subunits, each containing one (proto-)haem group (Haas & Brehm, 1993). Their active centre and NADPH-binding site have also been described in detail (Fita & Rossmann, 1985a, b). Comparison of the deduced amino acid sequences of these enzymes indicates that typical catalases share regions that are highly conserved among microbial, plant and mammalian enzymes (Switala *et al.*, 1990; Von Ossowski *et al.*, 1993).

In *S. aureus*, a typical catalase with high levels of enzymic activity and formed by four identical subunits of approximately 60 kDa has been described (Rupprecht & Schleifer, 1979; Ruiz Santa Quiteria *et al.*, 1992). However, the gene encoding the apoenzyme of staphylococcal catalase has not yet been studied. Previous studies on catalase deficiency of *S. aureus* subsp. *anaerobius* have shown that this bacterium synthesizes haem (De la Fuente *et al.*, 1987), the prosthetic group of catalase and cytochromes, but that no protein reacting with purified immunoglobulins against *S. aureus* catalase is detected in crude and partially purified cellular extracts (Ruiz Santa Quiteria *et al.*, 1992). These findings suggest that the catalase deficiency of *S. aureus* subsp. *anaerobius* could be due to mutations in the structural gene of the apoenzyme, or to alterations in the sequences that control its expression. The aim of this work was to establish the molecular basis of the catalase deficiency of *S. aureus* subsp. *anaerobius* by means of a genetic approach. Cloning, sequencing and characterization of the catalase gene from *S. aureus* was a prerequisite to analysing the corresponding gene from *S. aureus* subsp. *anaerobius*.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains, vectors and recombinant plasmids used in this work are described in Table 1. *Escherichia coli* and *S. aureus* strains were grown overnight aerobically with agitation at 37 °C in Luria–Bertani (LB) broth (tryptone, 10 g l^{-1} ; yeast extract, 5 g l^{-1} ; NaCl, 5 g l^{-1} ; pH 7.2). *S. aureus* subsp. *anaerobius* strains were grown in microaerophilic conditions without agitation. When it was needed, ampicillin was added to a final concentration of 100 $\mu\text{g ml}^{-1}$. For solid media, 15 g agar l^{-1} was added.

Purification of *S. aureus* catalase and isolation of internal peptides. *S. aureus* catalase was purified according to the method described by Ruiz Santa Quiteria *et al.* (1992). Purified catalase was digested with trypsin and the internal peptides obtained were separated by HPLC. Amino acid sequence was determined for two of the internal peptides (CAT 30, GSGAFGTFTV; CAT 21, QDDNYFEQP); from these sequences the degenerate oligonucleotides cat30 and cat21 (Table 2) were designed and were used to locate the *S. aureus* and *S. aureus* subsp. *anaerobius* catalase genes.

Nucleic acid extraction and molecular techniques. Routine DNA manipulations were carried out by standard procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1991). Total DNA from *S. aureus* and *S. aureus* subsp. *anaerobius* strains was extracted by the cetyltrimethylammonium bromide method after pretreatment of bacteria with lysostaphin (1 mg ml^{-1}) for 1 h at 37 °C in Tris/EDTA/sucrose (Ausubel *et al.*, 1991). Total RNA was prepared by the method of Kornblum *et al.* (1988). All restriction endonuclease digestions and ligations were performed as recommended by the suppliers. Plasmids were extracted from *E. coli* with the plasmid purification kit from Qiagen. When required, DNA restriction fragments or PCR products were separated by agarose gel electrophoresis and purified with the Qiaquick gel extraction kit (Qiagen).

Southern blot analysis. DNA was digested with different restriction enzymes (*Hind*III, *Hind*III/*Sac*I, *Hind*III/*Sal*I) in accordance with the manufacturer's suggestions (New England Biolabs), fractionated on 1% agarose gels and

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Source or reference
<i>S. aureus</i>		
ATCC 12600	Type strain	
MN 42	Clinical isolate from gangrenous mastitis	
MN 73	Clinical isolate from gangrenous mastitis	
<i>S. aureus</i> subsp. <i>anaerobius</i>		
MVF 10	Strain isolated from a lamb affected by abscess disease in 1981	
MVF 89	Strain isolated from a lamb affected by abscess disease in 1984	
MVF 213	Strain isolated from a lamb affected by abscess disease in 1988	
MVF 229	Strain isolated from a lamb affected by abscess disease in 1992	
<i>Escherichia coli</i>		
CC118	Cloning host strain	Dr de Lorenzo*
MOSBlue	Cloning host strain	Amersham
Plasmids		
pUC18	Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pMOSBlue	T-vector for cloning of PCR products	Amersham
pKat1	pMOSBlue, 1·1 kb internal PCR product from <i>katA</i> gene	This work
pKatA	pUC18, 1·9–2·2 kb <i>SacI</i> – <i>HindIII</i> fragment with <i>katA</i> gene	This work
pKatA1	pMOSBlue, 1582 bp PCR product with <i>katA</i> gene	This work
pKat2	pMOSBlue, 1·1 kb internal PCR product from <i>katB</i> gene	This work
pKatB	pUC18, 5·0–6·0 kb <i>HindIII</i> fragment with <i>katB</i> gene	This work
pKatB1	pMOSBlue, 1582 bp PCR product with <i>katB</i> gene	This work
pKatB2	pMOSBlue, 900 bp internal PCR product from <i>katB</i> gene	This work
pKatB3	pMOSBlue, 1245 bp internal PCR product from <i>katB</i> gene	This work
pKatAB	pMOSBlue, <i>katA</i> 5' end (1033 bp) and <i>katB</i> 3' end (570 bp)	This work
pKatBA	pMOSBlue, <i>katB</i> 5' end (1033 bp) and <i>katA</i> 3' end (570 bp)	This work

