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Efeito da terapia fotodinâmica sobre os fatores de virulência de *Staphylococcus aureus*

Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus*

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Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Maria do Amparo Ferreira Faustino, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery also has its beauty. Neither do I believe that the spirit of adventure runs any risk of disappearing in our world. If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity."

Maria Skłodowska-Curie

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Palavras-chave

Resumo

Staphylococcus aureus é uma espécie bacteriana Gram-positiva que integra a microbiota humana. No entanto, as bactérias desta espécie podem tornar-se patogénicas para os humanos. Devido ao aumento de ocorrência de *S. aureus* resistentes a antibióticos tornam-se necessárias novas abordagens terapêuticas no controlo deste organismo patogénico.

O processo antimicrobiano de inativação fotodinâmica (PDI) é baseado no uso combinado de luz, oxigénio e um agente fotoativado (designado por fotossensibilizador). A interação destes três componentes leva à formação de espécies reativas de oxigénio, altamente citotóxicas, que danificam, de forma irreversível, componentes vitais das células microbianas, podendo culminar na morte celular. A inativação fotodinâmica tem-se mostrado, de facto, uma alternativa promissora na inativação de microrganismos patogénicos. Ainda assim, o conhecimento sobre o efeito que esta abordagem tem sobre os fatores de virulência ainda é escasso. O objetivo deste trabalho de dissertação foi avaliar os efeitos da PDI sobre fatores de virulência de *S. aureus*, Para tal, recorreu-se ao tetra-iodeto de 5,10,15,20-tetraquis(1-metilpiridínium-4-il)porfirina (Tetra-Py$^+$-Me), usado como fotossensibilizador, e estudou-se o seu efeito seis estirpes de *S. aureus* (uma estirpe de referência, uma estirpe que expressa uma enterotoxina, duas estirpes com três enterotoxinas e duas estirpes resistentes à meticilina, MRSA, uma expressa cinco enterotoxinas e a segunda não enterotoxica). O efeito da fotossensibilização foi verificado na atividade da catalase, beta hemólise, lípases, termonuclease, produção de enterotoxinas e da enzima coagulase, bem como na resistência à meticilina.

Os resultados indicaram que a expressão de alguns fatores de virulência das células sujeitas ao processo fotodinâmico são afetados pela PDI. Adicionalmente verificou-se que a suscetibilidade das estirpes bacterianas à PDI não diminui ao longo de vários tratamentos consecutivos.

Keywords

Abstract

Staphylococcus aureus are Gram-positive bacteria who integrate the human microbiota. Nevertheless, these bacteria can be pathogenic to the humans. Due to the increasing occurrence of antibiotic-resistant *S. aureus* new approaches to control this pathogen are necessary.

The antimicrobial photodynamic inactivation process (PDI) is based in the combined use of a light source, an oxidizing agent like oxygen and an intermediary agent (a photosensitizer). These three components interact to form cytotoxic reactive oxygen species that irreversibly damage vital constituents of the microbial cells and ultimately lead to cell death. In fact, PDI is being shown to be a promising alternative to the antibiotic approach in the inactivation of pathogenic microorganisms. However, information on effects of photosensitization on particular virulence factors is strikingly scarce. The objective of this work was to evaluate the effect of PDI on virulence factors of *S. aureus*. For this, as photosensitizer the 5,10,15,20-tetrakis(1-methylpyridi-nium-4-yl)porphyrin tetra-iodide (Tetra-Py$^+$-Me) and six strains of *S. aureus* (one reference strain, one strain with 1 enterotoxin, two strains with 3 enterotoxins and two strains resistant to methicillin, MRSA – one with 5 enterotoxins and the other without enterotoxins) were used. The effect of photosensitization on catalase activity, beta hemolysis, lipases, thermonuclease, enterotoxins, coagulase production and resistance to methicillin was assessed.

The results indicate that the expression of some virulence factors in the cells subjected to this therapy is affected. Additionally the susceptibility of the strains to PDI did not decrease upon successive treatments.

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List of Acronyms

CPS, coagulase-positive staphylococci
CoNS, coagulase-negative staphylococci
DNA, deoxirybonucleic acid
MSCRAMM, microbial surface components recognizing adhesive matrix molecules
ROS, reactive oxygen species
SE, staphylococcal enterotoxins
Agr, accessory gene regulator
Sae, staphylococcal accessory element
sarA, staphylococcal accessory regulator A
σ^A, transcription factor sigma A
σ^B, alternative transcription factor sigma B
RNA, ribonucleic acid
MRSA, methicillin-resistant *Staphylococcus aureus*
PS, photosensitizer
PDI, photodynamic inactivation
$^1O_2$, singlet oxygen
O_2, molecular oxygen
PG, phosphatidylglycerols
CL, cardiolipins
Tetra-Py$^+$-Me, 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide
CFU, colony forming units
PBS, phosphate buffered saline
PCA, plate count agar
BAP, blood agar plates
BPA, Baird Parker agar
MSA, mannitol salt agar
Abs, absorbance

Chapter 1

Introduction

1.1 Context

The development of this study is founded on joint work that has been developed between the Organic Chemistry (QOPNA) group from the Chemistry Department (dqua) and the Laboratory of Applied and Environmental Microbiology (LMAA) from the Biology Department (dbio), both research groups from University of Aveiro.

One of the main goals of Organic Chemistry group in the field of organic synthesis and natural compounds focuses on improvement, synthesis and characterization of new tetrapyrrolic macrocycles obtained by isolation from natural sources or by organic synthesis and their applicability mainly in the fields of biology, nanotechnology, medicine and solar cells. Tetrapyrrolic macrocycles, particularly porphyrin derivatives, are one of the most exciting class of organic compounds due to their biological functions and potential applicability.

The evolution of the new synthetic porphyrin derivatives obtained in Organic Chemistry group have been done in collaboration with national and international research groups, including the Laboratory of Applied and Environmental Microbiology, dbio UA. Therein, have been assessed the potential applications for these new compounds as new antimicrobial approaches.

There is already a wide range of work conjointly carried out between the two research groups. In this follow-up, this work is a new moment of collaboration.

1.2 Motivations

This work theme appeared as consequence of several studies already conducted in cooperation between the LMAA and QOPNA research groups (UA). The antimicrobial approach addressed in this study has been more intensively studied thanks to the advantages it has shown over typical and commonly used antibiotic approaches.

1.3   Objectives

Through the development of this work, our main goals were to evaluate the potential application of the tested porphyrinic compound as new antimicrobial approach in the inactivation of microorganisms resistant to already implemented antimicrobial methods and to identify if the new approach shows to have a direct effect on the virulence factors expressed by several *Staphylococcus aureus* strains.

1.4   Guide of document organization

The present document is structured in four chapters, which are summarized as follows:

Chapter 1. Introduction. This chapter frames the work that was carried out under the dissertation theme: the context in which the issue arose, the motivations to explore this topic and the main objectives we wanted to achieve by exploring this topic.

Chapter 2. State of the Art. In this chapter are presented generalities about the underlying concepts to the theme of this work, including the microorganism species and the therapeutic approach studied.

Chapter 3. Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus.* This chapter is structured as an Original Research manuscript, including a brief introduction to the topic, the description of the materials and methods used; the obtained results are here shown and discussed. The presented manuscript was already submitted to the date of this document presentation and is "in revision" in the journal Frontiers in Microbiology.

Chapter 4. Additional communications. In this final chapter are presented additional communications made as result of the developed work exposed in this document.

Chapter 2

State of the Art

2.1 *Staphylococcus aureus*

2.1.1 *Staphylococcus* genus

Staphylococcus genus belongs to *Staphylococcaceae* family (Winn Jr et al. 2006). This genus is constituted for Gram-positive bacteria (Winn Jr et al. 2006; Hennekinne et al. 2010; Costa et al. 2013), characterized by individual cocci, whose dimensions vary between 0.5 and 1.5 μm in diameter (Santos et al. 2007; Costa et al. 2013). Its cells tend to form clusters with grape-like form (Hennekinne et al. 2010), once cell division occurs in more than one plane (Plata et al. 2009; Costa et al. 2013). This group of microorganisms does not have motile components (Hennekinne et al. 2010; Bien et al. 2011), does not form spores (Hennekinne et al. 2010) and, characteristically, are facultative anaerobic (Hennekinne et al. 2010), capable of generate energy through aerobic respiration and fermentation (Plata et al. 2009; Costa et al. 2013). *Staphylococcus* spp. are oxidase-negative, resistant to heat, have a complex nutritional requirement for growth (Plata et al. 2009; Costa et al. 2013) and are catalase-positive (Hennekinne et al. 2010). This is an important aspect once catalase is a hemeprotein that decomposes hydrogen peroxide into water and oxygen and may function as a protector enzyme to the bacteria, once the degradation of hydrogen peroxide is a bactericidal mechanism mediated by leukocytes (Mandell 1975). Actually, some studies suggested that staphylococcal catalase, by breaking down hydrogen peroxide produced by phagocytes, protects intraphagocytic bacteria and subsequently plays a role as a significant bacterial virulence factor (Mandell 1975).

Hennekinne et al. in 2010 reported that fifty staphylococci species and subspecies had been already described. Usually, *Staphylococcus* genus organisms are divided in two groups, according to their ability to produce the enzyme coagulase – an enzyme which action promotes blood clotting –, coagulase-positive staphylococci (CPS) (Hennekinne et al. 2010) and coagulase-negative staphylococci (CoNS) (Plata et al. 2009; Costa et al. 2013). The last mentioned group enwraps common *staphylococci* commensals of the skin, and to the first group, coagulase-positive group, belongs *Staphylococcus aureus* species (Costa et al. 2013).

2.1.1.1 Gram-positive bacterial structure

The principal structural components present in the bacterial cells (prokaryote beings) are the genome (deoxyribonucleic acid, DNA), ribosomes, cytoplasmic membrane and the cell wall (these two components form the cell envelope) and, in some cases, a cell capsule – an exterior layer to the cell envelope (Azevedo 2005).

Bacterial cells do not show an organized nucleus (in contrast to eukaryotic cells) (Azevedo 2005; Quintas 2008). Their genome is localized in a cytoplasmic region called nucleoid (Quintas 2008). Externally, involving cellular content, bacterial cells are constituted by a cell wall – majorly constituted by peptides and polysaccharides (Quintas 2008). Separating cell wall from cytoplasm, there is a cytoplasmic membrane which is adherent to the wall (Quintas 2008) and combined, these two cellular components ensure the isolation of the cytoplasmic content, protection to potential harmful agents and, mainly the cell wall, confer mechanic support to the cells (Azevedo 2005).

The cytoplasmic membrane is composed by a phospholipidic bilayer (Azevedo 2005), providing the permeability needed to these cells. Phospholipids are molecules with an amphiphilic nature. They have, in their structure, a negatively charged phosphate "head" attached to a glycerol molecule (hydrophilic components) by phosphoester bonds and a fatty acid "tail" (hydrophobic component) typically linked also to the glycerol molecule by ester bonds (Azevedo 2005). Additionally, cytoplasmic membrane plays an important role serving for the anchorage of proteins responsible for the communication with the external medium, as intercellular communication and signalization (Azevedo 2005).

