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Detection and expression of SapS a class C non-specific acid phosphatase with o-phospho-L-tyrosine-phosphatase activity in *Staphylococcus aureus* isolates from patients with chronic osteomyelitis

Detección y expresión de SapS, una fosfatasa ácida no específica clase C con actividad de o-fosfo-tirosina-fosfatasa en aislamientos de *Staphylococcus aureus* de pacientes con osteomielitis crónica

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Introduction. The identity of *Staphylococcus aureus* virulence factors involved in chronic osteomyelitis remains unresolved.SapS, Class C" Non-Specific Acid Phosphatase (NSAP, known virulence factors) was identified in *S. aureus* 154 from rotting vegetables.

Objective. SapS, Class "C" Non-Specific Acid Phosphatase (NSAP, known virulence factors) was identified in *S. aureus* 154 from rotting vegetables.

Materials and methods. *sapS* gene was isolated and sequenced from 12 clinical isolates of *S. aureus* and two reference strains; 49 *S. aureus* strains and 11 Coagulase-Negative *Staphylococci* (CoNS) were tested by *in silico* PCR. Culture media semi-purified protein extracts from the clinical strains were assayed for phosphatase activity with *p*-nitro-phenyl-phosphate (*p*-NPP), and o-phospho-L-tyrosine (o-p-L-tyrosine), o-p-L-serine, and o-p-L-threonine, testing them with various phosphatase inhibitors.

Results. *sapS* gene was detected in clinical and *in silico S. aureus* tested strains but not the *in silico* CoNS strains. Sec-type I lipoprotein-type N-terminal signal peptide sequences characteristic of *S. aureus* secreted proteins and aspartate bipartite catalytic domains coding sequences were found by *sapS gene* nucleotide and amino acid sequence analysis. SapS dephosphorylated p-NPP and o-p-L-tyrosine selectively; these reactions were resistant to tartrate and fluoride but sensitive to vanadate and molybdate.

Conclusion. *sapS* gene was found in clinical isolates and *in silico S. aureus* strains. SapS shares biochemical similarities with known virulent bacterial protein tyrosine phosphatases (PTPs) which suggests it may be a virulence factor in chronic osteomyelitis.

Keywords: *Staphylococcus aureus*, virulence factors, osteomyelitis.

Introducción. La identidad de los factores de virulencia de *Staphylococcus aureus* (*S. aureus*) implicados en la osteomielitis crónica sigue sin resolverse. SapS, una Fosfatasa Ácida No Específica Clase C (NSAP, factores de virulencia conocidos) fue identificada en *S. aureus* 154 de vegetales podridos.

Objetivo. Buscar el gen sapS y caracterizar la actividad de la fosfatasa SapS de cepas de *S. aureus* causantes de osteomielitis crónica y en *S. aureus* de una base de datos *in silico.*

Materiales y métodos. Se investigó y secuenció el gen sapS en 12 aislados clínicos de *S. aureus* y dos cepas de referencia; *in silico* en 49 cepas de *S. aureus* y 11 estafilococos coagulasa negativos (CoNS). Se analizó la actividad de la fosfatasa SapS de los extractos de los sobrenadantes de cultivos de cepas clínicas con p-nitro-fenil-fosfato (p-NPP) y o-p-L-tirosina, serina y treonina y usando varios inhibidores de fosfatasas.

Resultados. *sapS* se encontró en el genoma de las cepas clínicas y en 49 cepas de *S. aureus* pero no en CoNS. La secuenciación de SapS revelaron un péptido señal N-terminal de proteínas extracelulares y los dominios bipartita de aspartato (DDDD) de su sitio catalítico, hidroliza p-NPP y o-fosfo-tirosina selectivamente es resistente a tartrato y fluoruro, pero sensible a vanadato y molibdato.

Conclusión. sapS se encuentra en el genoma de aislados clínicos de *S. aureus* SapS es específica para o-p-L-tirosina comparte similitudes bioquímicas con las proteínas tirosina fosfatasas (PTP) bacterianas por lo que puede formar parte de la red de factores de virulencia en la osteomielitis crónica.

Palabras clave: *Staphylococcus aureus*, factores de virulencia, osteomielitis.

Staphylococcus aureus (*S .aureus*) is an opportunistic pathogen for humans and the main cause of osteomyelitis, which can evolve from an acute to a chronic stage, the latter characterized by bone loss and destruction. The temporary or permanent disability that ensues, in addition to substantial increases in hospitalization times and health care costs, are heavy social and institutional burdens (1,2).

S. aureus pathogenicity is due to the plasticity of its genome and the expression of an arsenal of virulence factors which can be located in the bacteria or are released into the extracellular space (3,4). The identity of active *S. aureus* virulence factors in chronic osteomyelitis remains unresolved. Although focus is currently on the discovery of new factors, there is also interest in exploring the virulence in osteomyelitis of well-known *S. aureus* factors such as Protein A, Panton-Valentine leukocidin, and coagulase, recently shown to play a role in bone loss and destruction (5).

Various genera and species of prokaryotes express Non-Specific Acid Phosphatases (NSPAs), classes A, B and C, which can be attached to cell membranes or as soluble extracellular enzymes and they are capable of “non-specifically” dephosphorylating a broad range of unrelated organic substrates, initially it was attributed as a way to acquire inorganic phosphate an essential substrate for bacterial metabolism; it has become clear that these phosphatases, in fact, have a role in bacterial pathogenicity (6,7).