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transferred to nylon membranes (N+, Amersham) by the capillary blot technique. DNA probe was radiolabelled with [³²P]dCTP by using the Multiprimer DNA labelling system (Amersham Life Science). Prehybridization reactions were carried out at 56 °C for 1 h in a solution containing 7% (w/v) SDS, 1 mM EDTA and 0·5 M sodium phosphate (pH 7·2). Hybridization was performed for 24 h at 56 °C. The nylon membranes were washed at 45 °C in 5 × SSC (1 × SSC is 0·15 M NaCl plus 0·015 M sodium citrate)/0·1% SDS for 30 min and exposed to X-ray films at –70 °C for 1–24 h.

Northern blot analysis. Total RNA was separated by electrophoresis on 1% agarose gels containing 2·2 M formaldehyde. Nucleic acids were vacuum-transferred to nylon membranes (N+, Amersham) and cross-linked by UV light. RNA probe was synthesized by *in vitro* T₃ polymerase transcription of pKat1 with digoxigenin-labelled UTP (Boehringer Mannheim) as substrate. Hybridizations were performed at 68 °C in 5 × SSC plus formamide 50% (v/v). Detection was performed with the DIG kit (Boehringer Mannheim) according to the manufacturer's instructions and signals were detected by chemiluminescence. Staphylococcal 16S and 23S rRNAs were used as size markers.

PCR amplification. Oligonucleotide primers (Table 2) were constructed by Isogen Bioscience. PCR was carried out in a 50 µl volume containing 50 ng genomic DNA with reagents and protocols supplied by the manufacturer (Perkin Elmer). Thermocycler reaction conditions were 1 min at 94 °C, 1 min at 52 °C and 1 or 1·5 min at 72 °C for 30 cycles. All PCR amplifications included preliminary denaturation at 94 °C for

10 min and a final incubation at 72 °C for 10 min. Amplified PCR products were analysed by electrophoresis on 1% agarose gels.

Cloning of catalase. Digested total DNA from *S. aureus* ATCC 12600 or *S. aureus* subsp. *anaerobius* MVF 213 was recovered from agarose gels with the Qiaquick gel extraction kit (Qiagen) and ligated with the T4 ligase (Amersham Life Science) into pUC18. Calcium chloride competent *E. coli* CC118 cells were transformed as described by Sambrook *et al.* (1989). Transformants were selected on LB agar containing ampicillin (100 µg ml⁻¹). Colonies were screened by colony hybridization and by their oxygen formation when they were overlaid with 3% H₂O₂. *kat* gene fragments amplified by PCR from *S. aureus* or *S. aureus* subsp. *anaerobius* genomic DNA were cloned in *E. coli* by using the pMOSBlue T-vector kit (Amersham).

Construction of *kat* hybrid genes. Recombinant hybrid genes were constructed by the method of gene SOEing or splicing by overlap extension (Higuchi, 1989). Oligonucleotides cat3 and cat4 (Table 2) are complementary and represent nucleotides from 992 to 1011 of the *katA* and *katB* genes. In separate reactions (30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min), oligonucleotides cat1 and cat3 and oligonucleotides cat2 and cat4 were used to amplify, from the *katA* and *katB* genes, PCR products representing sequences from each end of these genes. Samples of the products of each reaction were combined and subjected to a subsequent reaction with oligonucleotides cat1 and cat2 to amplify a full-length recombinant product

