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The antimicrobial peptide Temporin-L induces vesicle formation and reduces the virulence in *S. aureus*

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ABSTRACT

The evolution of methicillin-resistant *Staphylococcus aureus* (*MRSA*) has required the development of new antimicrobial agents and new approaches to prevent and overcome drug resistance. AntiMicrobial Peptides (AMPs) represent promising alternatives due to their rapid bactericidal activity and their broad-spectrum of action against a wide range of microorganisms. The amphibian Temporins constitute a well-known family of AMPs with high antibacterial properties against both Gram-positive and Gram-negative bacteria. In this paper, we evaluated the in vivo effect of Temp-L on *S. aureus* performing morphological studies using Transmission Electron Microscopy (TEM) that revealed the occurrence of protrusions from the cell surface. The formation of vesicle-like structure was confirmed by Dynamic Light Scattering (DLS). The global effect of Temp-L on *Staphylococcus aureus* (*S. aureus*) was deeply investigated by differential proteomics leading to the identification of up-regulated proteins involved in the synthesis of the cell membrane and fatty acids, and down-regulated virulence factors. GC-MS analysis suggested a possible protective response mechanism implemented by the bacterium after treatment with Temp-L, as the synthesis of fatty acids was increased. Adhesion and invasion assays on eukaryotic cells confirmed a reduced virulence of *S. aureus* following treatment with Temp-L. These results suggested the targeting of virulence factors as novel strategy to replace traditional antimicrobial agents that can be used to treat infections, especially infections caused by the resistant pathogen *S. aureus*.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (*MRSA*) is one of the most studied and wide-spread Gram-positive pathogens responsible for several severe infections, such as bacteremia, pneumonia and toxic shock syndrome [1]. Globally, the prevalence of *MRSA* infections is increasing at alarming levels worldwide, and the emergence of antibiotic resistance makes more difficult to treat severe MRSA infections. The limited number of antibiotics available for the treatment of infections and the ongoing development of antimicrobial agent resistance represent a major issue that should be carefully addressed [2].

S. aureus exhibits a variety of resistance mechanisms against many classes of antibiotics, underscoring the urgent need for the discovery of new antimicrobial compounds to treat bacterial infections. Hystorically, the glycopeptide vancomycin was first used to treat nosocomial MRSA infections However, mutations in multiple chromosomal genes have led to the cell wall reorganization and thickening, thus limiting drug access and resulting in resistant strains [3]. Consequently the rise in multi-drug-resistant *S. aureus* strains has intensified research efforts to identify innovative therapies with mechanisms different from those currently available using conventional antimicrobial drugs. Currently, more than 95 % of *S. aureus* strains are resistant to penicillin, 60 % to methicillin, and several cases of vancomycin-resistant *S. aureus* have been documented [4]. Current therapies for *S. aureus* infectious diseases often face significant limitations, including the development of resistance, adverse side effects, and limited efficacy against multidrug-resistant strains. These challenges underscore the necessity for novel therapeutic agents with distinct mechanisms of action.

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The ability of *S. aureus* to produce an astounding amount of virulence factors makes this bacterium different from other pathogens which often produce only one or a few toxins to promote disease [5]. These features prompted researchers to the development of a new strategy for disarming *S. aureus* by addressing bacterial virulence factors. Anti-virulence agents can suppress virulence phenotypes without affecting bacterial growth and/or enhancing the antibacterial response to conventional drugs [6]. This approach requires the identification of the proteins or genes involved in bacterial virulence that should be targeted to increase bacterial susceptibility to the immune system and antibiotics. However, the role of various virulence factors in the pathogenesis of *S. aureus* infections remains poorly understood [7].

Among the novel classes of antibiotics under development, Antimicrobial Peptides (AMPs) constitute a new field of investigations, since they are widely distributed throughout the animal and plant kingdoms, and show a broad spectrum of activity against Gram-positive and Gram-negative bacteria, yeast, fungi, viruses, and protozoa [8].

The antimicrobial mechanism of AMPs varies from membrane permeabilization to interactions with certain intracellular targets, including proteins and nucleic acids. Increasing evidences indicate that some AMPs might induce cell damages by interfering with vital physiological processes, such as the inhibition of cell wall biosynthesis, DNA and RNA functions, and protein synthesis [9,10].

Most studies on amphibian Temporins, a largest family of AMPs with potent antibacterial properties, have demonstrated their activity against Gram-positive bacteria, including clinically isolated methicillin-resistant *S. aureus*, at low inhibitory concentrations [11]. Temporin-L (Temp-L), a naturally occurring AMP, presents a promising alternative due to its broad-spectrum antimicrobial activity. The unique properties of Temp-L, such as its rapid bactericidal action and low propensity for resistance development, directly address the limitations of existing treatments, making it a compelling candidate for further research and potential clinical application [12].

Unfortunately, the use of Temp-L as an antimicrobial agent is not without challenges. One significant issue is its potential cytotoxicity at higher concentrations, which could limit its therapeutic window. Additionally, the stability of Temp-L in physiological conditions is a concern, as it may be susceptible to proteolytic degradation by enzymes present in the human body, thereby reducing its efficacy. Furthermore, the high cost of peptide synthesis and potential immunogenicity could pose barriers to its widespread clinical use [13]. Addressing these challenges through chemical modification, formulation strategies, and delivery systems will be crucial to fully realizing the therapeutic potential of Temp-L. Alternatively, understanding the molecular mechanisms of action of Temp-L and identification ot its specific intracellular target(s) can lead to the design of novel analogues with enhanced stability and reduced cytotoxicity, that minimize adverse effects and maximize therapeutic efficacy [14,15]. Moreover, elucidating these mechanisms can assist in overcoming resistance mechanisms and tailoring treatments to specific pathogens, thereby enhancing the clinical utility of Temp-L.