Du Plesiss et al. (8) characterized a Soluble acid phosphatase (SapS) from the culture supernatant of *S. aureus* strain 154 isolated from rotting vegetables. The phosphatase is MgCl2-dependent and was affected by EDTA and sodium molybdate. The corresponding gene sapS encodes a protein with an estimated molecular mass of 30 kDa and 296 amino acids with a 31-residue signal peptide, four conserved

sequence motifs could be identified and his structural homology corresponding to belongs to the bacterial class C family NSAPs.

Our aim here is to investigate the presence of the *sapS* gene in 12 strains of *S. aureus* isolated from bone infected samples of patients treated for chronic osteomyelitis and from 49 *S. aureus* strains from an *in silico* database, as well as to determine the presence and activity of SapS in partially purified protein extracts from supernatants of culture media of the clinical strains, and compare with those described previously (8).

sapS gene is present in our 12 clinical strains of *S. aureus, in silico* in 49 strains of *S. aureus* but not in 11 coagulase-negative strains. SapS N-terminal domains belong to the Sec-type I system of extracellular proteins of *S.aureus*, presents their bipartite "DDDD" catalytic domain. Partially purified SapS of clinical strains was shown to dephosphorylate O-phospho-tyrosine but not O-phospho-serine nor O-phospho-threonine, was resistant to sodium tartrate, but sensitive to tyrosine phosphatase inhibitors.

Our results show that the *sapS* gene is present in the genome of clinical strains and that extracellular acid phosphatase preferentially dephosphorylates o-p-tyrosine and suggests the possibility that during chronic osteomyelitis this enzyme may interact with bone tissue cells favoring the pathological bone resorption. For this end, detailed studies must elucidate its possible involvement in bone pathogenicity

Materials and methods

Isolation and identification of S. aureus clinical strains

The 12 *S. aureus* strains included in this study were isolated between 1997 and 1998 from infected bone chips (6-10 mm^2 approx.) of adult patients during surgical debridement treatment of tibiae and femurs in the Bone Infection Service at the then

National Institute of Orthopedics, now the National Institute of Rehabilitation in Mexico City. Mexico. Bone specimens were washed twice and suspended in 1.0 ml phosphate-buffered saline, ground for 1 min in a tissue grinder and centrifuged at 4000 xg. Supernatants were streak seeded in Blood agar media, Mannitol salt phenol red agar (Merck) and Baird-Parker agar (Merck) incubated aerobically at 37°C 24h. Characteristic bacterial isolates were identified by the standard biochemical tests and antibiotic susceptibility for *S. aureus* (Uniscept 20GP System). Gram staining, coagulase (Bactident Coagulase, Merck) and catalase (Catalase Assay Kit, Merck) were performed. Clinical strains are methicillin sensitive (MSSA) and were grouped according to infection description: chronic osteomyelitis, 17 CPJ, 69 GGT, and 92 HM; infected pseudarthrosis, 54 SL, 68 FFC, 88 VTM, 89 RTC, and 93 EMC; and chronic bacterial osteitis 76 IQM, 101 AOC, 105 IMO, and 107 FMR.

Clinical and reference *S. aureus* ATCC-6538 and *S. epidermidis* ATCC-12228 strains were preserved in casein peptone-soymeal peptone broth (CASO, Merck) with 10% glycerol at -80 °C.

Bacterial DNA extraction

Genomic DNA was extracted as described by Novick (9) with modifications. Bacterial suspensions grown in BHI (0.8 Absorbance at 620 nm) were centrifuged for 5 min at 10,000 x g; bacterial pellets were resuspended in 20 µL of a mixture lysostaphin-lysozyme /Tris-EDTA buffer (TE) (5.0 µg/µL each, Sigma Chem). After 30 min at 37 °C, DNA was extracted using the Genomic DNA Purification Kit (Promega). DNA was resuspended in 50 µL TE buffer; purity was tested by agarose gel electrophoresis and its concentration by UV spectroscopy. Extracted DNA was stored in 20 µL aliquots at -20 °C.

Molecular detection of sapS gene

sapS gene (Gene Bank AY061973) in genomic DNA extracts was detected by end point polymerase chain reaction (PCR) with the *sapS*-forward (F) (5'-3') GGCATGAATAAAATTTCAAAG and *sapS*-reverse (R) (5'-3') GGCTGCAGTTATTTAACTTCGCCTGT oligonucleotide primers, previously reported (5), with an expected amplicon size of 891 bp (location, 1-891), and amplified with oligonucleotide primers designed by us using the Primer3Plus software, namely *sapS2*-F (5'-3') CCAAAAGTTCTGCTGAAGTTC and *sapS2*-R (5'-3') TTATTTAACTTCGCCTGTTTT, complementary to the nucleotide sequence encoding the mature extracellular peptide with acid phosphatase activity of the *sapS* gene (8), with an expected amplicon size of 800 bp (gene location 92-892).

