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Resistance in antimicrobial photodynamic inactivation of bacteria

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Antibiotics have increasingly lost their impact to kill bacteria efficiently during the last 10 years. The emergence and dissemination of superbugs with resistance to multiple antibiotic classes have occurred among Gram-positive and Gram-negative strains including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* strains. These six superbugs can "escape" more or less any single kind of antibiotic treatment. That means bacteria are very good at developing resistance against antibiotics in a short time. One new approach is called photodynamic antimicrobial chemotherapy (PACT) which already has demonstrated an efficient antimicrobial efficacy among multi-resistant bacteria. Until now it has been questionable if bacteria can develop resistance against PACT. This perspective summarises the current knowledge about the susceptibility of bacteria towards oxidative stress and sheds some light on possible strategies of the development of photodynamic inactivation of bacteria (PACT)-induced oxidative stress resistance by bacteria.

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Introduction

Antibiotic resistance is not a new phenomenon, because bacteria themselves have developed antibiotics to fight against

each other as part of evolution. However bacteria have learnt to develop appropriate resistance against every single class of antibiotics in a short time. In general bacteria have a multiplicity of different pathways to evoke antibiotic resistance. Some bacteria naturally have a phenotype of low susceptibility to antibiotics (intrinsic resistance), acquired before these antibiotics were used extensively in the healthcare system.^{1–3} Antibiotic resistance can be induced by mutations of genetic elements and plenty of these genetic elements (plasmids and extrachromosomal elements) are mobile or transmissible, so bacteria can share them with each other.⁴ Furthermore bacteriophages can shuttle chromosomal- or plasmid coded resistance genes from one bacterium to another (transduction). Naked DNA, released from dead bacteria, can be taken up to a new bacterial host (transformation). Furthermore an efficient efflux-pump transport system can be expressed by bacteria to reduce the antibiotic concentration below threshold. Szczepanowski, R. *et al.* showed that up to 140 different resistance-gene-specific amplicons can be coded in a plasmid metagenome of antibiotic-resistant bacteria of wastewater treatment plants.⁵ The genes detected included aminoglycoside, lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim and sulfonamide resistance genes as well as multidrug efflux and small multidrug resistance genes.⁵ When the first bacteria of a colony have learnt how to become resistant to an antibiotic, it takes only a short time before all of the colony know how to survive the action of an antibiotic and to grow.⁶ In 1942 the first antibiotic, penicillin, was on the market worldwide. A few years later, in 1945, however, penicillin resistance of *Staphylococcus aureus* was

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already reported. In 1949 approximately 59% of the *S. aureus* strains were resistant to penicillin.^{7–9} Within a decade penicillin resistance had become a notable problem in clinical practice. This antibiotic breakthrough was not the only one. After penicillin, methicillin was developed in 1959; only two years later, 1961, the first MRSA strains were characterised. Within the next 40 years two additional steps of increasing antibiotic resistance were observed. Until now four waves of antibiotic resistance of *Staphylococcus aureus* have occurred.¹⁰ Several different factors led to the current situation of antibiotic resistance:^{11,12}

- (i) Selective pressure when antibiotics were used
- (ii) Dose and duration of antibiotic treatment
- (iii) Compliance with the use of antibiotics
- (iv) Inappropriate prescription of antibiotics for viral infections
- (v) Availability of antibiotics without any prescription in many countries (e.g. first aid antibiotic ointment containing bacitracin zinc/polymyxin B sulfate/neomycin sulfate)
- (vi) Antibiotics in livestock and the environment

(vii) Persistence of clinically relevant pathogens on dry inanimate surfaces for long periods (up to weeks, months and years¹³)

These aforementioned factors as well as the pollution of the environment by antibiotic contaminated sewage water, animal feed, foodstuffs and animals have favored the selective pressure of bacteria to become resistant (Fig. 1). Fig. 1 shows various routes by which bacteria and antibiotics can spread between livestock, humans and the healthcare system. So far more than 140 antibiotics have been developed for use in humans, but within the last 25 years the total number of new classes of antibiotics has decreased continuously.^{14,15}

From that point of view, the recent 2014 report of the World Health Organisation about the global surveillance of antibiotic resistance announced clearly that “antibiotic resistance is no longer a prediction for the future; it is happening right now, across the world, and is putting at risk the ability to treat common infections in the community and hospitals. Without urgent, coordinated action, the world is heading towards a post-antibiotic era, in which common infections and minor