Recently, the mechanism of action of Temp-L against *E. coli* was completely elucidated, identifying the FtsZ protein as its specific intracellular target [16]. However, the specific molecular mechanism exerted by Temp-L on *S. aureus* is still largely unknown. In this study, we investigated the global effect of Temp-L at sub-MIC concentration on *S. aureus* to provide insights into the bacterial processes affected by the peptide, without causing immediate cell death. This approach provides insight into the mechanisms of action and the peptide's potential role in preventing resistance development. Ultrastructural changes in *S. aureus* induced by Temp-L were evaluated by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS), revealing the occurrence of several regularly distributed protrusions on the cell surface with vesicle-like structures, and size and shape in treated cells. The proteomic profile of Temp-L treated *S. aureus* was examined using differential proteomics, which suggested an increase in the production of proteins involved in cell wall and fatty acids biosynthesis and a clear decrease of cell adhesion and virulence factors. The results were confirmed by GC-MS analysis of the fatty acid content and adhesion and invasion assays on eukaryotic cells.

These findings suggest that *S. aureus* might trigger a protective response against Temp-L, characterized by a substantial production of proteins involved in cell membrane and vesicle formation, along with a reduced synthesis of virulence factors.

2. Results

2.1. Morphological effect of Temp-L on S. aureus

Transmission Electron Microscopy (TEM) was employed to investigate the impact of sub-MIC concentrations of Temp-L on *S. aureus*, focusing on potential alterations to the bacterial cell surface induced by the peptide. In this experiment as well as in all the others performed in this study a Temp-L concentration well below the previously determined MIC value was used. Fig. 1 shows the TEM analyses of treated and untreated *S. aureus* cells following Temp-L incubation. TEM images revealed the occurrence of several regularly distributed protrusions from the cell surface similar to bubbles or vesicle-like structures. These findings indicated that the peptide exerted a peculiar effect on *S. aureus* cells, observable even after just 1h of incubation.

2.2. Detection of vesicle in S. aureus by Dynamic light scattering

The unanticipated effect of Temp-L treatment on *S.aureus* cell membranes prompted us to investigate the formation of these protrusions in greater details. Due to the dehydration of the sample required to for TEM imaging which did not provide reliable data on the size of these "bubbles", DLS measurements were performed.

The results from the TEM images are confirmed by comparing the treated (black line in Fig. 2B) and untreated (grey line in Fig. 2A) system, clearly indicating an increase in size following Temp-L treatment. Furthermore, with increased incubation time, we can observe how the representative population of *S. aureus* decreases in size and becomes steadily less abundant. Conversely, a smaller population, about 40 nm after 2 h of incubation, is increasing in size. Interestingly, this population increases over time, reaching a size of about 120 nm after 5 h of incubation with Temp-L (Fig. 2B). These results may indicate a mechanism of production of these "bubbles" by *S. aureus.* Temp-L causes an increase in the bacterium size, then these protrusions detach from the bacterium, causing a simultaneous presence of two populations, one of about 400/500 nm imputable to the bacterium and the other of about 40/60 nm attributable to the "bubbles", which then tend to aggregate into objects of about 120 nm. To verify whether the observed evolution of the sample was due to Temp-L treatment, measurements were performed on the untreated sample at different time points (Fig. 2A).

Membrane vesicle overproduction is considered a typical bacterial defence mechanism as it has been detected in most cases of stress-activated conditions [17].

2.3. Differential proteomic analyses of Temp-L treated S. aureus

We were then stimulated to investigate the global effect of Temp-L on *S. aureus* at the molecular level. The expression profile of *S. aureus* was examined by differential proteomic approaches according to the label-free procedure to highlight proteomic changes following treatment with sub-MIC concentration of the peptide. The experiment aimed to elucidate the molecular mechanisms leading to vesicle formation.

S. aureus cells were cultured both with and without Temp-L under the same conditions used for the TEM analysis. Cells were lysed, and the protein content was purified using solid-phase extraction. Proteins were digested with trypsin and analyzed by nanoLC-MS/MS. Proteins identification and the quantification of their expression levels were

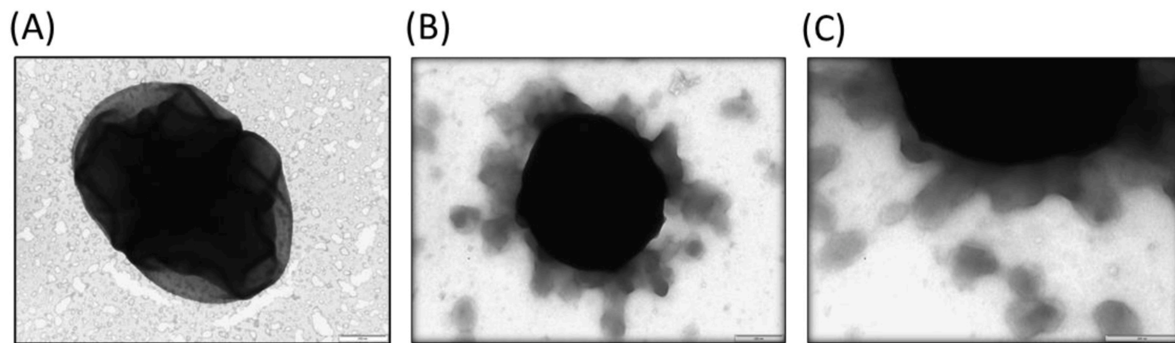

Fig. 1. Morphological analysis of *S. aureus* in the presence and absence of sub-MIC concentrations of Temp-L. Panels B and C show *S. aureus* cells treated with Temp-L. Panel A shows the cells without treatment. Scale bar 200 nm.