The reaction mixture (20 µl) consisted of 30 ng target DNA, 200 µM deoxynucleotide triphosphates (dNTPs) (100 mM dNTP set, Invitrogen), 0.5 µM of each oligonucleotide primer pair, 2.0 mM $MgCl_2$, and 1.0 U thermostable DNA polymerase (Platinum Taq DNA Polymerase, Invitrogen). Amplifications were performed in a thermal cycler (Eppendorf Mastercycler gradient), programmed as follows: an initial 3 min cycle at 94 °C, 35 cycles of 1 min steps at 94 °C/52 °C/72 °C, and a final 5 min cycle at 72 °C. After agarose gel electrophoresis, PCR products were analyzed under UV trans-illumination.

sapS gene detection in _S. aureus_ and coagulase-negative _Staphylococci_ strains (CoNS) from the _in silico_ database

in silico PCR were performed with the DNA of 49 *S. aureus* and 11 coagulase-negative *Staphylococcus* strains (CoNS) found in the *in silico* database (10) using the same primer pairs described above. However, because of requirements to fill out the FASTA format for the *sapS* gene oligonucleotide primer sequences, restriction sites

for both forward (F: 5'GGC3') and reverse (R: 5'GGCTGCA3') primer sequences were deleted. Thus, the trimmed oligonucleotide primer sequences used for *in silico sapS* gene amplification were F: 5'- ATGAATAAAATTTCAAAG-3', and R: 5'-GTTATTTAACTTCGCCTGT-3'.

Experiments were designed to allow for 2 nucleotide mismatches at the 5' end and 0 mismatches at the 3'end, as well as a maximum number of 3000 nucleotides for the amplicons. Results were obtained 60 seconds after starting the program. A screen capture was made showing the number of amplicons obtained with their corresponding molecular sizes.

Sequencing of PCR products and molecular analysis of sapS gene

Sequencing was performed with an Abi Prism 310 sequencer (Genetic Analyzer, Applied Biosystems) using a commercial reagent kit (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) with 120 ng of each PCR product (template DNA) and 0.5 µM of the sense (forward) *sapS* oligonucleotide primer. Electropherograms and nucleotide sequences were stored and analyzed using the Chromas V2 program.

A comparative multiple nucleotide sequence paired global alignment analysis was performed among the nucleotide sequences previously reported Gene Bank AY061973 (5) using the Clustal Omega Program1 (11). Nucleotide sequences from *S. aureus* clinical and reference strains were translated using the *in silico* Web page (3), the amino acid sequences of the SapS protein were used to analyze with the SignalP 5.0 server platform (12) the N-terminal signal peptide domain, its cleavage sequence, and the type of protein secretion system it might belong to. The subcellular location of SapS for Gram positive bacteria was analyzed on the Gpos-

mPloc platform (13). Bipartite signature "DDDD" motifs of class "C" non-specific acid phosphatases (NSAPs) were searched for using BLAST (14).

Isolation and concentration of protein extracts from bacterial culture media.

All bacterial strains were grown in a casein peptone-soymeal peptone broth (CASO, Merck) for 18 h at 37 °C under constant shaking. Bacteria were then separated by centrifugation at 10,000 xg for 10 min at 4 °C; supernatants were sterilized by filtration through 0.22 µm membranes (S-Pak Millipore), aliquoted, and stored at -80°C. Supernatant (or uninoculated CASO culture medium) protein extracts were obtained by mixing one volume of each supernatant with four volumes of cold (-20°C) ultrapure acetone (Merck EM Science); protein was allowed to precipitate at -75 °C for 1 h, recovered by centrifugation at 14,000 xg for 15 min at 4 °C, dried at 4 °C, and resuspended in 1.0 mL 0.1M sodium acetate pH 5.0 (buffer A). Aliquots (200 µL) were stored at -80°C. Protein was measured using a commercial kit (DC Protein Assay, BioRad).

Acid phosphatase activity

Acid phosphatase activity in bacterial culture media was measured as described by Golovan et al. (15) with *p*-nitro-phenyl phosphate (*p*-NPP disodium salt, hexahydrate, Sigma Chem). Reaction mixtures (100 µL) containing 10 or 20 µL of the protein extracts and 200 µL of 10 mM p-NPP in buffer A, were incubated for 30 min at 37 °C; reactions were stopped with 100 µL of 1.0 M NaOH; *p*-nitrophenol (p-NP) formed was measured by absorbance at 405 nm (Beckman DU 800 spectrophotometer). Specific acid phosphatase activity was defined as µmoles *p*-NP/min/mg protein. All measurements were made in triplicate

To 10 µL protein extracts were added one of the following phosphatase inhibitors: 20 mM dibasic dihydrate sodium tartrate, 10 mM sodium fluoride, 2.0 mM ammonium

molybdate tetrahydrate, and 10 mM sodium ortho-vanadate, dissolved in buffer A. Reaction mixtures were adjusted to a final volume of 100 µL, incubated first at 37 °C for 15 min, then 200 µL of 10 mM *p*-NPP were added and reaction mixtures were further incubated for 30 min at 37 °C; reaction was stopped with 100 µL of 1.0 M NaOH. Specific acid phosphatase activity was calculated as above.

Identification of extracellular SapS in protein extracts of culture media supernatants of clinical and reference strains.