Table 2. Oligonucleotide DNA primers utilized in this study

Oligonucleotide	Sequence (5'–3')	Description
cat30	GGWTCWGGWGCWTTYGGWACWTTYACWGTW	Degenerate oligonucleotide used for detecting and cloning <i>katA</i> and <i>katB</i>
cat21	WGGYTGYTCGAARTARTTRTCRTCRTCYTG	Degenerate oligonucleotide used for detecting and cloning <i>katA</i> and <i>katB</i>
Cat1	TATAAATTGTGGAGGGATGAT	Nucleotides –22 to –1 relative to the 5' end of <i>katA</i> used for amplifying the full-length <i>katA</i> and <i>katB</i> genes and for constructing <i>kat</i> hybrid genes
Cat2	TCATAAACTGCTCAACTACGC	Nucleotides 1562 to 1541 relative to the 3' end of <i>katA</i> used for amplifying the full-length <i>katA</i> and <i>katB</i> genes and for constructing <i>kat</i> hybrid genes
cat3	GAATAAACGCCCTTGCAGC	Nucleotides 992 to 1011 of <i>katA</i> used for constructing <i>kat</i> hybrid genes
cat4	GCTGCAAGGGCGTTTATTC	Complementary to cat3
cat5	TTGTTGCGTTCTAAAATGGAG	Nucleotides 641 to 662 used for amplifying an internal fragment from <i>katB</i>
cat6	TGGGTCAGCTTTGTAACA	Nucleotides 1400 to 1418 used for amplifying an internal fragment from <i>katB</i>

representing the 5' end of *katB* with the 3' end of *katA* (*katBA*) and the 5' end of *katA* with the 3' end of *katB* (*katAB*). Amplified products were cloned into *E. coli* by using the pMOSBlue T-vector kit (Amersham) (Fig. 1). Recombinant hybrids thus generated were confirmed by automatic nucleotide sequencing.

DNA sequencing. DNA sequencing was carried out on double-stranded plasmid DNA templates by the dideoxy chain-termination method of Sanger *et al.* (1977) with a T₇ sequenase 7-deaza-dGTP DNA sequencing kit (Amersham Life Science). Sequencing was performed with the universal primers or synthetic oligonucleotide primers designed from newly obtained DNA sequences.

Data research and computer analysis. Genetic data were analysed using the University of Wisconsin Genetics Computer Group program library (GCG) (Devereux *et al.*, 1984). CLUSTALX (1.5b) was used for comparison of analysed sequences. Phylogenetic relationships were inferred from a multiple amino acid sequence alignment and a consensus tree was constructed with the program TreeView 1.5.

Preparation of cell lysates. Bacterial lysates were prepared by resuspending cell pellets in 3 ml extraction buffer A (50 mM Tris/HCl, pH 8.0; 0.5 mM EDTA; 50 µg sodium azide ml⁻¹; 0.25 mg lysozyme ml⁻¹). *S. aureus* and *S. aureus* subsp. *anaerobius* cells were lysed with 0.2 mg lysostaphin ml⁻¹. The suspensions were incubated at 37 °C for 15 min. Following incubation, 0.3 ml extraction buffer B was added (1.5 M NaCl, 0.1 M CaCl₂, 0.1 M MgCl₂, 0.02 mg DNase I ml⁻¹; 0.05 mg ovomucoid protease inhibitor ml⁻¹). After incubation at room temperature for 5 min, the suspensions were centrifuged at 10000 g for 1 h. The supernatant was retained and stored in an ice bath or frozen at –20 °C until needed. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis and immunoblotting. Protein extracts were separated on 10% (w/v) polyacrylamide

gels under native and denaturing conditions. Denaturing gels were stained with Coomassie blue and nondenaturing gels were stained for enzyme activity as described below. The purified anti-catalase immunoglobulins for Western blot analysis were obtained as described by Ruiz Santa Quiteria *et al.* (1992).

Determination of enzymic activity. Quantitative determination of catalase activity present in extracts was performed by the colorimetric assay of Sinha (1972). Catalase activity present in extracts was also determined by staining non-denaturing polyacrylamide gels according to the method of Clare *et al.* (1984) using 5 mM H₂O₂.

RESULTS

Localization and cloning of *S. aureus* and *S. aureus* subsp. *anaerobius* catalase genes

Purified *S. aureus* catalase was used to design the degenerate oligonucleotides cat21 and cat30 (Table 2) from internal peptides obtained by HPLC. Using these oligonucleotides, a 1100 bp PCR product was amplified from total DNA of *S. aureus* ATCC 12600 and *S. aureus* subsp. *anaerobius* MVF 213. To determine whether the amplified fragments were derived from a catalase gene, the 1100 bp PCR products from the two bacteria were cloned into the PCR vector pMOSBlue, generating the plasmids pKat1 and pKat2, respectively (Fig. 2), and sequenced on both strands. The DNA sequences of both fragments had a high degree of homology with other catalases previously described. Thus, the 1100 bp PCR product was used as a probe to locate the *S. aureus* and *S. aureus* subsp. *anaerobius* catalase genes. To accomplish this, total DNA of both bacteria was digested with different enzymes (*Hind*III, *Hind*III/*Sac*I, *Hind*III/*Sal*I) and a unique band was detected by hybridization

Fig. 1. Strategy for creating *kat* hybrid genes. Oligonucleotide primers are listed in Table 2. (A) Locations of primers (arrows) in relation to the *katA* and *katB* genes. Primers *cat3* and *cat4* are complementary. (B) Separate PCRs amplified two fragments from each gene corresponding to the 5' and 3' ends. These fragments have overlapping sequences. (C) The products depicted in (B) were combined and amplified with *cat1* and *cat2*, the outer pair of primers. This step resulted in the constructions shown: *katAB*, which contains the *katA* 5' end and the *katB* 3' end, and *katBA*, which contains the *katB* 5' end and the *katA* 3' end. (D) The constructions shown in (C) were cloned into vector pMOSBlue.