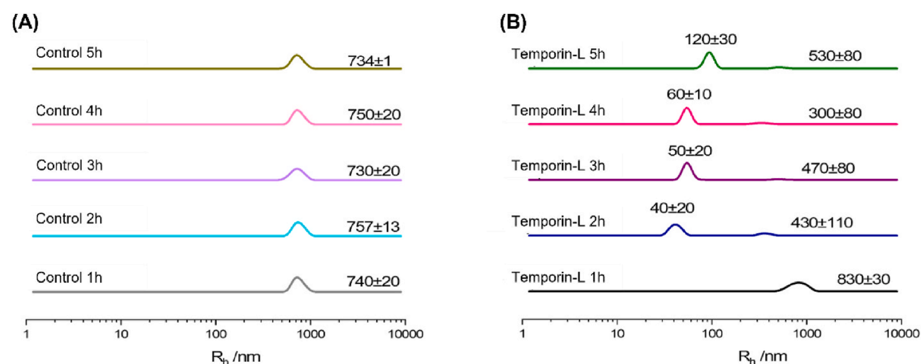

Fig. 2. DLS analysis of Temp-L treated and untreated *S.aureus* cells. Panel A: control cells. Panel B: *S.aureus* cells treated with 4 μM Temp-L.

performed using the MaXQuant software, and statistical significance was assessed.

A total of 304 differentially expressed proteins were identified, 67 down- and 237 up-regulated; Table S1 and Table S2 in the Supplementary Materials summarizes the results. The most interesting proteins were analyzed within the bioinformatic tool STRING (version 12.0) which predicts Protein-Protein Interactions (PPIs) network based both on functional and physical interaction. The confidence of each interaction was set as high and the over-representation analysis was done considering statistically significantly enriched categories carrying a p-value <0.05. Network analyses indicated that most of the up-regulated proteins belonged to cellular pathways involved in cell wall organization, proteoglycan and fatty acids biosynthesis (Fig. 3).

The up-regulated proteins involved in cell wall and fatty acid biosynthesis are reported Table 1 together with their SwissProt code, gene name, and Fold Change (FC). This observation suggested the hypothesis that Temp-L treatment stimulates cell wall biosynthesis and septum formation, possibly triggering the membrane extroflexion originating the vesicle-like structures observed in TEM analyses. This consideration was further supported by the up-regulation of protein

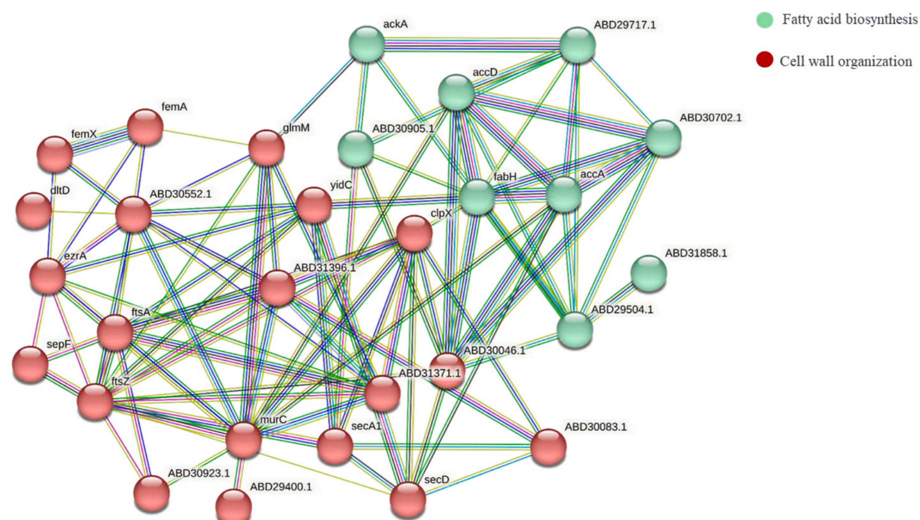

Fig. 3. STRING analysis of the up-regulated proteins following Temp-L treatment. The confidence of each interaction was set as high and the over-representation analysis was done considering statistically significantly enriched categories carrying a p-value <0.05.

Table 1
Up-regulated proteins involved in cell wall and fatty acid biosynthesis in *S. aureus* following Temp-L treatment.