SapS presence in protein extracts from culture media supernatants was analyzed by zymography: 10 µL of each clinical and reference protein extract and of the non-inoculated culture medium together with a standard pre-stained protein MW marker (Precision Plus Protein, All Blue Prestained Protein Standards, BioRad) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) (SDS-PAGE) under non-reducing conditions basically as described by Hamilton et al (16). After electrophoresis SDS was removed washing gels twice with de-ionized water and once with 1.0% Triton X-100. Gels were then incubated in re-naturing buffer (100 mM Tris HCl, 2.0 mM MgSO4.6H2O, Triton X-100 1.0%, pH 7.0) for 15 min at room temperature, equilibrated in buffer A. Acid phosphatase activity was assessed by adding to the buffer 0.1 % α-naphthyl phosphate and 0.2% Fast Garnet GBC (Sigma. Chem) and further incubating at 37°C for 45 min. α-naphthol appeared as a brownish-red precipitate on the protein band with phosphatase activity. Reaction was stopped by washing the gel twice with de-ionized water. Protein bands on parallel gels run under similar conditions were stained with Brilliant Coomasie R-250, (BioRad).

SapS specificity for phopho-L-amino-acids and the effect of phosphatase inhibitors

Substrate specificity of SapS for o-phopho-aminoacids was also determined by zymography according to Slotnick and Gottlieb (17) with modifications. After SDS-PAGE and re-naturation as decribed above, gel slabs were incubated at 37°C for 45 min in 10 mL with each of the following substrates: o-phospho-L-serine, o-phospho-L-threonine, and o-phospho-L-tyrosine (Sigma Chem), at a final concentration of 4 mM in buffer A. Gels were then washed twice with de-ionized water. The inorganic phosphate released was detected with a mixture of 0.045% Malachite Green (oxalate salt, Sigma Chem) and 4.2% ammonium molybdate for 10 min, at room temperature; the reaction was stopped with two washes of de-ionized water followed by 5% acetic acid. A positive phosphatase reaction was a green precipitate on the SapS protein band stained with Coomassie Brilliant Blue in a parallel gel run under similar conditions.

Similarly, phosphatase inhibitors on SapS catalysis were tested by zymography. Re-natured gels were incubated at 37°C for 30 min in 10 mL of 10 mM (final concentration) sodium tartrate, sodium fluoride, sodium ortho-vanadate or ammonium molybdate (Sigma Chem). Gels were then washed once in buffer A and further incubated at 37°C for another 30 min in 10 mL of 0.1 % α-naphthyl phosphate or 4 mM of the three o-phospho-amino acids as substrates. Corresponding reaction products, α-naphthol and released phosphate, were stained as described above with 0.2% Fast Garnet GBC or Malachite green/ammonium molybdate, respectively.

Statistical analysis

Statistical significance of differences among the means of acid phosphatase measurements with and without enzyme inhibitors was determined with the ANOVA

test and the comparative Tukey post-hoc test using the GraphPad Prism 5.0 program. Significance was set at $p<0.05$.

Results

sapS gene is present in the genomes of isolated _S. aureus_ clinical strains from chronically osteomyelitic patients

Using the oligonucleotide primers designed for *S. aureus* 154 (8) we detected the presence of the complete *sapS* gene as a single 891 bp-long amplicon in the DNA template of the 12 clinical strains and *S. aureus* ATCC-6538, but not for *S. epidermidis* ATCC-12228. With sapS2 oligonucleotide primers we obtained a single 805 bp-long amplicon that matched the nucleotide sequence of the extracellular mature peptide with acid phosphatase activity. These results show that the *sapS* gene and the nucleotide sequence that encodes the extracellular phosphatase are present in clinical and reference

S. aureus strains, but not *S. epidermidis* ATCC-12228.

sapS gene is present in _S. aureus_ strains but not in CoNS from the in silico database

in silico PCR confirmed the presence *sapS* gene only in the *S. aureus* species. Using the original set of oligonucleotide primers (8), the complete gene 892 bp-long amplicon was obtained for all 49 *S. aureus* strains tested from the *in silico* Web page (figure 1A) and none for any of the 11 CoNS strains. Similarly, with the sapS2 primers only a single 800 bp-amplicon was obtained for *S. aureus* strains (figure 1B); no amplicons were obtained with either oligonucleotide primers for any of the plasmids contained in the *in silico* database.

sapS gene nucleotide and amino acid sequences reveal the presence of protein SapS' catalytic domain and signal peptide's cleavage site

Multiple nucleotide sequence analysis performed with the *sapS* gene nucleotide sequences of *S. aureus* 154 (8), the 12 clinical *S. aureus* strains**,** and *S. aureus* ATCC 6538 reference strain**,** showed a sequence percent identity of 92-96% (table 1), suggesting that the *sapS* genes from the *S. aureus* strains tested here are homologous**.** The phylogenetic tree we built (data not shown) revealed the closeness among sequences implying they could share a common ancestor.

Amino acid sequence analysis of the *sapS* gene on the SignalP 5.0 server showed the presence of the signal peptide in the N-terminal amino acid sequence (27-STAF**AK**SSAEVQQ-39) in the 12 clinical *S. aureus* strains (table 2), as previously reported (7). It includes the recognition site for signal peptide cleavage between residues Ala 31 and Lys 32 (**AK).**

Predictive analysis on the Gpos-mLoc server suggests that Saps is in fact a typical extracellular protein belonging to the *S. aureus* secretome (19) of which the bipartite motifs, A (101-AL**D**L**D**ETVLDNSPY-114) and B (213-LVMLFG**D**NLL**D**F-224), with the four invariant aspartic residues associated with dephosphorylating enzymatic activity of class "C" NSAPs (20), as previously reported (18), were also found to be highly conserved in our 12 clinical *S. aureus* strains (table 2).