The bacterial cell wall is composed by a rigid heteropolymer – the peptidoglycan – constituted by linear chains of two amino sugars units: the *N*-acetylmuramic and *N*-acetylglucosamine acid alternately arranged and linked by glycosidic linkages β-(1 → 4) (Azevedo 2005). Crosslinked bonds are formed between the *N*-acetylmuramic acid molecules by short peptide chains (usually, tetrapeptide chains), which confers the rigidity to this macromolecule and, consequently to the cell wall.

The structural differences that occur in bacterial cell wall, observed among bacterial species, are responsible for the success of a well-known differential coloration technique – the Gram coloration, developed by Christian Gram, in 1884 (Azevedo 2005) – so, according to the structural organization of the bacterial cells, these microorganisms are divided into two sub-groups: Gram-positive and Gram-negative bacteria, which mainly differs in the peptidoglycan layer thickness, the inexistence of an outer cell membrane in the Gram-positive organisms, the presence of teichoic and lipoteichoic acid in Gram-positive cells and lipopolysaccharides in Gram-negative cells (Figure 1).
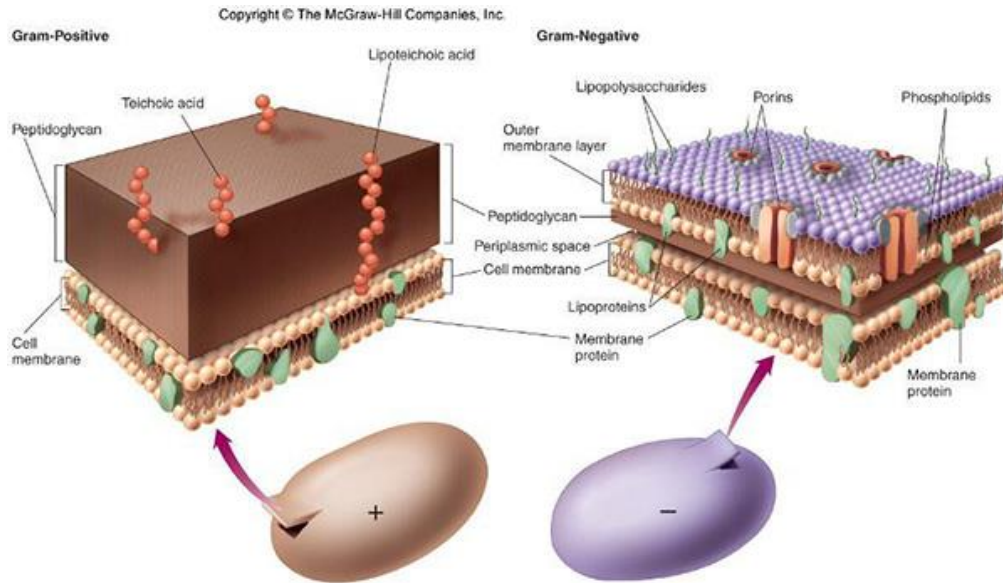
Figure 1. Structural differences of membrane and cell wall in Gram-positive and Gram-negative bacteria. (Prescott et al. 2005)

The thickness of the peptidoglycan layer may vary between different bacterial groups – it is considered that the constituents of peptidoglycan are arranged as a monolayer in Gram-negative bacteria, which has interruptions in the adhesion zones to the cytoplasmic and the outer membranes (Azevedo 2005). The peptidoglycan layer is thinner in Gram-negative bacteria when compared to Gram-positive bacteria (Figure 1).

2.1.2 *Staphylococcus aureus* species

The *S. aureus* bacterium was described for the first time in 1880 by Alexander Ogston (Santos et al. 2007), and the specific epithet assigned to this microorganism refers to the fact that the colonies formed by multiplicative division in nutritionally rich solid medium agar have a golden color, caused by the presence of carotenoid pigments (Costa et al. 2013).

S. aureus are the most pathogenic of the *Staphylococcus* genus microorganisms (Cheung et al. 2004; Winn Jr et al. 2006; Costa et al. 2013). Generally, this microorganism integrates, asymptomatically (Bien et al. 2011; Baptista et al. 2015(b)), the skin and the skin glands as well as the mucous membrane microbiota of healthy individuals, as commensal bacteria (Cassettari et al. 2005; Winn Jr et al. 2006; Plata et al. 2009; Costa et al. 2013). It is estimated that 20 - 30% of the population is colonized by this microorganism in a permanent and asymptomatic way, while 30% is estimated to be transient carriers of *S. aureus* (Costa et al. 2013). These numbers represent an increased risk of infection if the immune defenses of the host organism are compromised due to a trauma (Winn Jr et al. 2006; Costa et al. 2013). Once host defenses are impaired *S. aureus* has the ability to become lodged in the tissue, causing an inflammatory response (Santos et al. 2007).

After invasion of the host organism, and due to the synthesis and release of staphylococcal virulence factors, this bacterium has the ability to trigger many infectious processes: from chronic skin infections to toxinoses such as food poisoning outbreaks (SFPOs) (Hennekinne et al. 2010) and toxic shock syndrome to systemic and life-threatening infections as brain abscesses, meningitis and bacteremia (Winn Jr et al. 2006; Santos et al. 2007; Bien et al. 2011; Baptista et al. 2015(b)).

Currently, *S. aureus* is the most common microorganisms in pyogenic infections worldwide (Bronner et al. 2004; Santos et al. 2007). This is also a species of greatest importance in community-acquired and also in nosocomial environment (Bien et al. 2011), once it is often associated to the development of numerous infections in humans in the hospital environment (Cassettari et al. 2005). Its ability to cause such a wide range of infections is closely related to its ability to express a variety of factors involved in the pathogenesis of infection widely referred as virulence factors (Costa et al. 2013; Baptista et al. 2015(a)). These act at two levels: the adhesion to host cells/tissues and the infliction of toxic effects in the host organism (Bien et al. 2011; Costa et al. 2013).

2.1.3 *Staphylococcus aureus* strains, the population heterogeneity

Being a pathogenic bacterium, *S. aureus* is subjected to selective pressure imposed both by the action of the host immune system and the antibiotic treatments. It is known that bacteria can survive and adapt by the joint action of the gene expression regulatory systems to stress situations (Costa et al. 2013). However, this type of regulation may not be sufficient in certain cases of unpredictable stress. Thus, to overcome extreme situations, the bacterial cells tend to use alternative mechanisms such as the occurrence of mutations, which produces microbial diversity (Costa et al. 2013). As a result of this diversity, variants are generated (strains) and they tend to differ in their ability to adapt to new environments, compared with other population members, as different virulence factors expression (Winn Jr et al. 2006). Thus, the species ensure their survival, maintenance and/or improvement of their functions in response to environmental changes, as the antibiotic resistance development (Costa et al. 2013).

2.1.4 Virulence factors expressed by *Staphylococcus aureus*

The *S. aureus* virulence factors can be divided into factors of the cell surface and secretion factors. Cell surface factors include components of microbial cell surface (MSCRAMM), capsular polysaccharides and staphyloxanthin (Bien et al. 2011; Costa et al. 2013), which act in recognition and adhesion of bacterial cells to cells/tissues of the host, a critical step in the initiation of the colonization process (Burke et al. 2010; Bien et al. 2011). The MSCRAMM are covalently anchored to the peptidoglycan (Winn Jr et al. 2006; Bien et al. 2011) and include the staphylococcal protein A (SpA), fibronectin (FnbpA and FnbpB) and collagen binding proteins (Cheung et al. 2004; Burke et al. 2010; Bien et al. 2011), and clumping factor proteins (ClfA and ClfB) (Bien et al. 2011; Costa et al. 2013). Respectively, these factors are responsible for binding to immunoglobulin G (IgG) –

which inhibit the opsonization and phagocytosis processes; for binding to fibrinogen, fibronectin (Bien et al. 2011) and elastin (Burke et al. 2010) – which are adhesive proteins involved in adhesion of cells to the matrix and in coagulation, cicatrization and phagocytosis processes; for adherence to collagen of the tissues; for mediation of agglutination and the adhesion to fibrinogen in the presence of fibronectin (Bien et al. 2011; Costa et al. 2013). The capsular polysaccharides reduce the phagocytosis capacity by neutrophils and increase the rate of bacterial colonization and its permanence on mucosal surfaces (Costa et al. 2013), while staphyloxanthin (Lin and Peterson 2010; Costa et al. 2013; Baptista et al. 2015(b)), a carotenoid pigment, confers resistance to phagocytosis by neutrophils (Costa et al. 2013) – a host defense major aspect against staphylococcal infection (Mandell 1975) – being able to absorb energy from reactive oxygen species (ROS) and so protecting the bacteria against these ROS, such as hydrogen peroxide (Baptista et al. 2015(b)), once the hydrogen peroxide-myeloperoxidase-halide system is a potent bactericidal mechanism for organisms ingested by phagocytes (Mandell 1975).

The secreted factors, or exotoxins, are generally responsible either for the impairment of the integrity of the cell/tissues of the host – which result in the release of nutrients to the extracellular medium, facilitating bacterial growth (Bien et al. 2011) – as for the weakening of effective activity of the immune system (Lin and Peterson 2010). These include superantigens, toxins and cytolytic exoenzymes (Bien et al. 2011; Costa et al. 2013).

The synthesized superantigens by *S. aureus* include more than twenty staphylococcal enterotoxins (SE) – SE A, B, C1, C2, C3, D, E, G, H, I, R, S, T, U2 and V –, SE-like (SEl) – J, K, L, M, N, O, P, Q and lU –, (Hennekinne et al. 2010; Baptista et al. 2015(b)) and also the toxic shock syndrome toxin-1 (TSST-1), responsible for stimulating lymphocyte proliferation, lymphokine production even present at low concentration, making them massive immune T cell activators (Johnson et al. 1991; Hennekinne et al. 2010; Costa et al. 2013), which can lead to the immune system failure. Staphylococcal enterotoxins are a family of small (24 – 30 kDa) and single-chain proteins with a role in pathogenicity on food poisoning (Johnson et al. 1991; Hennekinne et al. 2010). These globular proteins can be encoded in prophages, plasmids and chromosomal pathogenicity islands (Hennekinne et al. 2010). They are highly hydrophilic, show a low content in α-helix and a high content in β-sheet – what suggest that these proteins have an accessible and flexible structure –, and are pH resistant, acid and heat stable (Johnson et al. 1991; Baptista et al. 2015(b)). These proteins are also resistant to digestive tract proteolytic enzymes activity, as pepsin (Hennekinne et al. 2010), whereby these proteins maintain their function in the digestive tract (Baptista et al. 2015(b)).

Cytolytic toxins include the leukocidin family (Badarau et al. 2014) and hemolysins α, β and γ (cytolysins) (Bien et al. 2011). Leukocidins, which includes Panton-Valentine leucocidin (PVL) (Bien et al. 2011), play an important role in the staphylococcal virulence, once they promote the lysis of phagocytic cells (Bien et al. 2011), contributing to immune evasion (Badarau et al. 2014). The cytolisins induce the lysis of a wide range of cells as monocytes and platelets (Bien et al. 2011; Costa et al. 2013), the hemolysis of sphingomyelin present in the cytoplasmatic membrane of cells such as monocytes, neutrophils, lymphocytes and erythrocytes and increase the susceptibility of host cells to other lytic agents, the lysis of erythrocytes (Bien et al. 2011; Costa et al. 2013;

Badarau et al. 2014). This group of toxins form pores, with a β-barrel structure (Winn Jr et al. 2006; Bien et al. 2011), in the membranes of target cells, causing the output of the cell content when present in low concentrations and cell lysis when present in high concentrations (Lin and Peterson 2010; Costa et al. 2013), providing nutrients required for bacterial growth (Bien et al. 2011).