Swissprot Code	Protein name	Gene	FC	Network
Q2G1C0	Ribitol-5-phosphate cytidylyltransferase 1	tarI	1.26	Cell Wall
Q2G1C0	Ribitol-5-phosphate cytidylyltransferase 1	tarI	1.26	Cell Wall
Q2G0J0	Phosphate acetyltransferase	SAOUSCH_00574	1.34	Fatty Acid Biosynthesis
Q2FZR9	3-oxoacyl-[acyl-carrier-protein] synthase 2	SAOUSCH_00921	1.38	Fatty Acid
Q2FXM6	Acetyl-coenzyme A carboxylase carboxyl transferase	accD	1.43	Fatty Acid
Q2FYI0	Penicillin-binding protein 2	SAOUHSC_01467	1.48	Cell Wall
Q2FXL5	Acetate kinase	ackA	1.61	Fatty Acid Biosynthesis
P0C0V7	Phosphoglucosamine mutase	glmM	1.64	Cell Wall
Q2FZS0	3-oxoacyl-[acyl-carrier-protein] synthase 3	fabH	1.65	Fatty Acid
Q2FY42	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	SAOUHSC_01624	1.69	Fatty Acid
Q2FWD4	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	murA	1.73	Cell Wall
Q2FV77	3-hydroxy-3-methylglutaryl coenzyme A reductase	SAOUHSC_02859	1.8	Fatty Acid
Q2FVZ4	Lipid II:glycine glycyltransferase	femX	1.82	Cell Wall
Q2FXI9	FtsK domain-containing protein	SAOUHSC_01857	1.85	Cell Wall
O07325	Cell division protein FtsA	ftsA	1.85	Cell Wall
Q2FXQ7	ATP-dependent Clp protease ATP-binding subunit ClpX	clpX	1.85	Cell Wall
Q2FYR2	Aminoacyltransferase FemA	femA	1.97	Cell Wall
Q2FXM7	Acetyl-Co A carboxylase carboxyl transferase sub alpha	accA	2.8	Fatty Acid Biosynthesis
Q2FXT8	Multifunctional fusion protein	secD	2.19	Cell Wall
O06446	Protein translocase subunit SecA 1	secA1	2.23	Cell Wall
Q2FZW3	Protein DltD	dltD	2.29	Cell Wall
Q2FXK8	Septation ring formation regulator EzrA	ezrA	2.29	Cell Wall
Q2FXJ7	1-acyl-*sn*-glycerol-3-phosphate acyltransferases domain protein	SAOUHSC_01837	2.38	Fatty acid Biosynthesis
Q2G124	Probable acetyl-CoA acyltransferase	SAOUHSC_00336	2.5	Fatty Acid Biosynthesis
Q2FZ89	Cell division protein FtsZ	ftsZ	2.7	Cell Wall
Q2FWG4	Membrane protein insertase YidC	yidC	2.85	Cell Wall
Q2FWF4	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	murA	2.92	Cell Wall
Q2FZ86	Cell division protein SepF	sepF	3.31	Cell Wall
Q2FXJ0	UDP-N-acetylmuramate–l-alanine ligase	murC	3.38	Cell Wall

machineries involved in fatty acid biosynthesis needed for lipidic bilayer formation (Table 1). Components of both the Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthetase (FAS II) complexes were found among the up-regulated proteins. ACC is a multisubunit enzyme that catalyzes the first reaction in the fatty acid biosynthetic pathway while FAS II is the enzymatic machinery involved the elongation cycle of the fatty acid chain [18].

Surprisingly, Temp-L treatment also showed the down regulation of several proteins involved in the regulation of virulence factors expression and adhesion and invasion mechanisms, as shown in the Fig. 4 and in the Table 2.

Among the identified proteins, Ebh, IsdA ClfB, SdrC and SdrD play an important role in adhesion to eukariotyc cells by promoting bacterial attachment to both components of the extracellular matrix and human fibrinogen and fibronectin inducing the formation of bacterial clumps [19] while SarA, SarX, and MsrR are involved in regulating the expression of several specific genes which are critically associated with bacterial virulence [20]. Finally, TRAP (Target of RNAIII Activating Protein) is a major regulator of the *S. aureus* pathogenesis. TRAP phosphorylation leads to the activation of the quorum-sensing Agr system the master regulator for *S. aureus* virulence factors [21]. These data suggested that incubation with the Temp-L peptide might affect *S. aureus* virulence capabilities.

2.4. Analysis of fatty acid content

We then aimed to confirm the results of the Differential Proteomic analysis by independent approaches. The increase in the expression of the enzymatic machinery involved in fatty acid biosynthesis prompted us to monitor the amount of fatty acids in *S. aureus* cells following incubation with Temp-L. Total fatty acids were extracted from treated and untreated *S. aureus* cells, converted into their corresponding methyl ester derivatives, and directly analyzed by GC-MS. The observed increase in total fatty acids in the treated sample compared to the negative control (Fig. 5) indicated a substantial alteration in the lipid composition of *S. aureus* membranes, consistent with the observed vesicle formation. This effect is further supported by the observation that clinical isolates of MRSA increased their membrane fluidity [22].

The over-expression of the ACC and FAS II enzymatic systems and the corresponding over production of fatty acids support the hypothesis that Temp-L increased membrane production in *S. aureus* possibly leading to vesicle formation [23].

2.5. Invasion assays

A number of down-regulated proteins identified in the Differential Proteomic analysis are involved in regulating the expression of adhesion and invasion factors in *S. aureus*.