SapS resistance to sodium tartrate and sodium fluoride and sensitivity to sodium ortho-vanadate and ammonium molybdate.

The average acid phosphatase specific activity was 25.35 mmoles *p*-NP/min/mg protein at pH 5.0 (figure 2) of protein extracts from the culture media of 12 *S. aureus* clinical strains with *p*-NPP as substrate. *S. aureus* ATCC-6538 showed the lowest level of activity (4.5 mmoles p-NP/min/mg protein) and *S. epidermidis* ATCC-12228

strain showed no activity at all. Moreover, this extracellular acid phosphatase activity was resistant to inhibition by both sodium tartrate, a general phosphatase inhibitor, and sodium fluoride, an inhibitor of acid phosphatases (figure 3). However, this activity was strongly inhibited by sodium ortho-vanadate (~50%) and ammonium molybdate (80-95%) (figure 2).

SapS is present in semi-purified culture media supernatant protein extracts of *S. aureus* clinical strains

Non-reducing PAGE of partially purified culture media supernatant protein extracts of *S.aureus* clinical strains and *S. aureus* ATTC-6538 (figure 3A), showed remarkably similar extracellular protein profiles (Coomassie Brilliant Blue-staining) ranging from 30 to 100 kDa. Zymographic analysis showed only one extracellular protein band with phosphatase activity from clinical *S. aureus* and *S.aureus* ATTC 6538, as revealed by the appearance of α-naphthol at the same migration distance as a protein band run in parallel in a neighboring gel stained with Coommassie Brilliant Blue. Both bands show an approximate molecular mass of 30 kDa (figures 3A and 3B), thereby strongly suggesting its identity with SapS. The protein extract from the *S. epidermidis* ATCC11228 (figures 3A and 3B, lane 15) showed no 30 kDa protein band nor associated enzymatic activity, respectively.

SapS exhibits o-p-L--tyrosine phosphatase activity and is sodium vanadate and ammonium molybdate sensitive.

Zymographic analysis of SapS o-phospho-amino-acids phosphatase activity of the culture media protein extracts of all *S.aureus* clinical strains and *S.aureus* ATTC-6538 strain showed a green malachite/molybdate/phosphate complex precipitate at the same migration distance as the 30 kDa Coomassie protein stain and the

brownish-red α-naphthol/Fast Garnet GBC precipitate staining revealing SapS' activity (figure 3C).

However, SapS showed o-p-L-tyrosine phosphatase activity, neither o-p- -serine nor o-p-L-threonine were dephosphorylated. It thus appears that SapS and the o-p--L-tyrosine phosphatase are one and the same enzyme.

Furthermore, in another set of parallel zymograms which included in the gels' reaction mixtures one of the four phosphatase inhibitors mentioned above, the green malachite/molybdate/phosphate complex precipitate seen after o-phospho-tyrosine dephosphorylation, was resistant to sodium tartrate and sodium fluoride but did not appear in the presence of sodium vanadate and ammonium molybdate.

Discussion

Bone destruction in chronic staphylococcal osteomyelitis is due to an inflammatory process induced by the expression of many *S. aureus* exoproteins whose number and function remain unresolved (1). Notably, however, since its initial characterization in the *S. aureus* 154 strain, the presence of the *sapS* gene and the activity of the extracellular acid phosphatase SapS has not been examined in the context of bone infections caused by *S. aureus*. Here we have identified and characterized *sapS* in our 12 clinical strains and 49 others from an *insilico* data base and identified and characterized SapS in the partially purified protein extracts from the culture media of the same clinical strains.

sapS and the region coding for the extracellular mature peptide (8) found in our clinical strains and *in silico* in a group of clinically relevant *S. aureus* strains of diverse origin, suggest that this gene could be part of an *S. aureus* "core" genome shared by all strains in the species, and likely to encode for functions related to the bacteria's basic cellular nutrition (20. *S. aureus* "core" genome has been studied in

large groups of *S. aureus* strains and no significant genotypic differences have been found between strains from asymptomatic carriers and patients with invasive infections (21).

NSAPs, the super family of acid phosphatases present in prokaryotes and eukaryotes exhibit nonspecific activity on many structurally unrelated phosphoesters. Prokaryote NSAPs are usually grouped into three classes, namely A, B and C, based on their amino acid sequence relatedness, and the highly conserved bipartite sequences of their greater signature motif “DDDD”, the two couples of invariant aspartate residues present in each domain, essential for its enzymatic activity (7).

Our *sapS* gene amino acid sequence analysis corroborated the presence of this bipartite domain in the 12 chronic osteomyelitis-causing *S. aureus* strains and in others of clinical importance, and further disclosed the presence of one of the four known types of staphylococcal N-terminal signal peptide domains for SapS (22) suggesting its secretion from the cell. Moreover, its predictive analysis further indicates with high probability (95.56%) that because of its N-terminal signal peptide domain, SapS would be expected to be released as an extracellular class C NSAP lipoprotein with a protein component of roughly 30 k Da and to become a part of the *S. aureus* secretome (23).