(Fig. 3), which demonstrated that a single copy of the catalase gene was present in both *S. aureus* and *S. aureus* subsp. *anaerobius* chromosomes. A 1900–2200 bp *SacI*–*HindIII* fragment from *S. aureus* total DNA (Fig. 3) was purified and cloned into pUC18, generating the plasmid pKatA (Fig. 2). In the case of *S. aureus* subsp. *anaerobius*, a 5000–6000 bp *HindIII* fragment from total DNA (Fig. 3) was purified and cloned into pUC18, generating the plasmid pKatB (Fig. 2). To analyse the *kat* region of *S. aureus* subsp. *anaerobius* further, different internal fragments from pKatB were amplified by PCR using oligonucleotide DNA primers deduced from the *katA* sequence and cloned into pMOSBlue. The PCR amplification strategy is summarized in Fig. 2.

When *E. coli* clones were screened for a change in oxygen production upon addition of 3% H_2O_2 , clones transformed with *katA* showed an increase in oxygen formation, but those transformed with *katB* did not show any appreciable increase in oxygen production in comparison with the untransformed control.

Analysis of the catalase nucleotide sequence

The nucleotide sequence of an internal 1756 bp fragment carried by pKatA was determined on both strands. A 1518 bp single ORF initiating at ATG (base-pair 166) and terminating at TAA (base-pair 1683) was identified within this sequence. The ORF encodes a protein of 505 amino acids with a calculated molecular mass of 58347 Da. A –10 region identical to the *E. coli* consensus sequence was detected upstream of the start

codon [TATAAT in *S. aureus* ATCC 12600 and *E. coli* (Horwitz & Loeb, 1990)]. A putative –35 region similar to the corresponding region in *E. coli* [TTGTAA in *S. aureus* ATCC 12600 and TTGAAG in *E. coli* (Horwitz & Loeb, 1990)] was also observed. Finally, a potential ribosome-binding site sequence was found at positions –12 to –8 upstream of the ATG initiation codon (GGAGG). The codon usage was similar to that detected in previously described *S. aureus* genes (Wada *et al.*, 1992). The 65.5 mol% A + T content of *katA* is consistent with the high percentage A + T of base-pairs found in other *S. aureus* genes.

In the case of *S. aureus* subsp. *anaerobius*, sequencing and analysis of a 1747 bp fragment revealed a single ORF of 1368 bp (nucleotides 166–1533). This ORF encodes a protein of 455 amino acids with a predicted molecular mass of 52584 Da. Potential –10 and –35 promoter sequences and the putative ribosome-binding site detected in this sequence were identical to those described for *S. aureus*.

Comparison of amino acid sequences

The predicted amino acid sequences obtained for KatA and KatB were compared to other catalase proteins in the GenBank-EMBL/SWISS-PROT databases. The search revealed significant similarities between the analysed catalases and large regions of numerous eukaryotic and prokaryotic catalases. Considering both identical and conservative replacements of amino acids, KatA and KatB showed more than 50% identity with *Bacteroides fragilis* catalase KatB, *Streptomyces coelicolor* catalase CatA, *Haemophilus influenzae* catalase HktE, *Bordetella pertussis* catalase KatA, *Pseudomonas fluorescens* catalase and *Neisseria gonorrhoeae* catalase (Fig. 4). The degree of similarity was greater in the core of the deduced amino acid sequences than in the N-terminal and C-terminal regions.

Comparison of the deduced amino acid sequence of KatA with the sequence of bovine liver catalase (BLC) revealed that the three residues involved in the active site of the BLC, His-74, Ser-113 and Asn-147 (Fita & Rossmann, 1985b; Murthy *et al.*, 1981), were conserved in *S. aureus* KatA at the His-56, Ser-95 and Asn-129 positions. Moreover, the proximal haem site, which comprises Pro-335, Arg-353 and Tyr-357 as ligands in BLC, was localized as Pro-317, Arg-335 and Tyr-339 in KatA. Of the five distal haem-site ligands in BLC, Val-73, Arg-111, Thr-114, Phe-152 and Phe-160, the Val-73 residue was replaced by Met-55 while the others were conserved as Arg-93, Thr-95, Phe-134 and Phe-142. In addition, the NADPH-binding site residues of BLC (Fita & Rossmann, 1985a), Arg-202, His-234 and His-304, seemed to be conserved in KatA as Arg-184, His-216 and His-286, but Asp-212, Tyr-214 and Lys-236 have been replaced by His-194, Phe-196 and Arg-218, respectively.