The exoenzymes group includes lipases (responsible for fatty acids hydrolysis), nucleases (accountable for nucleic acids strands cleavage), serine and cysteine proteases, hyaluronidase (Bien et al. 2011) and staphylokinase SAK (Costa et al. 2013), whose activity is centered on disruption of the cells and tissues of the host and inactivation of some of its antimicrobial immune mechanisms (Lin and Peterson 2010; Costa et al. 2013).

2.1.5 *Staphylococcus aureus* virulence factors regulation

The intervention of several virulence factors seems to be related to the various stages of the infection process: colonization, immune system components inactivation, bacterial proliferation and spread (Bien et al. 2011; Costa et al. 2013). The regulation of the virulence factors expression involves several regulatory systems that react to quorum sensing and to the conditions of the cellular surrounding environment (Cheung et al. 2004; Bien et al. 2011; Costa et al. 2013) – as the early expression of adhesins and the late release of toxins during infection process (Bien et al. 2011) –, which includes regulator *loci agr* (accessory gene regulator) and *sae* (staphylococcal accessory element), the staphylococcal accessory regulator (*sarA*) (Bischoff et al. 2001; Bronner et al. 2004), transcription sigma factor A (σ^A) and the alternative transcription factor B (σ^B) (Costa et al. 2013). These virulence factors regulators require, at first, the upregulation expression of genes coding to cell surface proteins, most of them responsible for the adhesion to the host cells and the inactivation of the immune system elements, as *sae* and *sarA loci* and, at last, the overproduction of toxins responsible for the later stages of infection, as *agr locus* (Bischoff et al. 2001; Bronner et al. 2004; Bien et al. 2011; Costa et al. 2013). Additionally, the sigma factors are responsible for regulating the expression of housekeeping genes essential for bacterial growth (σ^A), and by the expression of genes involved in cellular functions, such as the stress-response trigger (σ^B) (Bischoff et al. 2001; Bischoff et al. 2004; Cheung et al. 2004; Costa et al. 2013).

Sigma factors are of great importance in bacterial survival under extreme conditions and in expression of virulence (Bischoff et al. 2004). They bound to the enzymatic core of RNA polymerase (E) – who catalyze the transcription of DNA into RNA (ribonucleic acid) – directing the formed holoenzyme (E-σ) to specific promoter elements, allowing the initiation of transcription process (Bischoff et al. 2004; Bronner et al. 2004). It has been shown in *in vitro* studies that σB also influences (directly or indirectly) the expression of genes associated with virulence (such as coagulase, lipases, proteases, clumping factor, α-hemolysin and thermonuclease), the expression of others global virulence factor regulators, as *sarA*, and even in the mediation of antibiotic resistance and in the pigmentation (which can increase the bacterial resistance to hydrogen peroxide) (Bischoff et al. 2004; Cebrián et al. 2009). Bischoff et al. (2004) suggested that *S. aureus*

σB factor has influence in the expression of, at least, 251 genes – 198 genes positively controlled, 53 genes repressed when the alternative factor is present.

Agr and *sae loci* are part of the group of two-component regulatory systems (Bronner et al. 2004; Cheung et al. 2004; Costa et al. 2013). This type of regulatory systems has shown to be sensitive to environmental signals and requires the presence of two proteins, a sensor (a histidine kinase) and a response regulator (Bronner et al. 2004). The transcription regulation begins with the binding of the extracellular ligand to the sensor (directly or through a receptor), which induce a phosphorylation cascade, culminating in the activation of the response regulator (Bronner et al. 2004). The response regulator, once activated, will bind to specific DNA sequences, regulating its transcription (Bronner et al. 2004) and subsequently the expression of required genes, including specific effectors of two-component systems. One hundred thirty-eight genes whose transcription is regulated by *agr* system were identified: 104 genes are up-regulated, while 34 are down-regulated (Bronner et al. 2004). The expression of *sae locus* regulates the expression of certain genes at the transcription level, as alpha-toxin (*hla*), β-hemolysin (*hlb*) and coagulase (*coa*) genes (Bronner et al. 2004).

The expression of *sarA* operon is regulated by multiple factors and additionally to its gene expression, the protein SarA (a monomer with 124 residues) and its homologues (*e. g.* SarR, SarS, SarT, SarU, that show a high level of identity with SarA) are DNA-binding proteins that recognize conserved A/T-rich motifs located in promoter regions of target genes (Bronner et al. 2004). SarA can directly regulate the expression of some genes involved in bacterial virulence, as various cell wall-associated proteins and exoproteins, and regulate virulence expression through the regulation of other operons, as *agr* operon (Bronner et al. 2004).

The above mentioned regulators form a cooperative and complex regulatory network, what ensures that the genes involved in the bacterial virulence are expressed when its function is required (Bischoff et al. 2001; Bronner et al. 2004; Costa et al. 2013). Moreover, it is reported that the occurrence of mutations in the *loci* regulators generate mutants with strongly attenuated expression of virulence determinants (Bischoff et al. 2001) when compared with their staphylococci counterparts.

2.1.6   The problem of antibiotic resistance development

The *S. aureus* resistance to penicillin, the first antibiotic used in the treatment of staphylococcal infections (Winn Jr et al. 2006), appeared in 1942, only few years after its introduction in the clinical field (Costa et al. 2013). The penicillin-resistant strains have a plasmid which encodes for penicillinase (a specific β-lactamase), an enzyme with the ability to hydrolyse the β-lactam ring of penicillin, causing the loss of its antibacterial property (Chambers and DeLeo 2009). It is described in *Koneman's Color Atlas* that *S. aureus* strains produce up to four different β-lactamases, which was evidenced by studies of specificity to the substrate and by molecular weight studies.

More than 80% of *S. aureus* strains have shown to be resistant to penicillin (Winn Jr et al. 2006). In order to treat infections caused by *S. aureus* penicillin resistant strains, semi-synthetic antibiotics oxacillin and methicillin, penicillin derivatives and resistant to inactivation by β-lactamase penicillinase, had been developed in 1959 (Chambers and DeLeo 2009; Costa et al. 2013). Though, in 1961, the first reported cases of methicillin-resistant *S. aureus*, MRSA, began to appear (Chambers and DeLeo 2009; Costa et al. 2013). The resistance to this type of penicillin derivatives results of the acquisition of a chromosomic gene, *mecA* (Winn Jr et al. 2006). The expression of this gene induces the synthesis of an altered protein (PBP2a), in contrast to PBP protein present in non-methicillin resistant strains to which penicillin binds (Winn Jr et al. 2006; Chambers and DeLeo 2009). The protein PBP2a shows very low affinity to β-lactam antibiotics, including cephalosporins and carbapenems (Winn Jr et al. 2006; Chambers and DeLeo 2009). Once the protein PBP2a is not inactivated by β-lactam, it maintains its normal function in peptidoglycan synthesis (Winn Jr et al. 2006), essential to bacterial growth and cell division.

Owing to the significance of infections caused by *S. aureus* and the growing occurrence of antibiotic resistant strains, this bacterium has become the staphylococcal species of greatest clinical relevance (Bien et al. 2011), and therefore the most studied (Costa et al. 2013).

In this context, recent research in the field of microbiology and related specialties has been done to develop innovative methods in order to generate therapeutic protocols non-invasive, non-toxic, fast, efficient, and using compounds to which the microorganisms do not develop the ability to acquire resistance. One of the methodologies under strong research today is photodynamic therapy (Calin and Parasca 2009).

2.2 Photodynamic therapy

2.2.1 Photodynamic therapy principles

The use of light in the treatment of diseases, known as phototherapy, is used since antiquity (Ackroyd et al. 2001). In the early twentieth century, Tappeiner and Jodlbauer (Ackroyd et al. 2001) defined the photodynamic effect concept, referring to the use of a light (sunlight or artificial light source), molecular oxygen, O_2 (dissolved in the reaction medium) and an intermediate agent (photosensitizer, PS) that is able to absorb and transfer energy from light to molecular oxygen, leading to the formation of highly cytotoxic species ROS that cause damage to living tissue, or even destruction - such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($OH^\bullet$) (Henderson and Dougherty 1992; Bonnett 2000; Alves et al. 2008).

In photodynamic inactivation (PDI), initially, the PS adheres to the bacteria cells, followed by an irradiation process with light with adequate wavelength (λ), preferentially coincident with one of the maximum absorption peaks of the used PS (Calin and Parasca 2009). During the irradiation process will be formed ROS that will oxidize various cellular components such as proteins and lipids (Alves et al. 2013(b)). Oxidative reactions on these cellular components lead to changes in their structure and consequently loss of function (Alves et al. 2013(b)).

The interaction between the agents that take part in the PDI process can occur via two different pathways: the interaction occurs between excited PS and the substrate – type I mechanism; the interaction occurs between excited PS and O_2, resulting in $^1O_2$ formation – type II mechanism (Wainwright and Crossley 2004).

When the PS absorbs light energy it is excited to its singlet state (Oliveira et al. 2015). At this point, the PS returns to its fundamental state of energy (with fluorescence emission) or, with the occurrence of the intersystem crossing process, the PS energy state will change to the triplet state and at this energy level the PS has the ability to transfer energy to O_2, originating $^1O_2$ – type II mechanism (Oliveira et al. 2015). The type I mechanism occurs when the absorbed energy from light is transferred to surrounding substrates by PS, leading to the formation of radicals species or peroxides (Oliveira et al. 2015). The occurrence of each type of mechanism mainly depends on the chemical structure of the PS used – porphyrin derivatives tend to generate ROS *via* type II (Alves et al. 2014).

ROS have an extremely short life time owing to their unstable electronic configuration. Oxygen singlet has a lifetime of 3 – 4 μs and its diffusion range depends on the surrounding medium, being less than 50 nm in a protein-rich lipid layers ambient (Alves et al. 2014). The PDI efficiency highly depends on the PS localization during the irradiation process, once the nearness of the PS to its potential targets is essential.

2.2.2 The importance of photosensitizer charge

Several PS classes have been studied and developed due to their photo-therapeutic ability, including porphyrins, chlorins and phthalocyanines (Oliveira et al. 2015).

Porphyrins are aromatic heterocycles constituted by four pyrrole type units linked to each other by methine bridges (also called *meso* position) (Figure 2). Characteristically, these molecules exhibit absorption bands in the visible region of the electromagnetic spectrum, with a higher intensity band in the region of 400 nm – the Soret band – and less intense absorption bands at wavelengths around 500 – 650 nm – the Q bands (Oliveira et al. 2015).
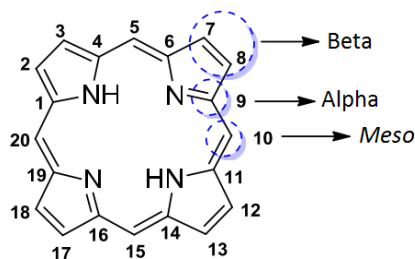
Figure 2. Porphyrin general structure, presenting alpha, beta and *meso* positions. (Oliveira et al. 2015)

The chlorins general structure is similar to the porphyrins. However, chlorins structure presents a reduced peripheral double bound that leads to changes in the corresponding absorption spectrum – chlorins exhibit strong absorption bands between 630 – 680 nm (Mesquita et al. 2014; Oliveira et al. 2015). Absorption bands around these wavelengths enlarge the applicability of chlorins type compounds when compared to porphyrins, once it allows the treatment of deeper lesions due to the high penetrance of red light in the tissues (Oliveira et al. 2015).