This feature differs from the targeted subcellular location anticipated for other class C NSAPs of pathogenic bacteria like *Clostridium perfringens* that are devoid of an N-terminal consensus lipoprotein-signal peptide-like motif (24). The same is true for the class C NSAPs of *Flavobacterium meningosepticum, Bacillus anthracis*, *Helicobacter pylori*, *Streptococcus pyogenes*, *Haemophilus influenza,* whose acid phosphatase N-terminal signal peptide sequences show a 98.75 % probability of them being

membrane bound lipoprotein-type enzymes, and only a 1.22% probability of them being extracellular phosphatases as expected for SapS.

Biochemical analysis of SapS activity in partially purified protein extracts from the culture media of the clinical strains of *S. aureus* described here showed the presence of the 30 k Da protein with a highly selective phosphatase activity for o-p-tyrosine, which was sensitive to vanadate and molybdate. Notably, these characteristics are shared with the extracellular 28 k Da type of class C NSAP SapM and their low molecular weight-phosphorylated-tyrosine protein phosphatases (LMW-PTP) MptpA of pathogenic *Mycobacterium tuberculosis*, proven virulence factors (23,25).

The genome of *S. aureus* contains all the information required for it to develop and function including an arsenal of virulence genes coding for proteins involved in their adherence and colonization of infected tissue, along with their immune evasive properties (26). Some of these proteins are well-known virulence factors with newly identified mechanisms of action, while the functional characterization of newer ones is currently under investigation (27).

Taking into account the structural and functional similarities between the SapS described here from our *S. aureus* osteomyelitis-causing strains and class C NSAPs and LMW-PTPs are virulence factors in various pathogenic strains, we are suggesting here that SapS may be also be a virulence factor. Considering that inorganic phosphate is a vital element for *S. aureus* metabolism, and that it is not freely available and is limited during infection, SapS might be the extracellular processing enzyme required by the bacteria to extract inorganic phosphates (Pi) from host organophosphates (phosphorylated nucleotides, sugars, and amino acids) which is then imported through Pi transporters to ensure its survival and enhance bone damage during infection (28,29).

Proving that SapS is in fact a virulence factor will require a detailed series of studies aimed at establishing the precise physiological function of the purified enzyme on the metabolism of bone tissue cells to determine if this newly identified and partially characterized class C NASP contributes to the pathogenicity of *S. aureus* in chronic osteomyelitis.

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Conflict of interests

The authors have no conflicts of interest.

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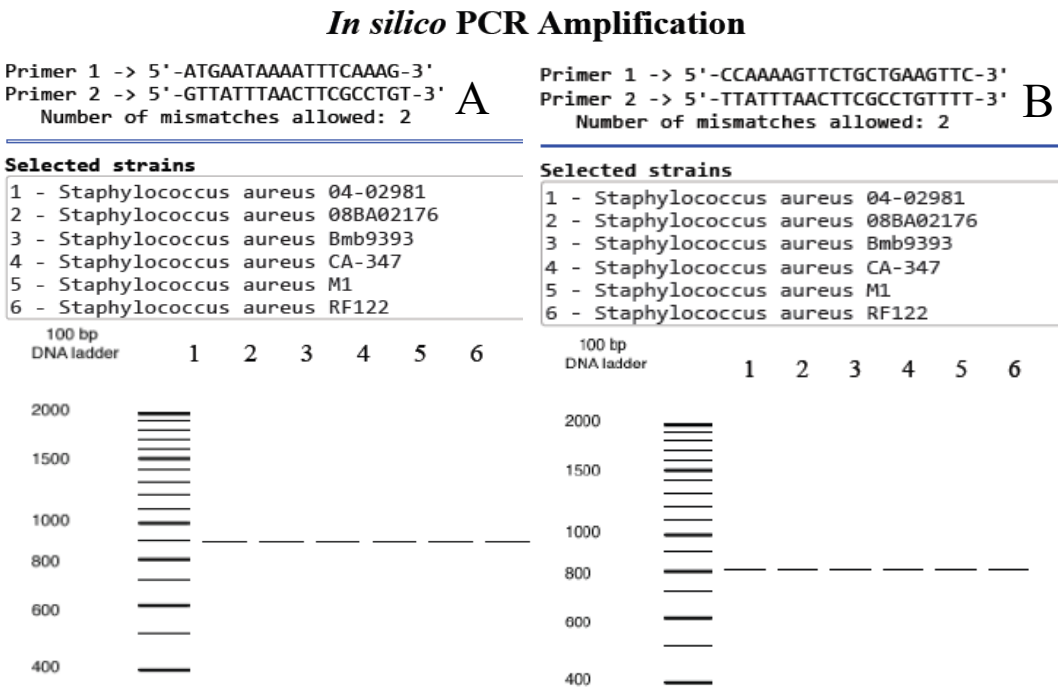

Figure 1. *In silico* PCR amplifications. A. *sapS* gene amplifications using the oligonucleotide primers described by du Plessis et al (5). B. Amplifications of the *sapS* gene region encoding for the mature extracellular acid phosphatase (sapS2*)* using our set of oligonucleotide primers. In both cases, DNA templates were 49 *S. aureus* strains and from 11 coagulase-negative *Staphylococcus* strains, as well as from plasmids housed in the in silico database (10).