In the case of the *S. aureus* subsp. *anaerobius* catalase, all the residues involved in the active site, haem-binding site and NADPH-binding site are identical to those

Fig. 2. Restriction map of recombinant plasmids containing the *S. aureus* and *S. aureus* subsp. *anaerobius* *kat* genes and subclones derived from these genes. The solid black lines represent cloned *kat* DNA. The dotted lines represent the flanking regions of the *kat* genes.

described for the *S. aureus* catalase KatA, except the residue Pro-317, involved in the proximal haem-binding site, which is replaced by a Ser residue.

Transcription of *katA* and *katB*

Northern blot assays with a *katA*-specific RNA probe revealed the presence of a transcript of approximately 1.8 kb in samples from early-exponential-phase cultures of *S. aureus* strains ATCC 12600, MN 42 and MN 73 (Fig. 5). Total RNA isolated from exponentially growing cells of *S. aureus* subsp. *anaerobius* hybridized with the same *katA*-specific RNA probe, showing the presence of a *kat*-related transcript larger than that observed in *S. aureus* (approx. 3.7 kb) (Fig. 5). In addition, other smaller positive hybridization signals were detected; these bands are probably degraded *katB*-transcript products. When samples were collected from stationary-phase cultures of *S. aureus* subsp. *anaerobius*, no hybridization was detected in the tested strains.

Expression of the recombinant *katA* and *katB* genes in *E. coli*

Protein extracts from *E. coli* harbouring pKatA showed a level of catalase activity 1000 times higher than the combined activity of *E. coli* HPI and HPII catalases under the same culture conditions [227×10^3 and 200 U (mg protein) $^{-1}$, respectively]. However, transformation of *E. coli* with pKatB did not produce any appreciable increase in catalase activity in relation to the parent *E. coli* strain. In crude extracts from *E. coli* CC118, two catalase bands were observed corresponding to the previously described *E. coli* catalases HPI and HPII, whereas crude extracts from *S. aureus* showed a unique band of catalase activity (Fig. 6). Crude extracts of *S. aureus* subsp. *anaerobius* did not show any band of catalase activity. Crude extracts of *E. coli* harbouring

pKatA showed one band of catalase activity with similar electrophoretic properties to that of the *S. aureus* purified catalase (Fig. 6). However, crude extracts from *E. coli* harbouring pKatB showed the same pattern of catalase activity as that observed in the parent *E. coli* strain.

Analysis of proteins in recombinant *E. coli*

An additional protein band with a molecular mass of approximately 60 kDa was detected in extracts of *E. coli* CC118(pKatA) in comparison with those of the *E. coli* CC118 parent strain (Fig. 7a). This additional protein showed an electrophoretic mobility similar to that of purified *S. aureus* catalase. The electrophoretic pattern of *E. coli* CC118(pKatB) was identical to that of *E. coli* CC118.

As shown in Fig. 7(b), purified immunoglobulins reacted with a single band of the same molecular mass as that of the electrophoretically pure *S. aureus* catalase in crude extracts of *S. aureus* ATCC 12600 and *E. coli* CC118(pKatA). However, no band with this electrophoretic mobility was detected in crude extracts either from untransformed *E. coli* CC118 or from *E. coli* CC118(pKatB). In the extract of *E. coli* CC118(pKatB), a reacting band corresponding to a molecular mass of approximately 20 kDa was detected. This protein was isolated and its N terminus was sequenced. The sequence obtained indicated that this protein corresponded to lysozyme C, which had reacted non-specifically with purified anti-catalase immunoglobulins.

Comparison of the *katA* and *katB* nucleotide sequences

The nucleotide sequence of *S. aureus* subsp. *anaerobius* *katB* showed 98.7% identity to that of *S. aureus* *katA*. Comparative analysis of the two sequences revealed the

Fig. 3. (a) Agarose gel electrophoresis of digested total DNA from *S. aureus* subsp. *anaerobius* (lanes A, B and C) and *S. aureus* (lanes D, E and F). Total DNA of both bacteria was cut with *Hind*III (lanes A and D), *Hind*III and *Sac*I (lanes B and E), or *Hind*III and *Sac*I (lanes C and F). (b) Southern hybridization analysis of the digested total DNA from *S. aureus* subsp. *anaerobius* (lanes A, B and C) and *S. aureus* (lanes D, E and F). The restriction fragments were transferred to nylon membrane and hybridized with the radiolabelled 1100 bp PCR product containing an internal fragment of the *katA* gene.

presence of several mutations in the *S. aureus* subsp. *anaerobius* *katB* gene compared with *S. aureus* *katA*. Firstly, a single base-pair deletion located at 1338 bp from the initiation codon was found. This deletion would originate a shift of the nucleotide reading frame and, as a consequence, a termination codon (TAA) is present at position 1368. In addition, 14 single base substitutions were found in *katB*, eight of which were silent mutations while six were mis-sense. Four of the mis-sense mutations detected in *katB* lead to a non-conservative replacement of the corresponding amino

acid (Arg108Ser, Asp238Gly, Pro317Ser and Asp440Gly) (Fig. 8), while those located at codons 215 and 313 produce a conservative replacement in the KatB amino acid sequence. The most relevant substitution was that located at the first nucleotide of codon 317 (CCA in *katA* → TCA in *katB*). As result of this mutation, the non-polar amino acid Pro-317, which is involved in the proximal haem-binding site, was replaced by the polar amino acid Ser-317 (Fig. 8).