In order to verify if Temp-L could affect MRSA virulence, we

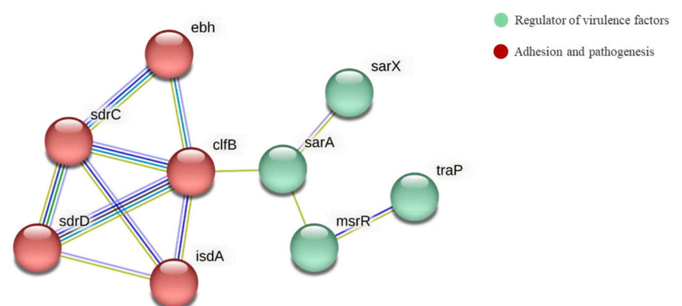

Fig. 4. STRING analysis of the down-regulated proteins following Temp-L treatment. The confidence of each interaction was set as high and the over-representation analysis was done considering statistically significantly enriched categories carrying a p-value <0.05.

Table 2
Down-regulated proteins involved in regulation of virulence factors and adhesion mechanisms in *S. aureus* following Temp-L treatment.

Swissprot Code	Proteins	Gene	FC	Network
Q2G2F3	Signal transduction protein TRAP	traP	0.38	Reg Virulence Factors
Q2G0L4	Serine-Asp repeat-containing protein D	sdrD	0.40	Adhesion
Q2G0L5	Serine-Asp repeat-containing protein C	sdrC	0.50	Adhesion
Q2FYJ6	Extracellular matrix-binding protein ebh	ebh	0.52	Adhesion
Q2FUY2	Clumping factor B	clfB	0.61	Adhesion
Q7BHL7	Regulatory protein MsrR	msrR	0.62	Reg Virulence Factors
Q2FZE9	Iron-regul. surface determinant protein A	isdA	0.62	Adhesion
Q2G0D1	HTH-type transcriptional regulator SarX	sarX	0.64	Reg Virulence Factors
Q2G2U9	Transcriptional regulator SarA	sarA	0.65	Reg Virulence Factors

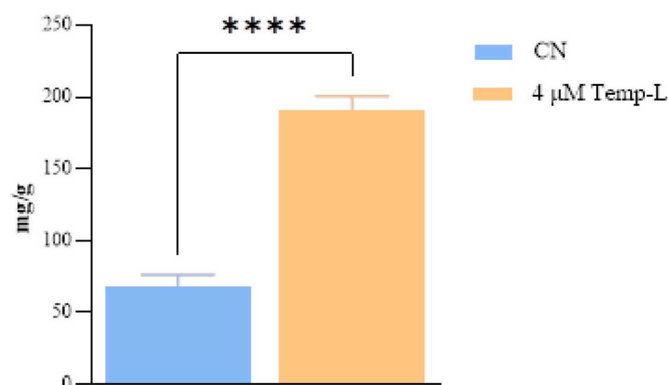

Fig. 5. The graph illustrates the concentration of total fatty acids in milligrams per gram of bacterial extract in the absence and the presence of Temp-L. Results are presented as the mean ± SD from two independent experiments. Statistical significance was determined through *t*-test analysis using GraphPad Prism 9 and p-value <0.0001 was indicated as **** in the graph.

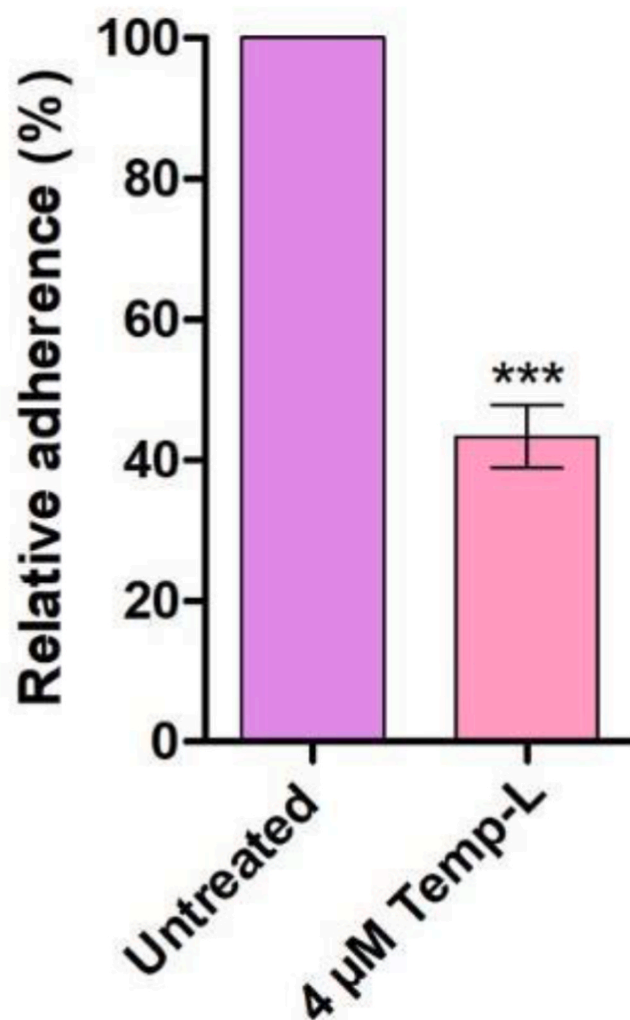

Fig. 6. Adhesion assay. Sub-inhibitory concentration of Temp-L reduced relative adherence (%) of *S. aureus* NCTC 12493 on A549 epithelial cells. Experiments were carried out in triplicate and repeated three times. The mean value is shown with standard deviation. ***P < 0.0001.

performed human cell adhesion/invasion assays using human type 2 pneumocytes A549 cells. We tested if sub-inhibitory concentration of Temp-L, which did not affect the viability of the pathogenic strain, decrease adherence of *S. aureus* on epithelial cells. To this end, A549 cells were infected with treated and untreated *S. aureus* NCTC 12493 and after removal of non-adherent bacterial cells, infected cells were lysed and colony counts were performed to determine the numbers of viable bacteria (both adherent and internalized).