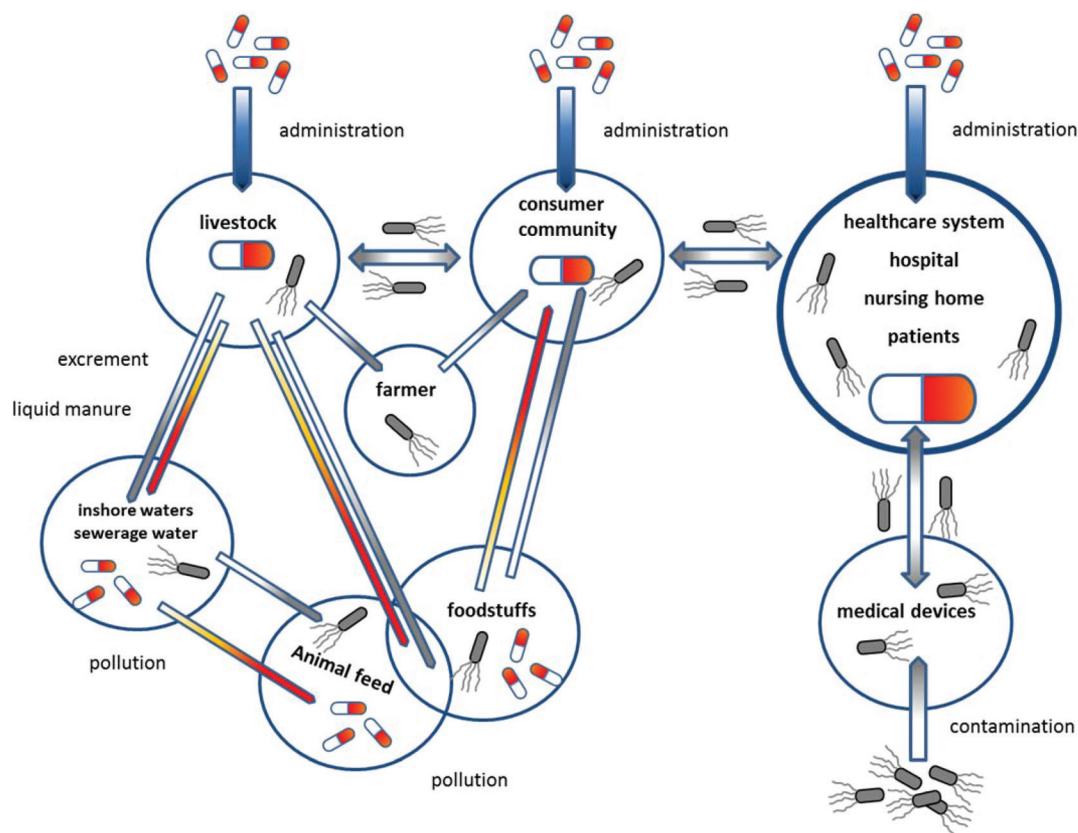


Fig. 1 Various routes by which bacteria and antibiotics can spread between livestock and humans. The transmission of bacteria from animals to humans can be either direct or indirect and vice versa. Routes for indirect transfer include contaminated sewage water, animal feed contaminated by excrement/manure, foodstuffs, and animals or humans colonized with resistant bacteria. Antibiotic administration to humans and livestock, as well as accumulation in the environment, creates selective pressure on bacteria that favor resistance. Medical devices can be colonized by pathogenic bacteria enhancing the nosocomial risk of infection. Arrows show transmission routes of bacteria and pollution of reservoirs by the overuse of antibiotics.



injuries, which have been treatable for decades, can once again kill".¹⁶

Therefore photodynamic antimicrobial chemotherapy (PACT) plays a crucial role when living in a world without antibiotics in the future.¹⁷ It is already accepted that PACT is an efficient antimicrobial approach to kill both antibiotic-sensitive and multi-resistant bacteria.^{18–21} In general the mechanism of action of PACT is a multi-target damaging process in contrast to those of antibiotics, which act very specifically to a definite target. For PACT's action no specific ligand (photosensitizer)–receptor interaction at or inside bacteria is necessary. Furthermore no specific extracellular or intracellular localization of a given photosensitizer is needed. No specific target structures (*e.g.* enzymes, chromosome or ribosome) are in focus for the oxidative burst induced by PACT after light activation of a given photosensitizer²² (Fig. 2). In contrast to PACT's action, the mechanism of action of antibiotics occurs *via* the so-called key-hole principle, acting very specifically toward one distinct bacterial target²³ (Fig. 2).

Until now it has been questionable if bacteria can develop resistance against PACT. This perspective summarises the current knowledge about the susceptibility of bacteria towards oxidative stress and possible strategies of bacteria developing PACT resistance.