To determine whether *katB* mutations were specific for strain MVF 213 or were a more general feature of *S. aureus* subsp. *anaerobius*, the *katB* genes of three additional strains isolated from different outbreaks (MVF 10, MVF 89 and MVF 229; Table 1) were amplified by PCR using oligonucleotides cat1 and cat2 (Table 2), cloned into *E. coli*, and sequenced. All of these strains showed the same alterations as described above for strain MVF 213.

Construction and characterization of *kat* hybrid genes

The *katAB* construction comprises the 5' end of *katA* (of approximately 1033 bp) and the 3' end of *katB* (of approximately 570 bp). In this construction all of the mutations described above have been repaired except the non-conservative substitution at position 1320 (GAC in *katA* → GGC in *katB*) and the deletion of the nucleotide T-1338. The *katBA* construction includes the 5' end of *katB* (of approximately 1033 bp) and the 3' end of *katA* (of approximately 570 bp). In *katBA* the deletion of the T-1338 nucleotide as well as the non-conservative substitution located at the second nucleotide of the codon 440 (GAC in *katA* → GGC in *katB*) have been repaired. Recombinant hybrids thus generated were confirmed by automatic nucleotide sequencing. Constructions were cloned into pMOSBlue in *E. coli*, yielding plasmids pKatAB and pKatBA, respectively.

As shown in Fig. 6, *E. coli* MOSBlue(pKatAB) and *E. coli* MOSBlue(pKatBA) showed the same pattern of catalase activity as that of the wild-type *E. coli* strain. In addition, levels of catalase activity showed by *E. coli*

Fig. 4. Phylogenetic relationship of *S. aureus* KatA and *S. aureus* subsp. *anaerobius* KatB and representative typical catalases. This tree was constructed from a multiple alignment using CLUSTALX (1.5b) and the tree topology was obtained by use of TreeView 1.5.

Fig. 5. Northern blot analysis. Whole-cell RNA from different strains of *S. aureus* and *S. aureus* subsp. *anaerobius* was hybridized with a digoxigenin-labelled RNA probe specific for *katA*. Lanes A, B, C, D and F correspond to RNAs from early-exponential-phase cultures and lanes E and G to RNAs from stationary-phase cultures. Lanes: A, *S. aureus* ATCC 12600; B, *S. aureus* MN 42; C, *S. aureus* MN 73; D and E, *S. aureus* subsp. *anaerobius* MVF 10; F and G, *S. aureus* subsp. *anaerobius* MVF 213. Positions of migration of 16S rRNA (1541 nt) and 23S rRNA (2904 nt) are indicated. The arrow indicates the *katB*-related transcript.

Fig. 6. Detection of catalase activity in crude extracts by the staining method of Clare *et al.* (1984). Total proteins from extracts of the following bacteria were separated on nondenaturing 10% polyacrylamide gels: *E. coli* CC118 (lane A), *S. aureus* ATCC 12600 (lane B), *S. aureus* subsp. *anaerobius* MVF 213 (lane C), *E. coli* CC118(pKatA) (lane D), *E. coli* CC118(pKatB) (lane E), *E. coli* MOSBlue(pKatAB) (lane F) and *E. coli* MOSBlue(pKatBA) (lane G). Crude extracts corresponding to lanes B and D were diluted 1000-fold due to their high catalase activity. The arrows indicate the position of *E. coli* HPI and HPII catalases and *S. aureus* KatA catalase.

harbouring *katAB* (185 U mg⁻¹) or *katBA* (190 U mg⁻¹) constructions were similar to that of the *E. coli* control (200 U mg⁻¹). The protein band pattern of cell lysates from the *E. coli* parent strain in SDS-polyacrylamide gels was similar to that of the *E. coli* strains harbouring

pKatAB or pKatBA (Fig. 7a). When cell extracts from *E. coli* harbouring pKatAB or pKatBA were analysed by Western blotting, no protein reacting with the purified immunoglobulins against *S. aureus* catalase was detected (Fig. 7b).