Phthalocyanines are constituted by four isoindole units linked by nitrogen atoms. These compounds present a large band around 350 nm and two more absorption bands around 600 – 750 nm, that comprehend the infrared region from electromagnetic spectrum (Oliveira et al. 2015).

According to literature, neutral PS does not inactivate Gram-negative bacteria as efficiently as they inactivate Gram-positive bacteria (Alves et al. 2009). Through the insertion of positively charged substituents to the macrocycle peripheral positions, PS can be converted into cationic compounds, increasing their amphiphilic character (Alves et al. 2009).

The *meso*-substituted cationic porphyrins are present as one of the most efficient PS in the inactivation of both Gram-negative and Gram-positive bacteria, which are believed to be due to increased interaction between the positive charge of these derivatives and the negative sites of lipopolysaccharides that constitute the external bacterial membrane of Gram-negative bacteria. In general, the combination of hydrophobic nature of the macrocycle with the hydrophilic character of the charged substituents results in the occurrence of an intramolecular polarity axis, that may facilitate the disorganization/penetration in cytoplasmic membrane by the PS, leading to a more effective interaction with important biological targets and consequently to a higher efficiency in bacterial photosensitization (Alves et al. 2008; Costa et al. 2008; Preuß et al. 2013; Alves et al. 2014). The photosensitizing porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py$^+$-Me), a *meso*-substituted cationic porphyrin, is one of the most studied porphyrin derivatives worldwide (Figure 3).

The effectiveness of antimicrobial photodynamic chemotherapy greatly depends to the broad spectrum of the photosensitizer action, the inactivation of the microorganisms strains whether they present or not antibiotic resistance (Taylor et al. 2002), the possibility of developing photodynamic therapy protocols that allow large reduction in the pathogens population with very limited damages in the host tissues and the reduction or inability to promote the appearance of mutagenicity, which could lead to the possible development of photo-resistance (Alves et al. 2014).
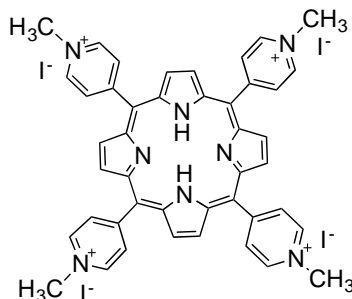
Figure 3. Structure of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py$^+$-Me)

2.2.3 Photodynamic therapy in the inactivation of *Staphylococcus aureus*

Methicillin-resistant *S. aureus* are among the multiresistant bacteria with great incidence (World Health Organization 2012). It has been demonstrated the photodynamic efficiency of different photosensitizers over diverse bacterial species, including *S. aureus*, such as toluidine blue O (TBO), functionalized fullerenes (Huang et al. 2010), hypericin (Yow et al. 2012), hypocrelin A (Du et al. 2012), protochlorophyllide (Walther et al. 2009), rose bengal, phloxine B and erythrosine B (Kato et al. 2012), hematoporphyrin derivative (Jin et al. 2010), 5,10,15,20-tetrakis(4-*N,N,N*-trimethylammoniumphenyl)porphyrin (TMAPP), 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TSPP) and Tetra-Py$^+$-Me (Komagoe et al. 2011), showing global effects in the chemical composition of the bacterial wall, leakage of cytoplasmic constituents and inhibition of membrane potential with consequent inactivation of membrane transport systems (Sahu et al. 2009; Jin et al. 2010; Komagoe et al. 2011). Specifically, photodynamic effect of cationic porphyrin Tetra-Py$^+$-Me seems to act at membrane potential and respiratory efficiency level (Komagoe et al. 2011).

Proteomic analysis of *S. aureus* membrane proteins and enzymes after photosensitization by Tetra-Py$^+$-Me (using sub-lethal doses) showed structural and functional damage in proteins involved in cell division, metabolic activities, oxidative stress response and sugar uptake (Dosselli et al. 2012).

The phospholipidic composition of *S. aureus* cytoplasmic membrane is rich in phosphatidylglycerols (PG), lysyl phosphatidylglycerols (LPG) and cardiolipins (CL) (Shireen et al. 2012). In a lipidomics based study, Alves et al (2013(a)) evaluated the effect of PDI on various classes of phospholipids present in *Staphylococcus warneri* membrane, including PG and CL, using a cationic porphyrin – 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py$^+$-Me-PF). They confirmed the formation of oxidized species of CL, which can lead to lethal damage to photosensitized bacterial cells, corroborating that membrane lipids are molecular targets to PDI (Alves et al. 2013(a)).

S. aureus did not develop PDI resistance after 25 consecutive cycles using methylene blue (MB) and it was shown that oxygen singlet has the ability to inactivate enzymes such as catalase

(Kim et al. 2001). However, studies about the effects of PDI on the *S. aureus* virulence factors are scarce. It is already known that the activity of secreted virulence factors V8 protease, α-hemolysin and sphingomyelinase is affected (Tubby et al. 2009), with exposure to laser light in MB presence, in a dose-dependent manner (Tubby et al. 2009).

To date, few studies were conducted to evaluate the effect of PDI in most of *S. aureus* virulence factors, which made us to want to explore this subject to get more answers.

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Chapter 3

Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a Gram-positive bacterium who integrates the human microbiota. Nevertheless, these bacteria can be pathogenic to the humans. Due to the increasing occurrence of antibiotic-resistant *S. aureus* strains, new approaches to control this pathogen are necessary. The antimicrobial photodynamic inactivation (PDI) process is based in the combined use of light, oxygen and an intermediary agent (a photosensitizer). These three components interact to generate cytotoxic reactive oxygen species that irreversibly damage vital constituents of the microbial cells and ultimately lead to cell death. Although PDI is being shown to be a promising alternative to the antibiotic approach for the inactivation of pathogenic microorganisms, information on effects of photosensitization on particular virulence factors is strikingly scarce. The objective of this work was to evaluate the effect of PDI on virulence factors of *S. aureus* and to assess the potential development of resistance of this bacterium as well as the recovery of the expression of the virulence factors after successive PDI cycles. For this, the photosensitizer 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py$^+$-Me**) and six strains of *S. aureus* (one reference strain, one strain with 1 enterotoxin, two strains with 3 enterotoxins and two strains methicillin resistant (MRSA) – one with 5 enterotoxins and the

other without enterotoxins) were used. The effect of photosensitization on catalase activity, beta hemolysis, lipases, thermonuclease, enterotoxins, coagulase production and resistance/susceptibility to methicillin was tested. To assess the development of resistance after successive cycles of treatment, three strains of *S. aureus* (ATCC 6538, 2065 MA and SA 3 MRSA) were used. The surviving colonies of a first cycle of PDI were collected from the solid medium and subjected to further nine consecutive cycles of PDI. The results indicate that the expression of some external virulence factors is affected by PDI and enterotoxin producing strains are more susceptible to PDI than non-toxigenic strains. The surviving bacteria neither developed resistance nor recovered the expression of the virulence factors after 10 cycles of treatment. PDI, contrarily to traditional antibiotics, inhibits the expression of virulence factors, inactivating even more efficiently highly virulent strains than low virulent *S. aureus* strains, inactivating also antibiotic susceptible and resistant strains, without development of resistance after at least 10 consecutive cycles of treatment, thus representing a strong promising alternative to antibiotics to control pathogenic microorganisms.

1   Introduction

Staphylococcus aureus is a Gram-positive bacterium that occurs on the surface of the skin and on mucous membranes of warm-blooded animals (Morikawa et al. 2001; Costa et al. 2013) as a commensal microorganism, asymptomatically colonizing the host (Bronner et al. 2004). Nevertheless, due to its invasiveness and taking advantage of host immune weaknesses, *S. aureus* is able to cause a wide broad of infections affecting any organ (Bronner et al. 2004; Baptista et al. 2015), from infections of superficial lesions to intoxications and life threatening systemic conditions (Bien et al. 2011). This opportunistic bacterium is a major human pathogen not only associated with community-acquired bacteremia but also nosocomial bacteremia (Morikawa et al. 2001; Cheung et al. 2004; Bien et al. 2011), being readily able to acquire antibiotic resistance (Morikawa et al. 2001). Its ability to survive under stressful circumstances, such as those imposed by host immunity system, is due to the activation of stress response mechanisms (Morikawa et al. 2001; Bronner et al. 2004; Cheung et al. 2004). These mechanisms involve the action of an interactive regulatory network that includes the accessory gene regulator (*agr*) and staphylococcal accessory element (*sae*) (Bronner et al. 2004; Novick and Geisinger 2008; Costa et al. 2013). These two components of the regulatory system regulate the expression of several exoproteins and cell wall-associated proteins related to virulence (Costa et al. 2013). The regulatory network also includes the staphylococcal accessory regulator A (*sarA*) and its homologues that regulate the expression of some virulence factors; and sigma factors (σ), as the primary sigma factor, σ^A, that may function in living process through the housekeeping genes expression, and the alternative sigma factor σ^B, which may participate on the bacterial stress response, by regulating the expression of several genes that are involved on this cellular function (Morikawa et al. 2001; Cheung et al. 2004; Costa et al. 2013).

The *S. aureus* pathogenicity involves a wide array of cell wall and extracellular components orderly expressed during the different stages of infection: colonization, avoidance or invasion of

the host immune defense, growth and cellular division culminating in bacterial dissemination, causing toxic effects to the host (Cheung et al. 2004; Bien et al. 2011; Costa et al. 2013; Ebrahimi et al. 2014). Some of the cell wall components are responsible for the recognition of adhesive matrix molecules, such as the **clumping factor proteins** (Clf) that mediates the adherence to fibrinogen (Costa et al. 2013) and the **carotenoid pigment staphyloxanthin** that acts as virulence factor once it is able to perform an antioxidant action against oxidant-based reactions (Clauditz et al. 2006; Liu and Nizet 2009; Costa et al. 2013). The extracellular components include the superantigen molecules such as the **staphylococcal enterotoxins** (SE), a family of a single chain proteins with small molecular-weight (24 - 30 kDa) (Johnson et al. 1991; Baptista et al. 2015); the cytolytic **β-hemolysin**, the clotting factor coagulase, besides more exoenzymes as **lipases** and **nucleases**, in which their main function is to disrupt the host cells/tissue and the inactivation of host immunity mechanisms of defense (Costa et al. 2013).