Amplifications of six coagulase-positive *S. aureus* strains are shown as examples.

no amplicons were found for coagulase-negative strains.

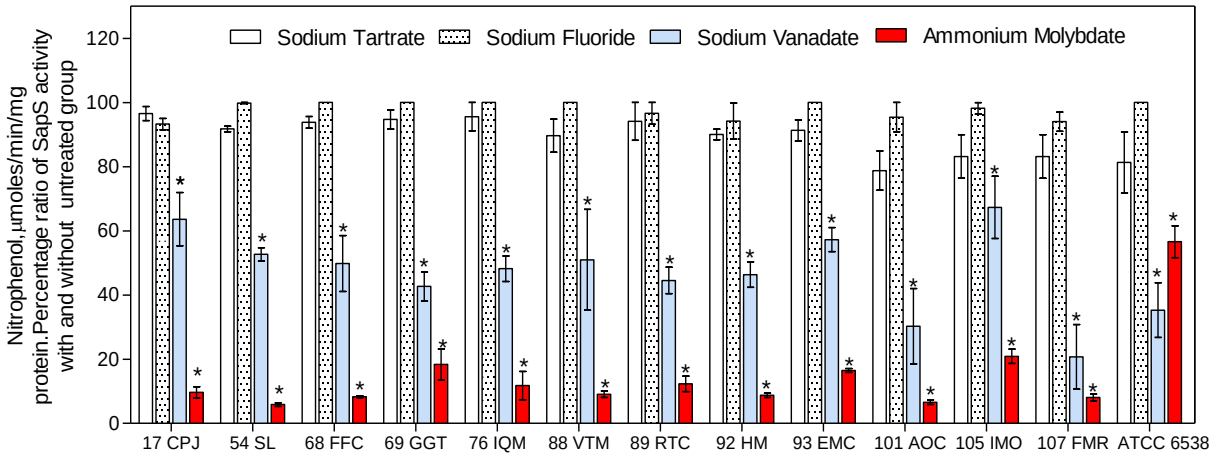

Figure 2. Percent ratio of SapS activity in the presence and absence of phosphatases inhibitors. SapS activity assessed from protein extracts of culture media of clinical strains; p-NPP was used as substrate. Assays were run in triplicate and are from three independent experiments; results are expressed as the mean ± SEM. Statistical analysis was performed using ANOVA and significance was set at *p<0.05.