DISCUSSION

Previous studies have demonstrated that *S. aureus* subsp. *anaerobius* synthesizes haem (De la Fuente *et al.*, 1987), the prosthetic group of catalase and cytochromes, and that polyclonal antibodies against *S. aureus* catalase do not recognize any protein in cellular extracts from *S. aureus* subsp. *anaerobius* (Ruiz Santa Quiteria *et al.*, 1992). Based on these results, we planned a genetic approach to clarify the catalase deficiency of this bacterium. As the gene encoding the apoenzyme of catalase had not yet been studied in any of the staphylococcal species, the isolation and sequencing of the *S. aureus* catalase gene was a necessary first step to determine the molecular basis of the deficiency of catalase activity in *S. aureus* subsp. *anaerobius*.

Expression of *katA* in *E. coli* and nucleotide sequence analysis indicated that the cloned DNA encodes the catalase of *S. aureus*. Multiple amino acid sequence alignment showed a strong relationship between KatA and the prokaryotic catalases from *Bacteroides fragilis* (65% identity), *Streptomyces coelicolor* (64% identity), *Proteus mirabilis* (62% identity) and *Haemophilus influenzae* (61% identity). However, the KatA sequence showed less than 50% amino acid identity with eukaryotic catalases; this is in agreement with data reported by Von Ossowski *et al.* (1993) and Klotz *et al.* (1997), who established that animal, plant and fungal catalases in general form a homogeneous group in terms of evolution whereas bacterial catalases appear to have a different phylogenetic origin.

The changes detected in KatA in comparison with BLC in both the proximal haem site (Val-73 in BLC → Met-55 in KatA) and the NADPH-binding site (Asp-212 → His-194, Lys-236 → Arg-218 and Tyr-214 → Phe-196) have also been described in the *Proteus mirabilis* catalase (Gouet *et al.*, 1995) and, according to biochemical results and molecular modelling, they do not appear to influence either the specific activity of catalase or the interaction of the enzyme with the NADPH molecule (Gouet *et al.*, 1995).

Northern blotting showed the presence of a *kat*-specific transcript in samples from exponential-phase cultures of all the *S. aureus* subsp. *anaerobius* strains tested, although *katB* is probably transcribed with other gene(s) since the observed transcript in *S. aureus* subsp. *anaerobius* is larger than that of *S. aureus* controls. The fact that all of the *S. aureus* subsp. *anaerobius* strains showed a *kat* mRNA transcript but no catalase activity could be due to a post-transcriptional defect. Western blot assays favour this hypothesis, as none of these strains showed a catalase-specific band with antisera against the purified *S. aureus* catalase. Similar results

Fig. 7. (a) Denaturing SDS-PAGE gel stained for proteins with Coomassie blue. (b) Detection of catalase in SDS-PAGE-separated crude extracts by immunoblotting. Crude extracts were incubated with purified immunoglobulins against *S. aureus* catalase. Lanes: A, electrophoretically pure catalase from *S. aureus* ATCC 12600; B, *S. aureus* ATCC 12600; C, *S. aureus* subsp. *anaerobius* MVF 213; D, *E. coli* CC118; E, *E. coli* CC118(pKatA); F, *E. coli* CC118(pKatB); G, *E. coli* MOSBlue(pKatAB); H, *E. coli* MOSBlue(pKatBA).

	10	108
KatA	MSQQDKKLTG.....	R.....
KatB	MSQQDKKLTG.....	S.....
	215 238	313 317
KatA	F.....D.....	A.....P.....
KatB	L.....G.....	V.....S.....
	440 446	455
KatA	D.....FTNTANAMEGVTD	DDVKRRHIRH CYKADPEYG
KatB	G.....LQIQMQWKA	
		505
KatA	KGVAKALGIDINSIDLETENDETYENFEK	

Fig. 8. Differences in the predicted amino acid sequence between KatA and KatB. Numbers indicate the relative amino acid positions.

have been reported in several human-pathogenic isolates of coagulase-negative *S. aureus*, which showed a *coa* RNA transcript but no detectable coagulase activity (Vandenesch *et al.*, 1994). Whether the lack of a *kat*-

related transcript in samples from stationary-phase cultures of *S. aureus* subsp. *anaerobius* is related to regulation of catalase gene expression was not investigated; this requires further analysis.