Additionally to the virulence factors already described, *S. aureus* has a notorious capacity to acquire antibiotic resistance (Guillemot 1999; Morikawa et al. 2001; Ito et al. 2003; Chambers and DeLeo 2009; Costa et al. 2013; Theuretzbacher 2013), by a bacterial gene mutation and horizontal transfer of resistance genes from external sources (Ito et al. 2003; Chambers and DeLeo 2009). The resistance to the penicillin emerged in the mid-1940s, only a few years after the introduction of this antibiotic in the clinical practice (Chambers and DeLeo 2009; Costa et al. 2013). Later, in 1959, the semi-synthetic antibiotic methicillin was introduced for the treatment of infections caused by penicillin-resistant *S. aureus* (Enright et al. 2002; Costa et al. 2013). Yet, in 1961 the first cases of methicillin-resistant *S. aureus* (MRSA) isolates (Chambers and DeLeo 2009; Costa et al. 2013) were reported and currently, only few compounds are still effective in the treatment of MRSA infections (Chambers and DeLeo 2009; Theuretzbacher 2011).

With the knowledge that the development of new classic antibiotics is not likely to solve the resistance drug problem for too long (Chambers and DeLeo 2009), non-traditional antimicrobial approaches to treat MRSA infections will be needed. Ideally, the new antimicrobial methods should be non-invasive and non-toxic to the hosts, but efficient and with fast action, avoiding the development of resistance (Calin and Parasca 2009; Kossakowska et al. 2013; Alves et al. 2014; Almeida et al. 2015). In this context, the photodynamic inactivation (PDI) arises as a photochemotherapeutic approach with forthcoming applications as antimicrobial therapy (Almeida et al. 2009; Carvalho et al. 2009; Costa et al. 2012; Alves et al. 2013; Melo et al. 2013; Alves et al. 2014; Almeida et al. 2015; Alves et al. 2015(b)). The photodynamic effect is based on the use of visible light and an agent (photosensitizer, PS) capable to absorb energy from light and transfer it to molecular oxygen, originating highly cytotoxic species, namely reactive oxygen species (ROS) as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), peroxide anion radical ($O_2^{\bullet-}$), and hydroxyl radical ($OH^\bullet$) (Alves et al. 2008; Calin and Parasca 2009; Alves et al. 2013; Melo et al. 2013; Alves et al. 2014). These species can cause irreversible damages to molecular cell constituents or even its destruction (Alves et al. 2014). Initially, the PS adheres to the microbial external structures and later, during the irradiation process, the photodynamic inactivation is initiated and cellular components, such as proteins and lipids will be exposed to oxidizing reactions which will alter their structure and, subsequently, affect the biological function in which they are involved (Alves et al. 2014).

As PDI acts *via* ROS, a high number of microbial targets are simultaneously affected, thus preventing the development of resistance (Costa et al. 2008; Tavares et al. 2010), and allowing the inactivation of a broad-spectrum of microorganisms, independently of their resistance profiles to classic antimicrobials (Tavares et al. 2010; Arrojado et al. 2011; Costa et al. 2011; Almeida et al. 2014). In addition, PDI affects the expression of virulence factors, also causing their degradation (Kömerik et al. 2000; Tubby et al. 2009; Kossakowska et al. 2013). The effects of PDI on virulence factors is of extreme importance as they may be present during the infection process, when the microorganism is present, but they can also be present when the microorganism is not present already, such as in the case of intoxications, causing severe damage to the host. Some studies showed that the biological activity of lipopolysaccharides from *Escherichia coli* and proteases from *Pseudomonas aeruginosa* were successfully reduced by toluidine blue (TBO)-mediated PDI (Tubby et al. 2009). Additionally, light-activated methylene blue (MB) showed to inhibit the expression of staphylococcal V8 protease, alpha-hemolysin and sphingomyelinase (Tubby et al. 2009). However, the information about the effects of PDI on virulence factor is still scarce. The objectives of this work were the evaluation of the effect of PDI on some virulence factors of *S. aureus* – catalase activity, beta hemolysis, lipases, thermonuclease, enterotoxins, coagulase – and the assessment of development of resistance to PDI treatment. For that, a cationic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py$^+$-Me**) was used as photosensitizer against on six *S. aureus* strains – ATCC 6538, 2153 MA, 2065 MA, 2095 M1A1, DSM 25693 MRSA and SA 3 MRSA.

2   Material and Methods

2.1   Experimental design

An experimental procedure was established in order to study the effects of PDI on some virulence factors expression/activity of *S. aureus* strains and to test the potential development of resistance to PDI by *S. aureus* strains after successive photodynamic cycles of treatment, testing also the recovery of the expression/activity of the virulence factors after the successive photodynamic cycles of treatment. Six different strains of *S. aureus*, including methicillin resistant and susceptible strains, as well as, enterotoxin and non-enterotoxin producing strains were tested. The effect of PDI on the expression/activity of virulence factors was tested in all strains. The potential development of resistance to PDI and the recovery of the expression/activity of the virulence factors after the successive cycles of treatment were tested only in three of the strains. For each of these three strains, a total of ten cycles of treatments were performed.

2.2   Characterization of bacterial strains and culture conditions

Six strains of *S. aureus* were used in this study: ATCC 6538, a non-enterotoxic strain; 2153 MA, the only strain used that does not ferment mannitol (Baptista et al. 2015), producing staphylococcal enterotoxin (SE) A; 2065 MA, with SE A, G, I and *S. aureus* 2095 M1A1 with SE C, G and I – the three strains isolated from food products and characterized in the Centre of Biotechnology and Fine Chemistry of the Faculty of Biotechnology of the Catholic University,

Portugal; *S. aureus* DSM 25693, a methicillin-resistant (MRSA) strain, positive for SE A, C, H, G and I; and a staphylococcal strain isolated from a biological sample from the lower respiratory tract of an hospitalized individual according to Gonçalves et al. (2014), a non-enterotoxic MRSA strain (SA 3 MRSA).

All the strains were grown in Brain-Heart Infusion (BHI, Liofilchem, Italy) at 37 ˚C for 18 h at 170 rpm, in order to reach the stationary phase, corresponding approximately to a concentration of $10^8 - 10^9$ colony forming units per mL (CFU mL^{-1}). Before each PDI assay, a colony of *S. aureus* was transferred to 30 mL of BHI and incubated as previously described. Subsequently, an aliquot was transferred to fresh medium, and grown in the same conditions. This procedure was repeated twice.

2.3 Photosensitizer

The photosensitizer 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py⁺-Me**) used in this study were prepared according to the literature (Carvalho et al. 2010). Their ^{1}H NMR and UV–vis spectra were consistent with the literature data. Their purity was confirmed by thin layer chromatography and ^{1}H NMR. ^{1}H NMR (DMSO-d^6): −3.12 (s, 2H, *N*H), 4.73 (s, 12H, CH$_3$), 9.00 (d, *J* = 6.5 Hz, 8H, Py-o-H), 9.22 (s, 8H, β-H), 9.49 (d, *J* = 6.5 Hz, 8H, Py-m-H). UV–vis (DMSO) λ$_{max}$ (log ε): 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30) nm. The stock solutions (500 μM) of this porphyrin was prepared using the polar aprotic solvent dimethyl sulfoxide (DMSO).

2.4 Antimicrobial photodynamic therapy (PDI) treatments

Bacterial cultures in stationary phase were tenfold diluted in phosphate buffered saline (PBS) and this bacterial suspension was distributed in sterilized glass beakers. The appropriate quantity of the photosensitizer **Tetra-Py⁺-Me** was added to achieve a final concentration of 5.0 μM. The total volume of final solution was 10 mL per beaker. During the experiments, light and dark controls were also performed: in the light control the beaker without **Tetra-Py⁺-Me** was exposed to light; in the dark control the beaker containing 5.0 μM **Tetra-Py⁺-Me** was protected from light with aluminium foil during the experiment. During the pre-irradiation period, the samples were incubated for 10 min with stirring, at room temperature, in order to promote the binding of the porphyrin to *S. aureus* cells. The samples were exposed to an artificial white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380-700 nm) with an irradiance of 40 W m^{-2} for 60 min, under stirring. During the experiment, aliquots of treated and control samples were collected at times 0, 5, 10, 15, 30 and 60 min.

2.5 Enumeration of viable cells

From each treated and control samples tenfold serial dilutions were prepared in sterile PBS (10^{-1} to 10^{-6}). Aliquots of 100 μL were pour-plated, in duplicate, in Plate Count Agar medium (PCA, Liofilchem, Italy). The plates were incubated at 37 ˚C for 48 h and the number of colonies was counted. Three independent assays were performed.

2.6 PDI resistance assays

In order to verify the development of resistance to PDI treatment with **Tetra-Py⁺-Me**, ten cycles of PDI were performed. After each cycle of a total irradiation time of 60 min, a new set of bacterial cultures were prepared from an isolated colony, surviving to the previous cycle of PDI (at 37 ˚C, 18 h, 170 rpm). The PDI treatment was repeated under similar conditions. Three independent assays were performed.

2.7 Virulence factors, mannitol fermentation and methicillin susceptibility

To assess if PDI treatments affected the virulence factors of *S. aureus*, treated and controls samples were tested for the presence or activity of virulence factors, according to literature (Baptista et al. 2015). One typical colony of *S. aureus* was selected from each strains. The **β-hemolysin** activity was detected by streaking Blood Agar Plates (Sheep Blood 7%) (BAP, Liofilchem, Italy) and observing the development of a clear/yellow zone surrounding *S. aureus* colonies. **Lipase** and **lecithinase** activities were assessed by streaking Baird Parker Agar (BPA, Liofilchem, Italy) in which *S. aureus* colonies appear in black, with an opaque precipitation zone (lipase activity) and a clear zone surrounding it (lecithinase activity). The **mannitol fermentation** was evaluated using Mannitol Salt Agar (MSA, Liofilchem, Italy), being the positive results detected by a change of color of the medium from pink to yellow. **Catalase** activity was assessed using Catalase/Oxy Test (Liofilchem, Italy), interpreting the formation of gas bubbles as a positive result. The activity of **bound coagulase** (**clumping factor**) was determined using Pastorex Staph Plus (Bio-Rad, USA) and the activity of **free coagulase** was detected using BBL Coagulase Plasma Rabbit (BD, USA). For this, 1.0 mL of supernatant of treated and non-treated samples was collected by centrifugation at 13 000 g for 10 min, at 4 ˚C, and 0.5 mL of BBL Coagulase Plasma reagent was added and the mixture was incubated at 37 ˚C for 24 h. The results were considered positive when the agglutination occurred. The rate of clot formation was evaluated according to the manufacturer's instruction. **Thermonuclease** activity was determined by D.N.A. Toluidine Blue Agar (Bio-Rad, USA) and positive results were detected as a change of color of the halos from blue to pink. The presence of **SE** was determined using SET-RPLA Kit Toxin Detection Kit (Thermo Scientific, United Kingdom), a kit based on reversed passive latex agglutination technique, according to the manufacturer's instruction. Treated and non-treated samples were centrifuged at 900 g for 20 min at 4 ˚C. The presence of enterotoxins H, G and I were not tested since SET-RPLA Kit Toxin Detection Kit only covers SEA, B, C and D, which are the most common enterotoxins of *S. aureus* (Baptista et al. 2015). The **susceptibility to methicillin** was determined using the cefoxitin disk screen test, accordingly to the Clinical and Laboratory Standards Institute (CLSI). Cultures with halos ≥ 22 mm were considered methicillin susceptible and cultures with halos ≤ 21 were classified as methicillin resistant (CLSI 2013). Carotenoid pigments (**staphyloxanthin**) were determined using a protocol adapted from Morikawa et al. (2001). Each strain was cultured in BHI medium at 37 ˚C for 72 h. Twenty milliliters of the culture were harvested by centrifugation (10,000 g, 10 min) and washed with purified water. The cells were suspended in 5.0 mL of methanol and heated in a bath at 55 ˚C for 15 min, until visible pigments have been extracted. Cellular debris were removed by centrifugation at 15,000 g for 10 min. The

absorbance at 465 nm of the methanol extracts were measured in a quartz cuvette in a spectrophotometer (Dynamica Halo DB-20, UK).