Our results show that the adhesion efficiency of *S. aureus* NCTC 12493 was significantly affected by treatment with Temp-L (Fig. 6). The adhesion capacity of *S. aureus* strain was reduced by roughly twofold in the presence of the peptide, in comparison to untreated bacterial cells. This finding is in accordance with decreased expression of proteins involved in staphylococcal adhesion and host cell invasion obtained with proteomic techniques (see Table 2).

3. Discussion

Most of AMPs active against Gram-positive ~~or~~ and Gram-negative bacteria exert their mechanism of action by directly interacting with the cell membrane and cell wall, disrupting the lipid bilayer and leading to bacterial death. However, recent studies have revealed that AMPs can also interfere with cellular processes and metabolic function by targeting intracellular molecules, including nucleic acids and proteins [24]. For instance, cathelicidin-derived short Pro-rich peptides have been shown to inhibit protein translation or the assembly of the large subunit of ribosomes by interacting with the 70S ribosome subunit [25]. Temp-L and novel Temp-L analogues inhibit cell division by binding to FtsZ in *E. coli* cells [16–26], while Magainin-2 interacts with the membrane protein BamA in *E. coli* impairing the correct folding of the Outer Membrane Proteins (OMPs) [10]. Although intracellular targets have been identified in Gram-negative microorganisms, little is known about such mechanisms in ~~on~~ Gram-positive bacteria, such as the pathogen *S. aureus*.

In this study, we analyzed the morphological changes in *S. aureus* induced by the antimicrobial peptide Temp-L using TEM and DLS analyses to elucidate the effect of the peptide on the bacterial phenotype. Several cell membrane protrusions occurred in response to Temp-L incubation, suggesting the formation of vesicle-like structures. DLS measurements of treated *S. aureus* cells over time showed a decrease in bacterial cell size and the emergence of a second population, possibly associated with extracellular vesicle.

Based on these morphological observations, we were stimulated to investigate the mechanism of vesicle formation at the molecular level. Differential proteomic experiments were designed to provide insight into the effect of Temp-L on *S. aureus* by monitoring changes in bacterial

protein expression in response to peptide treatment. The proteomic results revealed a significant increase in the expression of proteins involved in cell wall organization and fatty acid biosynthesis, supporting the previously detected vesicle formation.

The production of membrane vesicles to attenuate the destructive effects of antibiotic treatments has been previously reported in *S. aureus* [27]. Under antibiotic stress conditions, *S. aureus* greatly increased the secretion of extracellular vesicles compared to bacterial cells grown without stress. The activity of vancomycin against *S. aureus* was counteracted by vesicle formation, which greatly raised MIC values and increased the survival of bacterial cells pre-treated with the antibiotic [28]. Overall, the phenomenon of lipid-coated vesicle formation is considered a critical factor in antibiotic resistance, contributing to bacterial survival in the presence of antibiotics and to the pathogenesis of *S. aureus* [29].

These observations were supported by an increase in enzymatic machinery involved in the biosynthesis of fatty acid required for assembling the lipid bilayer surrounding the extracellular vesicle. Most of the enzymatic components of both the Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthetase (FAS II) complexes were identified among the proteins up-regulated following Temp-L treatment. ACC is a multi-subunit enzyme that catalyzes the first reaction in the fatty acid biosynthetic pathway, whereas the FAS II complex and the FabH enzyme catalyze the elongation step to produce both straight and branched chain fatty acids. The activity of these machinery constitutes the first step in bacterial membrane lipid synthesis. These data were confirmed by GC-MS analysis, showing an increase in total fatty acids extracted from the treated sample compared to the negative control. Genes involved in fatty acid biosynthesis were also found upregulated in a *S. aureus* mutant with a decreased telavancin susceptibility [30] and increased amounts of longer-chain, unsaturated fatty acids were found in *S. aureus* strains resistant to thrombin-induced platelet microbicidal proteins [31]. This is further supported by the observation that clinical isolates of methicillin-resistant *S. aureus* increased their membrane fluidity [32].

Surprisingly, besides the upregulation of cell wall and fatty acid biosynthetic pathways, proteomic data pointed out to a decrease in the expression levels of several proteins involved in the regulation of virulence factors. The expression of virulence proteins in *S. aureus* is controlled by a network of regulatory loci associated with the expression of cell wall-associated adhesins during exponential growth collectively known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) [33]. These proteins specifically interact with some major plasma or extracellular matrix protein components of normal tissues or those coating biomedical devices. Most of these MSCRAMMs, including the extracellular matrix-binding protein Ebh, the clumping factor ClfB, and the fibrinogen and fibrinonectin-binding protein IsdA, together with SdrC and SdrD which promote bacterial adhesion to host cells have been found down-regulated following Temp-L incubation [34]. Moreover, treatment with the AMP led to a decrease in the production of several transcriptional regulators involved in controlling the expression of virulence factors, including SarA, SarX, and MsrR.