Comparison of the *katA* and *katB* nucleotide sequences revealed significant identity (96.5% similarity) between the two catalase genes, but there are some mutations in the *S. aureus* subsp. *anaerobius* catalase gene. Firstly, a single base-pair deletion at position 1338 originates a shift of the nucleotide reading frame and would be responsible for the presence of an early termination codon at 1368 bp from the initiation codon. As a consequence of this deletion, the last 50 amino acids of the C-terminal region of KatB would be absent (Fig. 8). As shown by Rocha & Smith (1995), The C-terminal region of catalase seems to be essential for enzymic activity. These authors, studying the *Bacteroides fragilis* catalase gene, found that catalase activity was lost when the last 21 codons of the gene were deleted. Therefore, the loss of the last 50 amino acids from the C terminus in KatB could account for the catalase deficiency of *S. aureus* subsp. *anaerobius*. Based upon the three-dimensional structure of the *Proteus mirabilis* catalase (Gouet *et al.*, 1995) and a high degree of similarity between the amino acid sequences of *P. mirabilis* and *S. aureus* subsp. *anaerobius* catalases, a hypothesis about the role of this deletion in the lack of catalase activity in *S. aureus* subsp. *anaerobius* could be established. Gouet *et al.* (1995) determined the structure of the *P. mirabilis* catalase (484 amino acids) and established that each subunit of this enzyme is composed of four domains. The fourth domain is a bundle of four α -helices (α 10 to α 13) comprising residues 417 to 484. Residues in this region are present at the protein surface, exhibit a high temperature factor and could play a role in the protein stability (Fita & Rossmann, 1985b; Gouet *et al.*, 1995). In addition, these α -helices appear to form a pocket lined by several hydrophobic residues where NADPH is bound (Melik-Adamyany *et al.*, 1986), and they contribute to forming the hydrophobic channel leading to the haem group (Murthy *et al.*, 1981). In KatB, as a consequence of the deletion described above, the α 12 and α 13 helices and part of the α 11 helix are not present. The lack of these α -helices would produce an alteration of the NADPH peripheral site and could change the entrance of substrate to the active centre. Moreover, the loss of the α 11, α 12 and α 13 helices in the C-terminal region would sensitize the polypeptide chain to the action of proteases and other degrading enzymes due to the presence of structural changes in the protein surface.

In addition, 14 base substitutions were found in *katB*, six of which were mis-sense mutations. Four of the mis-sense substitutions in *katB* would lead to a non-conservative amino acid replacement (Fig. 8), the most significant being that located at position 951 (CCA in *katA* $\rightarrow$ TCA in *katB*) because the affected amino acid is involved in the proximal haem-binding site. As a result of this mutation, the non-polar amino acid proline is replaced by the polar amino acid serine. Fita & Rossmann (1985b) described the active centre of BLC

and proposed that the residue Pro-335 (Pro-317 in KatA) provides a non-polar face which hinders the movement of Tyr-357 (Tyr-339 in KatA). The phenolic hydroxyl group of Tyr-357 interacts with the haem iron and forms hydrogen bonds with Arg-353 (Arg-335 in KatA). Therefore, Pro-335 is involved in the binding of catalase to the haem group and influences the tertiary structure of catalase. The polar serine could thus modify the position of Tyr-339. The Pro → Ser replacement could influence binding of catalase to the haem group and could modify the tertiary structure of the protein, thus altering the formation of hydrogen bonds among other amino acids in the polypeptide chain.

With regard to the other non-conservative substitutions described for *katB*, it seems that these mutations are not involved in the active site or in the binding of catalase to the haem group and NADPH cofactor. Nevertheless, it cannot be ruled out that some of these amino acid replacements cause a conformational change of the protein such that biological activity of catalase is decreased or nullified.

All of the mutations described in the *katB* gene isolated from *S. aureus* subsp. *anaerobius* MVF 213 were also detected in three other strains of this bacterium isolated from outbreaks of abscess disease which occurred in different Spanish regions from 1981 to 1992. These results suggest that the mutations may be widely present in *S. aureus* subsp. *anaerobius* strains, and that the strains tested could have a common origin.

Two chimeras (*katAB* and *katBA*) were constructed for establishing whether the two main mutations described above are involved in the lack of catalase activity in *S. aureus* subsp. *anaerobius*. Both catalase activity and Western blot analysis of the recombinant hybrids were similar to those found in an *E. coli* wild-type strain. Consequently, each of the two main alterations found in *katB* (the deletion and the substitution in the 317 codon) seems to contribute to the lack of catalase activity in *S. aureus* subsp. *anaerobius*. Nevertheless, to clarify the individual role of each of the mutations detected in *KatB* in enzymic activity and in the tertiary structure of the protein, it would be necessary to replace the corresponding residue in *S. aureus* catalase by site-directed mutagenesis and to analyse the biological activity of each variant enzyme.

In conclusion, a 1368 bp gene isolated from *S. aureus* subsp. *anaerobius* with high homology to the *S. aureus* *katA* gene, described in this report, has been identified and designated as *katB*. Northern blotting has showed that *katB* is transcribed to mRNA and, therefore, the lack of catalase activity in this bacterium is probably due to a post-transcriptional defect. According to data described in this report and taking into account that transcription and translation occur almost simultaneously in bacteria, a protein similar to but smaller than *S. aureus* catalase is probably synthesized. It is possible that this protein is degraded after synthesis due to the alterations present in the structure and/or in the active site. That could provide an explanation for the

lack of reaction of anti-catalase immunoglobulins with crude extracts from *S. aureus* subsp. *anaerobius* or *E. coli* harbouring the *katB* gene or the *kat* hybrid genes.

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