The β-hemolysis, lipase and lecithinase, catalase, bound coagulase and thermonuclease activities, mannitol fermentation and methicillin susceptibility were tested in the PDI surviving cells, after plating and incubation at 37 ˚C. Methicillin susceptibility was inferred from the diameter of the inhibition zone around cefoxitin discs, and the results represent the average of the inhibition zones from three independent tests. Free coagulase and SE A and C activities were assessed in the supernatant of treated samples and controls after PDI assays.

The effect of PDI on the isolated toxins was also individually assessed. Purified SE A and C (available on SET-RPLA Kit Toxin Detection Kit as SE A and C controls) were subjected to the PDI treatment. The concentration of the PS was the same used before: 5.0 μM; the amount of SE used was 63 μL from the reconstituted control reagents (the kit control solution contains 25 ng of purified enterotoxin reconstituted in 0.5 mL of diluent from the kit as showed in Figure 1.1 of the Supplementary Material), and PBS was added to make up a total volume of 2.0 mL of solution, which was irradiated. Light and dark controls were included. At times 0, 5, 10, 15, 30 and 60 min aliquots of 25 μL were collected and the activity of the SE was tested using the SET-RPLA Kit Toxin Detection Kit.

2.8   Data processing

The PDI statistical significance of the differences was verified by analysis of variance (ANOVA) and the Turkey test, using GraphPad Prism 6.01 (GraphPad Software, Inc., USA). Normal distributions were checked by Kolmorov-Smirnov test and homogeneity of variances by Brown-Forsythe test. Differences corresponding to $p < 0.05$ were considered significant. The statistical analysis was performed considering the three independent assays performed with each strain.

3   Results

3.1   Bacterial inactivation by PDI

Cells suspensions of *S. aureus* strains were subjected to 60 min of PDI treatments (5.0 µM of **Tetra-Py$^+$-Me** and an irradiance of 40 W m^{-2}), and aliquots were taken before (0 min) and after 5, 10, 15, 30 and 60 min of treatment. All the strains were efficiently inactivated by PDI (Figure 1). After 60 min of treatment under the tested conditions reductions higher than 5 log CFU mL^{-1} were observed for all the tested strains. However, in general, the pattern of photoinactivation was different among the *S. aureus* strains (Table 1) (ANOVA $p < 0.05$).
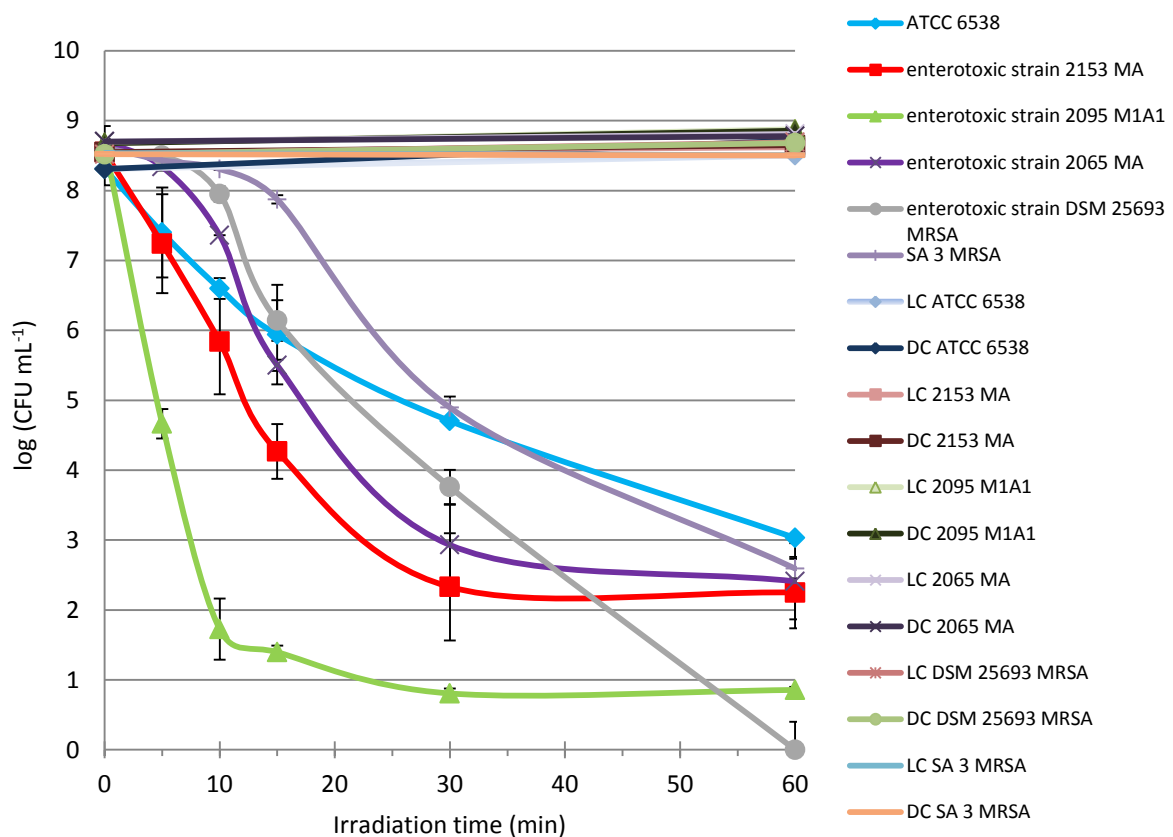
Figure 1. Survival curves of six *S. aureus* strains (ATCC 6538, enterotoxic strains 2153 MA, 2095 M1A1, 2065 MA and DSM 25693 MRSA and SA 3 MRSA) incubated with 5.0 µM of **Tetra-Py$^+$-Me** and irradiated with white light (380–700 nm) with an irradiance of 40 W m^{-2} for 60 min. Controls: light (LC) and dark (DC) controls. Values represent the mean of three independent experiments with two replicates each; error bars indicate the standard deviation.

Table 1. Two-way ANOVA performed to analyze the similarity between reduction efficiency from photodynamic inactivation assays (PDI) of six *S. aureus* strains.

Tukey's multiple comparisons test	Significantly different? (Adjusted p value)					
	Irradiation time (min)					
	0	5	10	15	30	60
ATCC 6538 *vs* 2065 MA	0.9930	0.8357	0.8176	0.9831	0.0736	0.9458
ATCC 6538 *vs* 2095 M1A1	0.9737	0.0014	< 0.0001	< 0.0001	< 0.0001	0.0270
ATCC 6538 *vs* 2153 MA	0.9993	0.9990	0.9302	0.1615	0.0121	0.8655
ATCC 6538 *vs* DSM 25693 MRSA	0.9997	0.6985	0.3005	0.9997	0.7430	0.0080
ATCC 6538 *vs* SA 3 MRSA	0.9977	0.7548	0.1063	0.0690	0.9998	0.9877
2065 MA *vs* 2095 M1A1	> 0.9999	< 0.0001	< 0.0001	< 0.0001	0.0578	0.2240
2065 MA *vs* 2153 MA	> 0.9999	0.6200	0.2543	0.5105	0.9853	0.9999
2065 MA *vs* DSM 25693 MRSA	0.9998	0.9999	0.9545	0.9260	0.7264	0.0932
2065 MA *vs* SA 3 MRSA	> 0.9999	> 0.9999	0.7382	0.0105	0.0361	0.9998
2095 M1A1 *vs* 2153 MA	0.9985	0.0049	< 0.0001	0.0012	0.2473	0.3392
2095 M1A1 *vs* DSM 25693 MRSA	0.9974	< 0.0001	< 0.0001	< 0.0001	0.0008	0.9984
2095 M1A1 *vs* SA 3 MRSA	0.9996	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1319
2153 MA *vs* DSM 25693 MRSA	> 0.9999	0.4607	0.0360	0.0857	0.3174	0.1572
2153 MA *vs* SA 3 MRSA	> 0.9999	0.5209	0.0080	< 0.0001	0.0051	0.9962
DSM 25693 MRSA *vs*. SA 3 MRSA	> 0.9999	> 0.9999	0.9952	0.1335	0.5678	0.0492

The enterotoxic strains were more efficiently inactivated than the non-enterotoxic ones. The toxigenic *S. aureus* strain 2095 M1A1 was particularly susceptible to PDI showing a reduction of ≈7 log within the first 10 min of irradiation. With equivalent irradiation time, the enterotoxic strain 2153 MA showed a reduction of only ≈2.5 log and all the other strains were inactivated less than 2 log. For DSM 25693 MRSA strain, also an enterotoxic, a linear decrease was observed after 15 min of treatment, reaching complete inactivation after 60 min of treatment (8.5 log). After 60 min of treatment, the inactivation factors for the other strains were 5.3 log for ATCC 6538, 6.0 log for SA 3 MRSA, 6.3 log for 2065 MA, 6.3 log for 2153 MA and 7.8 log for 2095 M1A1. In the controls, the concentration of viable cells did not vary significantly, indicating that the viability of bacterial cells was neither affected by light alone (light control), nor by the direct toxicity of the PS.