Finally, the TRAP protein, a well-known key molecule regulating the pathogenesis of *S. aureus*, was also found down-regulated upon Temp-L incubation. Expression and histidine phosphorylation of TRAP are necessary for the expression of most bacterial toxins and their global regulator agr. Decreased expression or inhibition of TRAP phosphorylation makes *S. aureus* unable to produce virulence factors, leading to a complete loss of staphylococcal pathogenesis [35]. Adhesion and invasion assays on eukaryotic A549 cells (human type 2 pneumocytes) infected with Temp-L-treated *S. aureus* confirmed that the peptide affected adhesion and invasion properties of *S. aureus*, thus reducing its infectious capabilities.

The evidence reported here, based on proteomic approaches combined with microscopic, spectroscopic, and cellular assays, showed that *S. aureus* triggers a defence mechanism based on the up-regulation of several proteins responsible for cell wall and fatty acids biosynthesis in the presence of the antimicrobial peptide Temp-L, along with a significant decrease in its virulence and pathogenicity.

The findings presented herein provide compelling evidence of Temp-L's efficacy in diminishing the virulence and pathogenicity of *S. aureus*. This assertion aligns with existing literature demonstrating similar outcomes with other AMPs. For instance, previous studies have highlighted the capacity of defence peptides such as human defensins to attenuate virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid in mouse and neonatal rat models [36].

The present study have significant implications for advancing Temporin-L as a promising therapeutic agent. By clarifying its effects at sub-MIC concentrations, we identify potential cellular pathways to use Temporin-L's antimicrobial properties while minimizing the risk of bacterial resistance emergence. Furthermore, understanding the mechanisms of action of Temporin-L can lead to the development of peptidomimetic drugs approaches to be used in innovative antimicrobial treatments. However, challenges persist in translating Temporin-L from laboratory research to clinical application, including issues related to formulation, pharmacokinetics, and toxicity profiles. Addressing these obstacles will be crucial in realizing the complete clinical potential of Temporin-L as an effective and safe therapeutic option against bacterial infections.

The current findings are highly motivating and contribute to the search for better strategies to counteract the health threats associated with this dangerous pathogen.

4. Materials and methods

4.1. Bacterial strain and eukaryotic cells culture conditions

Methicillin-resistant *S. aureus* NCTC 12493 was used for all the experiments. Planktonic cultures were grown in Luria broth (LB, Oxoid) at 37 °C under vigorous agitation (180 rpm). A549 cells were cultured in Dulbecco modified Eagle medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), I% glutamine, and 1 % penicillin/streptomycin in an atmosphere of 5 % CO_2. Monolayers were used 48 h after seeding.

4.2. Transmission Electron Microscopy (TEM)

S.aureus (MRSA) (NCTC 12493) were grown to 0.1 OD in the presence of 4 μM Temp-L in sub-MIC concentration for 1h at 37 °C. In this experiment as well as in all the others performed in this study a Temp-L concentration well below the previously determined MIC value was used. Cells were then centrifuged, washed with PBS, and prepared for TEM microscopy. After the incubation, bacterial cells were centrifuged at 3000 rpm for 15 min, washed with PBS and resuspended in PBS and 2.5 % glutaraldehyde to fix the samples. 10 μL of the sample were applied to a glow-discharged formvar/carbon film copper mesh grid and ~~leave~~ allowed to adsorb for 2 min. Excess liquid was removed by washing with water, and the sample was stained with 1 % uranyl acetate. The grids were then dried before TEM analyses. TEM was performed using a JEOL JEM-1400 TEM with an accelerating voltage of 120 kV. Digital images were collected with an EMSIS Xarosa digital camera with Radius software.

4.3. Dynamic Light Scattering (DLS)

S. aureus cells were grown to 0.5 OD/mL in the presence of 4 μM of Temp-L for 1h, 2h, 3h, 4h, and 5h. After incubation, bacterial cells growth at 1 $OD_{600\ nm}$ were centrifuged at 5,0~~.~~000 rpm for 15 min, washed three times with 1X PBS, resuspended in 1X PBS. Untreated cells at different time~~s~~ points were used as controls. Dynamic Light Scattering (DLS) measurements were performed with a homemade instrument

composed by a Photocor compact goniometer, an SMD 6000 Laser Quantum 50 mW light source (Quantum Laser, Heaton Mersey, UK) operating at 532.5 nm, a photomultiplier (PMT-120-OP/B), a correlator (Flex02-01D), and a thermostat bath for the temperature control. The experiments were carried out at a constant temperature of ~~(~~25.0 ± 0.1~~)~~ °C and at a scattering angle of 90°. The hydrodynamic radius R_h was determined from the diffusion coefficient of scattered particles through the Stokes-Einstein equation:

$$R_h = \frac{k_B\, T}{6\pi\eta D}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity [37,38].

4.4. Differential Proteomic

S. aureus cells were grown in 5 mL BHI medium and treated at 0,5 OD/mL with 4 μM of Temp-L for 1h at 37 °C under stirring. Untreated cells were used as control and the experiment was performed in duplicate. The pellets were recovered by centrifugation at 4 °C for 10 min at 5000 rpm, and washed ~~for~~ three times in PBS 1X, resuspended in 100 mM Tris HCl, 2 % SDS, 100 mM DTT, pH 7,6, and incubated for 5 min at 95 °C. The cell cultures were sonicated at 200 W power for 30 min on ice and then centrifuged at 4 °C for 10 min at 16,000 rpm to pellet unlysed cells and cellular debris. The recovered supernatant was quantified using the Bradford assay.