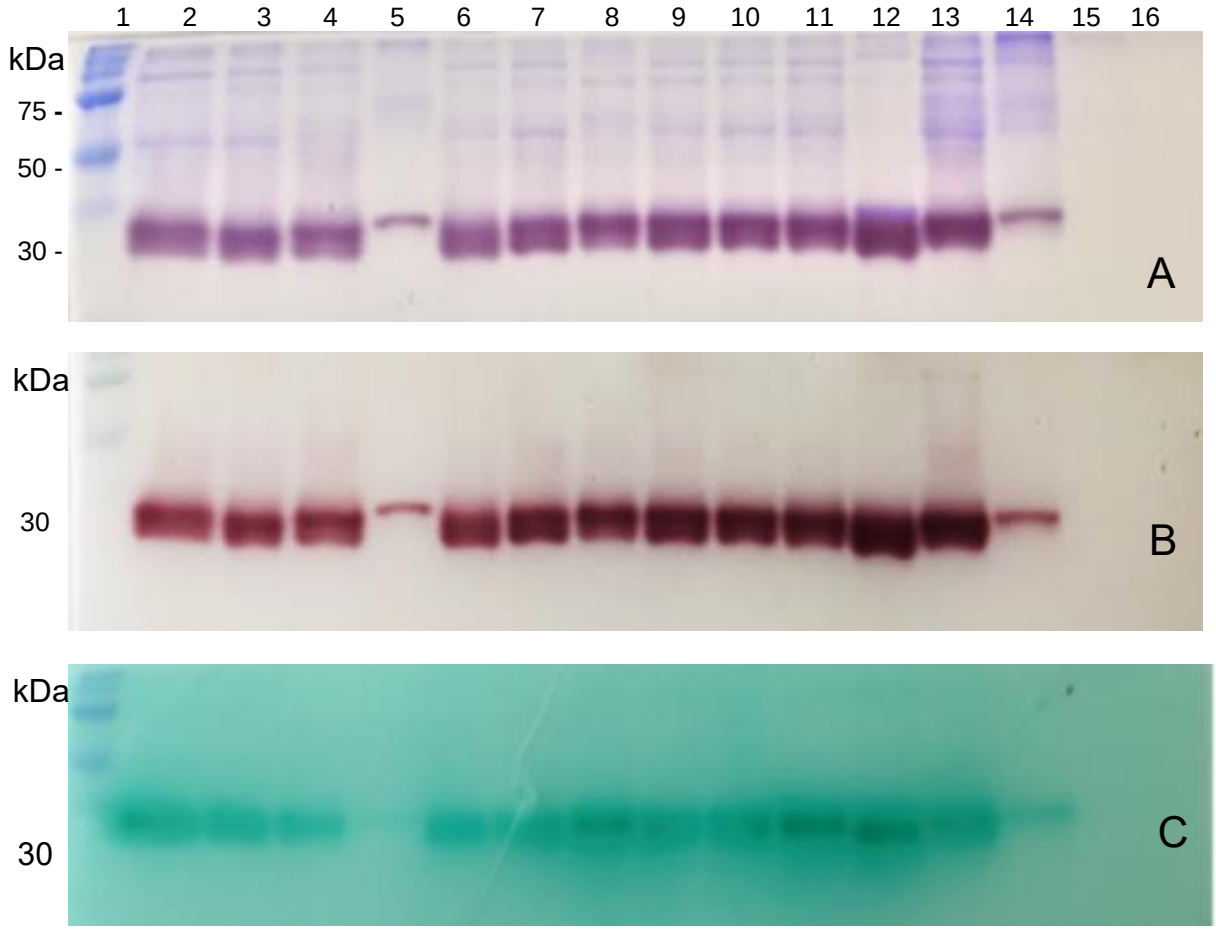

Figure 3**.** Non-reducing gel electrophoresis and zymographic analysis of protein extracts obtained from the culture media of clinical bacterial strains, reference strains and from the sterile culture medium (SCM).

Gel stains are: A. Coomassie Brilliant Blue for protein staining. B. Brownish-red α-naphthol-Fast Garnet GBC complex for SapS activity assessment, and C. phosphate/green malachite/ammonium molybdate complex for SapS' o-p-L-tyrosine phosphatase activity. Lanes depicted are from left to right: 1, pre-stained MW protein ladder marker; 2-13 clinical bacterial strains; 17 CPJ, 54 SL, 68 FFC, 69 GGT, 76 IQM, 88 VTM, 89-RTC, 92-HM, 93-EMC,101-AOC,105-IMO,107-FMR; 14, *S. aureus* ATCC 6538, 15 *S. epidermidis* ATC -12228, 16 SCM.

Table 1. Percent identity of nucleotide sequence similarities between gene *sapS*ofour12 clinical *S. aureus* strains, the *S. aureus* ATCC 6538 reference strain, and the *S. aureus* strain 154 (5) AY61973.1 sequence.

Clinical bacterial strains	105 IMO	76 IQM	17 CPJ	AY061973.1*	ATCC 6538*	54 SL	107 FMR	101 AOC	69 GGT	88 VTM	68 FFC	92 HM	93 EMC	89 RTC
105 IMO	100.0	95.01	94.73	96.28	94.48	95.30	95.19	94.20	94.77	92.48	92.37	91.93	91.98	91.68
76 IQM	95.01	100.0	96.76	95.89	96.29	96.76	95.95	96.42	96.30	92.06	93.34	92.60	93.67	93.67
17 CPJ	94.73	94.76	100.0	99.30	98.17	96.34	97.26	96.69	96.80	92.30	93.64	92.24	93.40	93.29
AY61973.1*	96.28	95.89	99.30	100.0	99.07	97.68	98.38	98.03	97.92	92.78	94.97	93.65	95.19	94.84
ATCC6538*	94.48	96.29	98.17	99.07	100.0	96.10	97.02	96.56	96.45	93.41	93.39	92.09	93.26	93.15
54 SL	95.30	96.76	96.34	97.68	96.10	100.0	96.46	96.23	96.80	92.49	93.63	92.81	93.97	94.09
107 FMR	95.19	95.95	97.26	98.38	97.02	96.46	100.0	96.70	97.27	93.08	93.53	92.70	93.99	93.99
101 AOC	94.20	96.42	96.69	98.03	96.56	96.23	96.70	100.0	96.60	92.17	92.55	92.48	96.55	93.00
69 GGT	94.77	96.30	96.80	97.92	96.45	96.80	97.27	96.60	100.0	92.62	93.23	92.94	93.56	93.68
88 VTM	92.48	92.06	92.00	92.78	93.41	92.49	93.08	92.17	92.62	100.0	93.16	93.36	94.19	94.19
68 FFC	92.37	93.34	93.64	94.97	93.39	93.63	93.53	92.55	93.23	93.16	100.0	94.72	93.39	92.62
92 HM	91.93	92.60	92.24	93.65	92.09	92.81	92.70	92.48	92.94	93.36	94.72	100.0	95.27	95.29
93 EMC	91.98	93.67	93.40	95.19	93.26	93.97	93.99	92.65	93.56	94.19	93.39	95.27	100.0	92.72
89 RTC	91.68	93.67	93.29	94.84	95.15	94.09	93.99	93.00	93.68	94.19	92.62	95.29	97.72	100.0

*Known sequences

Table 2. Conserved relevant SapS amino acid sequences in *S. aureus* clinical strains.

***S. aureus* strain**	**SIGNAL PEPTIDE**	**SIGNATURE MOTIF A**	**SIGNATURE MOTIF B**
154 (5)	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
17 CPJ	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
54 SL	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
68FFC	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
69 GGT	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
76 IQM	27-STAF**AK**SSCSCST-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
88 VTM	27-STAF**AK**SSGEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
89 RTC	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
92 HM	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
93 EMC	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
101 AOC	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
105 IMO	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
107 FMR	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224
ATCC 6538	27-STAF**AK**SSAEVQQ-39	101-AL**D**LDETVL**D**NSPY-114	213-LVMLFG**D**NLL**D**F-224