3.2 Development of resistance after repeated PDI cycles and recovery of viability between cycles

Three *S. aureus* strains (ATCC 6538, 2065 MA and SA 3 MRSA) were subjected to ten consecutive PDI cycles (Figure 2). The PDI efficiency of photosensitization with **Tetra-Py$^+$-Me** was not affected during the sequence of ten PDI cycles. No significant differences between cycles of

photosensitization were observed (ANOVA $p < 0.05$).
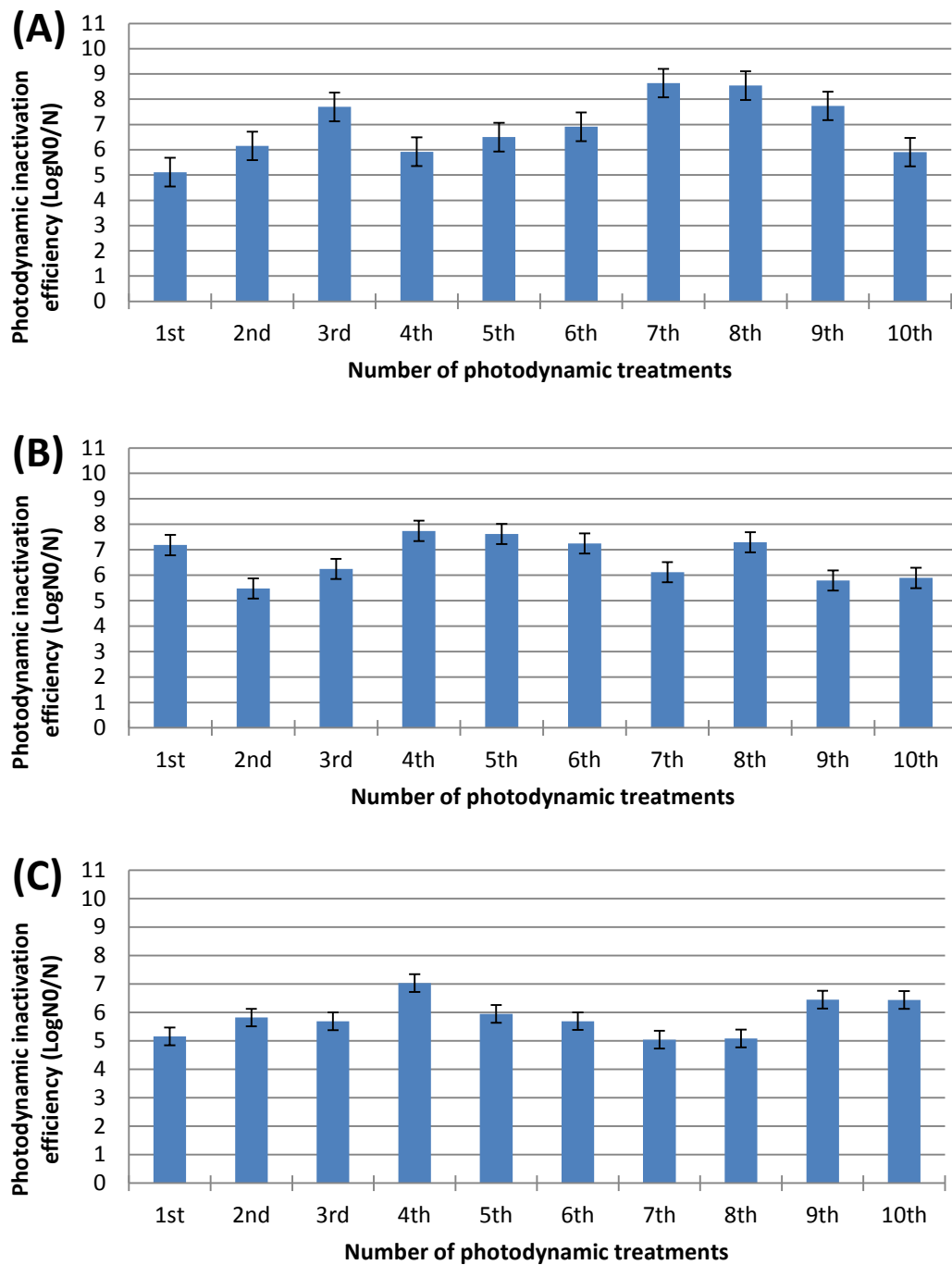
Figure 2. Photodynamic inactivation efficiency of ten consecutive cycles of *S. aureus* ATCC 6538 (A), 2065 MA (B) and SA 3 MRSA (C) by 5.0 µM of Tetra-Py$^+$-Me after 60 min of irradiation with white light (40 W m^{-2}). N_0 represents the plaque counts of bacterial cells before the irradiation; N represents the plaque counts after the cycle treatment; error bars indicate the standard deviations of bacterial reduction efficiency between the 10 cycles.

3.3 Effect of PDI on *S. aureus* virulence factors, methicillin susceptibility and mannitol fermentation

After each PDI assay, the activity and presence of virulence factors, mannitol fermentation and methicillin susceptibility were tested (Table 2).

Table 2. The activity of the virulence factors, mannitol fermentation and susceptibility to methicillin were tested, after PDI treatments, in presence of 5.0 μM of Tetra-Py$^+$-Me and irradiated with white light (380–700 nm) with an irradiance of 40 W m^{-2} for 60 min. These tests were performed in three independent assays for each strain. Cardinal symbol in the "Supernatant, SE" columns means that such tests have not been performed for the respective strains once these strains do not produce the mentioned virulence factors.

| *Staphylococcus aureus* strains | Samples | Surviving cells | | | | | | | Supernatant | | |
		β-hemolysis	Lipase and lecithinase	Mannitol fermentation	Catalase	Bound Coagulase	Thermonuclease	Susceptibility to methicillin (mm)	Free coagulase	SEs A	C
ATCC 6538	S	+	+	+	+	+	+	30	-	#	#
	LC	+	+	+	+	+	+	29	4$^+$	#	#
	DC	+	+	+	+	+	+	29	4$^+$	#	#
2153 MA	S	+	+	-	+	+	+	25	-	-	#
	LC	+	+	-	+	+	+	26	3$^+$	+	#
	DC	+	+	-	+	+	+	26	3$^+$	+	#
2065 MA	S	+	+	+	+	+	+	28	-	-	#
	LC	+	+	+	+	+	+	28	3$^+$	+	#
	DC	+	+	+	+	+	+	28	3$^+$	+	#
2095 M1A1	S	+	+	+	+	+	+	27	-	#	-
	LC	+	+	+	+	+	+	26	3$^+$	#	+
	DC	+	+	+	+	+	+	27	3$^+$	#	+
DSM 25693 MRSA	S	+	+	+	+	+	+	< 21	-	-	-
	LC	+	+	+	+	+	+	< 21	3$^+$	+	+
	DC	+	+	+	+	+	+	< 21	3$^+$	+	+
SA 3 MRSA	S	+	+	+	+	+	+	< 21	-	#	#
	LC	+	+	+	+	+	+	< 21	4$^+$	#	#
	DC	+	+	+	+	+	+	< 21	4$^+$	#	#

The surviving cells to PDI treatments retained the capacity to express all the virulence factors and to ferment mannitol. However, the activity of the extracellular virulence factors free coagulase and enterotoxins, assessed in the supernatant of treated samples, was affected (Figure 3 and Table 2). For the test of free coagulase, the BBL Coagulase Plasma reagent was added to an aliquot of supernatant and the resulting samples was incubated for 24 h. Clot formation was not detected in photosensitized samples. The SE test, performed in the supernatant of photosensitized cells by the SET-RPLA Kit Toxin Detection Kit test, revealed the formation of a tight button, interpreted as absence of SE or presence at a concentration below the detection limit. These two virulence factors persisted in light and dark controls.
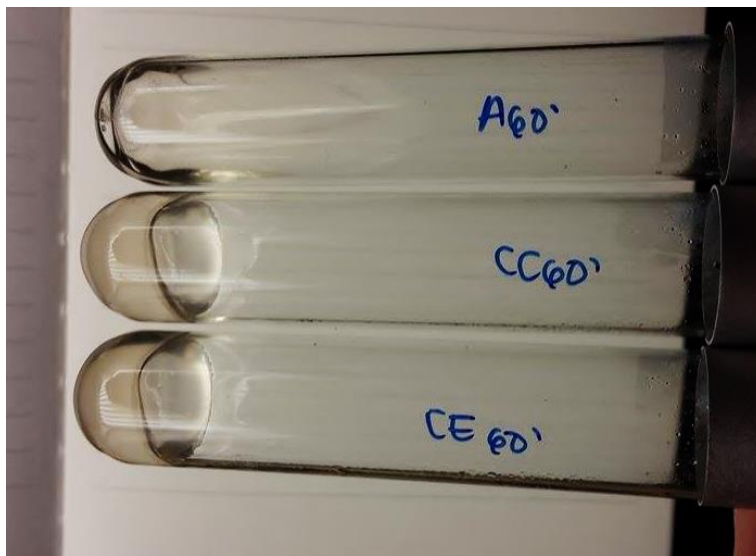

Figure 3. Testing the presence of free coagulase. After each PDI assay was assessed if free coagulase (an extracellular virulence factor) was present in the supernatant of samples involved in the assays. The clot formation indicates a positive result; a negative result occurs when there is no clot formation. [A60', treated sample (5.0 µM of **Tetra-Py$^+$-Me** and light**)**, after 60 min of treatment; CC60', irradiated but non-treated (only light); CE60', non-irradiated sample (only 5.0 µM of **Tetra-Py$^+$-Me**].

3.4   Susceptibility of staphylococcal isolated enterotoxins to PDI

The isolated enterotoxins A and C were directly treated by PDI (Figure 4 and Table 3). The positive result corresponds to agglutination, leading to the formation of a lattice structure and negative correspond to the formation of a tight button, which occurs if SE are absent or present in a concentration bellow the detection level (TD0900, SET-RPLA, Oxoid - Product Detail). Before the treatment, enterotoxins A and C were still detected (formation of a lattice structure). During the PDI , the formation of the lattice structure decreases (as seen in the first well of 15 and 30 min test, Figure 4 A, B) and the formation of a tight button (as seen in the first well of 60 min test),

begins to occur, indicating the shift to a negative result. After 60 min of irradiation, the inactivation was > 68% for both SE (SE A and C) (section 1.2 of Supplementary Material).
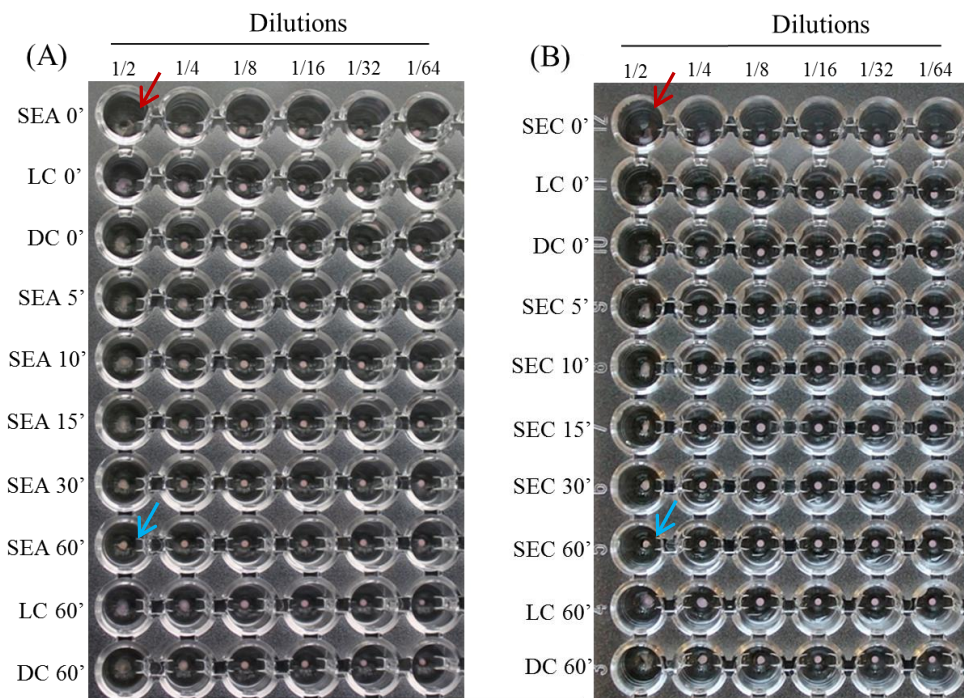

Figure 4. The purified SE A (A) and C (B) were subjected to photodynamic treatment, in presence of 5.0 µM of Tetra-Py$^+$-Me and irradiated with white light (380–700 nm) with an irradiance of 40 W m^{-2} for 60 min. Rows represent a different sample; columns represent a different dilution (doubling dilutions). In the first three rows was tested the presence of SE before the treatment (SE A 0' and C 0', red arrows); in the following four rows were tested the presence of SE after 5, 10, 15 and 30 min of treatment; in the last three rows were tested the presence of SE after the treatment (a total of 60 min) (SE A 60' and C 60', blue arrows). Three independent assays were performed for each SE.