50 μg of each sample was digested by trypsin onto to S-trap™ micro spin columns, following the standard protein digestion protocol of the manufacturer (Protifi, Huntington, NY). The resulting peptide mixtures were analyzed by Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS) using a LTQ Orbitrap XL coupled to a nanoLC system (ThermoFisher Scientific, Waltham, MA). All peptide mixtures were fractionated onto a C18 capillary reverse-phase column (200 mm lenght, 75 μm ID, 5 μm biosphere), using a non-linear 5 %–50 % gradient for eluent B (0.2 % formic acid in 95 % acetonitrile) in A (0.2 % formic acid and 2%acetonitrile in MilliQ water) over 260 min.

MS/MS analyses were performed using Data-Dependent Acquisition (DDA) mode by fragmenting the 10 most intense ions in CID modality. All samples were run in duplicates. Raw data obtained from nano LC-MS/MS were analyzed with MaxQuant (v.1.5.2.8) using UniProt *S. aureus* as database for Andromeda search [39]. The selected parameters for protein identification were the following: minimum 2 peptides, at least 1 unique; variable modifications allowed were methionine oxidation and pyroglutamate formation on N-terminal glutamine; accuracy for the first search was set to 10 ppm, then lowered to 5 ppm in the main search; 0.01 FDR was used, with a reverse database for decoy; retention time alignment and second peptides search functions were allowed. The fold changes (FCs) were calculated according to LFQ values.

The confidence of each experiment was assessed by statistical analysis using the MultiExperiment Viewer (MeV) software and a p-value <0.05.

4.5. GC-MS analysis

Fatty acids extracted from *S. aureus* cells were converted in methyl esters by adding MeOH, H_2SO_4 and chloroform and incubated for 16 h at 90 °C under stirring. The GC-MS analysis was performed with a HB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm film thickness). The flow rate of helium carrier gas was set at 1 mL/min 1 μl of sample was injected with a 3 min of solvent delay time and split ratio of 20:1. The ionization occurred in the electron impact (EI) mode at 70 eV. The MS data were acquired in full scan mode from m/z 40–400 with acquisition frequency of 12.8 scans per second. The identification of compounds was confirmed by injection of pure standards and comparison of the retention time and corresponding EI MS spectra. The content of fatty acids was calculated by external standard method.

4.6. Adhesion/invasion assays on A548 eukaryotic cell lines

The adhesion assays were carried out as previously described [40] with the following modifications. *S. aureus* NCTC 12493 from overnight cultures in LB broth, grown in the absence of Temp-L were further sub-cultured up to OD 600 of 0.5 at 37 °C in LB broth and treated with or without 4 μM of Temp-L for 1h under stirring. Cells were then pelleted, washed twice with DMEM (without FBS and 1 % penicillin/streptomycin) and re-suspended in DMEM (without FBS and 1 % penicillin/streptomycin) to a density of ~1 × 10 7 CFU/mL (defined as the original bacterial CFU). A549 human lung epithelial cells (ATCC CCL 185) were grown in complete DMEM medium, then were seeded into 12-well plates at a concentration of 2 × 10 5/ml in DMEM medium (without FBS and 1 % penicillin/streptomycin) and grown to 90 % confluence at 37 °C and 5 % CO_2. On the day of infection, cells were washed twice with PBS and infected with 1 ml of the prepared bacterial inoculum to produce a multiplicity of infection (MOI) of 50: 1 (1 x 10 7 bacteria: 2 × 10 5 cells). The plates were quickly centrifuged for 1 min at 500 g to allow bacterial adhesion to the cell monolayer and incubated for 60 min at 37 °C in 5 % CO_2 (v/v) atmosphere. A549 cells were then washed three times with PBS and lysed by the addition of 1 ml distilled water. To determine the total number of cell-associated bacteria, corresponding to adherent and intracellular bacteria, serial 10-fold dilutions of the resulting suspension were carried out in PBS and plated on LB agar incubated at 37 °C for 18 h.

The bacterial adhesion in each well was determined as the CFU that adhered to and invaded into the cells. The controls were pretreated with medium alone (without Temp-L) considered to have 100 % adhesion. Adhesion was then normalized against controls according to the equations:

Relative adherence = Adhered&Internalized bacteria CFU of sample/Original CFU of sampleAdhered&Internalized bacteria CFU of control/Original CFU of control.

Each experiment was performed in triplicate and almost three times. The relative adhesion values were statistically analyzed by Student's t-test, using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, United States). Differences between mean values were tested for significance using Student's t-test for paired samples. A P-value <0.0001 was considered to be statistically significant.

Data availability

All data are contained within the article.

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CRediT authorship contribution statement

Carolina Canè: Conceptualization, Investigation, Validation, Visualization, Writing – original draft. **Noemi Gallucci:** Formal analysis, Methodology, Validation, Writing – original draft. **Angela Amoresano:** Formal analysis, Methodology, Validation, Writing – original draft. **Carolina Fontanarosa:** Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Luigi Paduano:** Formal analysis,

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Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101808.

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