Table 3. The purified SE A and C were subjected to PDI for 60 min. The SE activity was assessed before (0 min), during (5, 10, 15 and 30 min) and after 60 min of treatment (see Figure 4), for treated (S) and non-treated samples (light control, LC, and dark control, DC). Three independent assays were performed.

Irradiation time		Isolated and purified SEs	
(min)		**A**	**C**
	S	+	+
0	LC	+	+
	DC	+	+
	S	-	-
60	LC	+	+
	DC	+	+

3.5 Carotenoid pigments content

Carotenoid pigments were detected in all the tested strains. The strains SA 3 MRSA with an absorbance at 465 nm of 0.995 ± 0.001 and the 2065 MA with an absorbance of 0.911 ± 0.017, display the highest concentration of pigments, followed by DSM 25693 MRSA (Abs of 0.788 ± 0.021), ATCC 6538 (Abs of 0.701 ± 0.005), 2153 MA (Abs of 0.480 ± 0.002) and 2095 M1A1 (Abs of 0.411 ± 0.013).

4 Discussion

According to literature, the susceptibility of *S. aureus* to PDI is strain-dependent and MRSA strains seem to have a lower susceptibility to PDI than methicillin-sensitive counterparts (Grinholc et al. 2008). The lower susceptibility of MRSA strains to PDI has been attributed to slime production by MRSA strains (Grinholc et al. 2008).

In this study, all the strains tested were susceptible to PDI, with a survival reduction above 5 log CFU mL^{-1}, which according to American Society of Microbiology is higher than the minimum required (reduction > 3 log CFU mL^{-1}) for a new approach to be termed as antimicrobial (ASM 2015). However, as observed in previous studies, a strain-dependent efficiency of inactivation was observed (Grinholc et al. 2008). Nonetheless PDI does not seem to be antibiotic-susceptible dependent, since DSM 25693 MRSA, an MRSA and enterotoxic strain, was the only strain that was inactivated to the limit of detection of the method, with a survival reduction of 8.5 log. Another MRSA strain, SA 3 MRSA, was not as efficiently inactivated, but the inactivation profile was similar to that of the reference ATCC 6538 strain ($p > 0.05$). The difference in PDI susceptibility of the two MRSA strains after 60 min of irradiation ($p < 0.05$) can be probably due to the presence of enterotoxins in the DSM 25693 MRSA strain. In fact, the four enterotoxic strains were more efficiently inactivated that the two non-enterotoxic strains (see Figure 1). However, significant differences in PDI inactivation between enterotoxic strains were observed, which means that other cell factors contribute to these differences in PDI efficiency.

Carotenoid pigment content is related with the resistance of *S. aureus* to several stress factors, such as external oxidative stress. Several studies have shown that mutant strains that are unable to produce carotenoids are more susceptible to those stress factors (Liu et al. 2005; Clauditz et al. 2006; Cebrián et al. 2007). In an attempt to verify if carotenoid pigments were able to attenuate oxidative damages and consequent cellular inactivation, the pigment content of each strain was measured. Although a direct relation between carotenoids content and PDI efficiency was not observed for all the strains, the least susceptible strains to PDI (ATCC 6538 and SA 3 MRSA) show a high relative concentration of this pigment, with absorbances of 0.701 and 0.995 respectively.

The results of this study suggest that the strain dependency of the PDI efficiency in *S. aureus* is related with more than one characteristic. Although it is not yet possible to identify the mechanisms underlying these differences, the σ^{B} factor is known to be implicated in *S. aureus*

resistance to stress factors by controlling 251 genes and/or operons (Bischoff et al. 2004; Cebrián et al. 2009).

Once the photodynamic action occurs through ROS generation (formed along the irradiation process), the damages (through oxidative process) can affect a variety of cellular components with great importance in the maintenance of bacterial stability, such as molecular components (proteins and lipids) of external structures, and enzymes (Alves et al. 2014; Almeida et al. 2015). The majority of virulence factors of *S. aureus* are proteins or enzymes that are prone to photodynamic oxidative damage. The results of this study show that the phenotypic expression of the of β-hemolysin, lipase, lecithinase, catalase and bound coagulase by surviving cells cultivated on PCA medium for 48 h at 37 ˚C was not affected by PDI. Mannitol uptake and fermentation also persisted. Previous proteomic analysis showed that the oxidative damage caused by PDI treatment affects the expression of functional proteins involved in cell division, metabolic activities, oxidative stress responses, and sugar uptake (Alves et al. 2014; Alves et al. 2015(a)). However, these studies were not performed in recovered surviving cells. Since this detection was performed in the surviving cells, it would be expected that the bacteria, even if injured during PDI treatments, would be able to continue producing those virulence factors and other enzymes. However, some of these virulence factors are released to the extracellular compartment thus becoming more exposed to the effects of the PDI process.

In this study, the presence/activity of two external virulence factors (free coagulase and enterotoxins A and C) in the supernatant of PDI treatment samples was assessed. The results show that both virulence factors are affected by PDI. This represents an advantage relatively to traditional antibiotics, which act only on bacterial cell and not on extracellular virulence factors. These results are in accordance with those observed in the previous studies by Kömerik et al. (2000) and Tubby et al. (2009).

In this study, the response of one of the most important *S. aureus* virulence factors, the staphylococcal enterotoxins which are the staphylococcal food poisoning causative agents, was assessed after PDI treatment in order to confirm the effect of PDI on extracellular virulence factors. Two isolated enterotoxins, SE A and C, were treated by PDI. The porphyrin **Tetra-Py$^+$-Me** at a concentration of 5.0 µM reduced at least in 68% the amount of active SE A and C. This discovery is of great importance since it is known that this family of proteins covers very stable, resistant to heat and to degrading enzymes molecules (Schelin et al. 2011). These results demonstrate that PDI is not only effective in the inactivation of microorganisms but also in the degradation of released external virulence factors.

Microorganisms express a variety of mechanisms of resistance to traditional antimicrobial drugs (Enright et al. 2002; Costa et al. 2013). One of the main advantages of PDI is that because of the nature of the process, development of resistance is very unlikely (Tavares et al. 2010; Costa et al. 2011). The results obtained in this study do not show evidence of resistance development in the three strains subjected to ten PDI cycles, which corroborates literature conclusions. Considerable reduction in the efficiency of photosensitization of *S. aureus* strains ATCC 6538, enterotoxic 2065 MA and SA 3 MRSA after ten consecutive photosensitization sessions of 60 min

with 5.0 µM of **Tetra-Py⁺-Me** was not observed. As in this study the viable bacterial colonies have been aseptically removed from the plate and resuspended in PBS after each PDI cycle, the cellular density obtained after the colony resuspension could be different. To avoid differences in the PDI efficiency due to different bacterial densities, this parameter was controlled in all the experiments by measuring the optical density of the bacteria suspension before each assay.

Overall, it can be concluded that 1) although the efficiency of PDI to inactivate *S. aureus* is strain-dependent, all the strains can be effectively inactivated, namely the enterotoxic strains; 2) PDI process is not only effective in the inactivation of microorganisms but also in the degradation of their external virulence factors after their release to the exterior and 3) *S. aureus* strains do not develop resistance to PDI treatment.

5   References

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Supplementary Material

1. Supplementary Data

1.1 Quantity of enterotoxins (ng) per kit

Thermo Fisher
SCIENTIFIC

The world leader
in serving science

To Whom It May Concern
OXOID LTD Wade Road, Basingstoke, RG24 8PW, Hants, UK

RE: Toxin Detection Kits

We hereby declare that the products listed below contain 25 ng of toxin per kit.

- TD0900A SET RPLA KIT
- TD0930A PET RPLA KIT
- TD0960A VTEC RPLA KIT

Yours sincerely,

Yannis Bekatoros
Regulatory Affairs Officer

10MAR15

Microbiology	Oxoid Ltd.	Hants	+44 (0) 1256 841144	www.thermofisher.com
	Wade Road	RG24 8PW	+44 (0) 1256 814626 fax	
	Basingstoke	UK		

1.2 Calculating the decrease (%) of enterotoxins after PDI assays

Amount of enterotoxin in control of the SET-RPLA Kit Toxin Detection Kit: 25 ng.
This amount is reconstituted in 0.5 mL of diluent. Hence, the concentration of enterotoxins in control solutions is 50 ng mL^{-1}.

63 µL of control solutions were used to integrate irradiated samples and the light (LC) and dark (DC) controls. In each 63 µL there are 3.15 ng of enterotoxin.

The total volume of irradiated samples was 2.0 mL (63 µL of enterotoxin control solution + 20 µL of **Tetra-Py$^+$-Me** of stock solution to achieve a concentration of 5.0 µM + 1917 µL of PBS) with a concentration of enterotoxin of 1.58 ng mL^{-1}.

Aliquots of 25 µL from the total volume of irradiated samples were collected to test the presence of enterotoxins. In the first well of the top three rows (SE A/C and LC and DC controls before treatment) in the Figure 4, the enterotoxins are present at a concentration of 1.58 ng mL^{-1}. In the first well of the 8th row (sample after 60 min of treatment) the result is negative (formation of a tight button) for the presence of enterotoxin. It means that the enterotoxin is absence or present at concentrations bellow the method detection limit.

In the Instruction leaflet of the kit it is written (in section Limitations of the Test) that "The sensitivity of this test in detecting the enterotoxins has been reported to be 0.5ng/mL in the test extract". So, assuming that in the first well of the 8th row the enterotoxins are present at a concentration ≤ 0.5 ng mL^{-1}, there was a decrease of enterotoxin concentration of at least three times along the 60 min of treatment.

In percentage, it means a decrease of at least 68%.

Chapter 4

Additional communications

4.1 Poster communications

The development of the work presented in this document allowed the exposé of its results in two congresses, the 16th Congress of European Society of Photobiology, which took place in University of Aveiro, and in the 6th joint Congress of Microbiology and Biotecnology, MicroBiotec'15, in University of Évora, organized by the Portuguese Societies of Microbiology and Biotecnology, as follows:

Bartolomeu M, Rocha S, Cunha A, Neves MGPMS, Faustino MAF, Almeida A. (2015) Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus*. 16th Congress of the European Society for Photobiology. 31st August to 4th September, Aveiro, Portugal. P.040

Bartolomeu M, Rocha S, Cunha A, Neves MGPMS, Faustino MAF, Almeida A. (2015) Photodynamic inactivation of *Staphylococcus aureus*: effects on virulence factors. 6th Portuguese Congress of Microbiology and Biotechnology 2015 – MicroBiotec'15. 10th to 12nd December, Évora, Portugal.

4.2 Additional work

In parallel to the work done and already presented in this document, a Research Paper was developed within the theme of PDI – the study of PDI effect on the genomic DNA of *Escherichia coli*. The laboratorial work was developed during Pesquisa, the annual curricular unit of Biology course, in co-contribution with a master student, Sónia Coimbra, in LMAA, dbio, UA. The manuscript is "in revision" in the Journal of Porphyrins and Phthalocyanines.