Oxidative stress in bacteria induced by the environment and by PACT

Susceptibility of bacteria to oxidative stress

Bacteria have to negotiate oxidative stress during their interaction with host cells in the context of an infection, colonization and persistence. The formation of reactive oxygen species like hydrogen peroxide (H₂O₂), superoxide radical anion (O₂^{•-}) and hydroxyl radical (•OH) *via* the Fenton reaction can occur in the presence of oxygen. These ROS are known to effectively oxidize a wide variety of biomolecules and ultimately cause substantial biological damage. Furthermore in the case of an infection reactive oxygen and nitrogen species can be produced intracellularly by phagocytic cells of the innate immune system. Therefore the ability to adapt to oxidative stress is of importance for bacterial survival in their natural environment. In general bacteria have developed several mechanisms to elude oxidative stress from the environment. This protective system consists of an enzyme network of proteins like catalase, peroxidase or superoxide dismutase, detoxifying reactive oxygen species. A more detailed review about bacterial responses to photo-oxidative stress was published by Ziegelhoffer, E. C. and Donohue, T. J.²⁴ Furthermore Wolf, C. *et al.* used proteomics analysis to investigate the response of *S. aureus* to various kinds of oxidative stressors under *in vitro* conditions.²⁵ In this study hydrogen peroxide, paraquat and diamide were used as appropriate oxidants. They analyzed the changes in the synthesis of cytoplasmic proteins of *S. aureus* in response to the above mentioned agents.²⁵ Paraquat, a quaternary ammonium bipyridyl herbicide, was used as an intra-

cellular inducer of superoxide, and of hydrogen peroxide and hydroxyl radicals by further subsequent redox cycling processes.²⁶ Diamide specifically oxidizes thiol groups in proteins, thereby changing the amount of disulfide bonds in cytoplasmic proteins, leading to protein misfolding and instability.²⁷ In general the imbalance of misfolded or unstable proteins leads to an increase of heat shock proteins.^{28,29} The exposure of *S. aureus* to H₂O₂ (10 mM) showed that the synthesis rate of proteins involved in the detoxification of organic and inorganic peroxides and mainly proteins involved in DNA metabolism, protection, and repair was increased.²⁵ A 10 nM paraquat stress induced the expression of marker proteins with a multitude of different biological functions ranging from sigma factor, biosynthesis and cofactors. Incubation of *S. aureus* with 1 mM diamide causes an overexpression of both heat shock protein and oxidative stress response which indicates the accumulation of misfolded proteins.²⁵ After diamide stimulation all of the identified proteins are known to be controlled by the main repressors of the heat shock response, CtsR (ClpB and ClpP) and HrcA (DnaK, GroEL, GroES, and GrpE).^{25,30} The alkyl hydroperoxide reductase subunit C (AhpC) was induced by all three oxidative stress agents. AhpC protects *S. aureus* from peroxide stress, and expression of AhpC is relevant for aerobic survival, environmental persistence (desiccation) and nasal colonization.³¹ A reduced nasal colonization by *ahpC* and *kataA* (catalase deficient) mutants compared to the wild type was observed which was linked to reduced development of *S. aureus* infection.^{31,32} Besides catalases and the Ahp peroxidase, which are the main scavengers of hydrogen peroxide in bacteria, additional enzymes have been proposed to play a role in the clearance of hydrogen peroxide.³³ Further enzymes are thiol peroxidase, bacterioferritin comigratory protein, glutathione peroxidase, cytochrome c peroxidase, and rubrerythrins.³³ All these enzymes play a role in the degradation of hydrogen peroxide, but why bacteria use so many enzymes for scavenging hydrogen peroxide is unclear.³⁴

Oxidative stress induced by PACT

Proteomics analyses of a membrane enriched fraction of *S. aureus* have shown that a sublethal PACT treatment affected the expression of several functional classes of proteins.³⁵ Dosselli, R. *et al.* found that most of the proteins were involved in metabolic activities, in oxidative stress response, in cell division and in the uptake of sugar.³⁵ In this study a sublethal dose of PACT caused a different protein pattern compared to a lethal dose where only 1% of bacteria survived. Expression of catalase KatA was induced by a factor of 1.9 after a sublethal dose of PIB *vs.* 7.9 after lethal PIB, respectively.³⁵ Therefore expression of catalase might help bacteria to survive in the case of photo-oxidative stress induced by PACT.

Hydroxyl radicals, superoxide anions, hydrogen peroxide and singlet oxygen are the most relevant molecules that can induce oxidative stress in bacteria by PACT. How much and in what quantity these reactive oxygen species are generated depends on the chemical origin of the given photosensitizers. Methylene blue (MB), porphyrins (TMPyP) or perinaphthe-



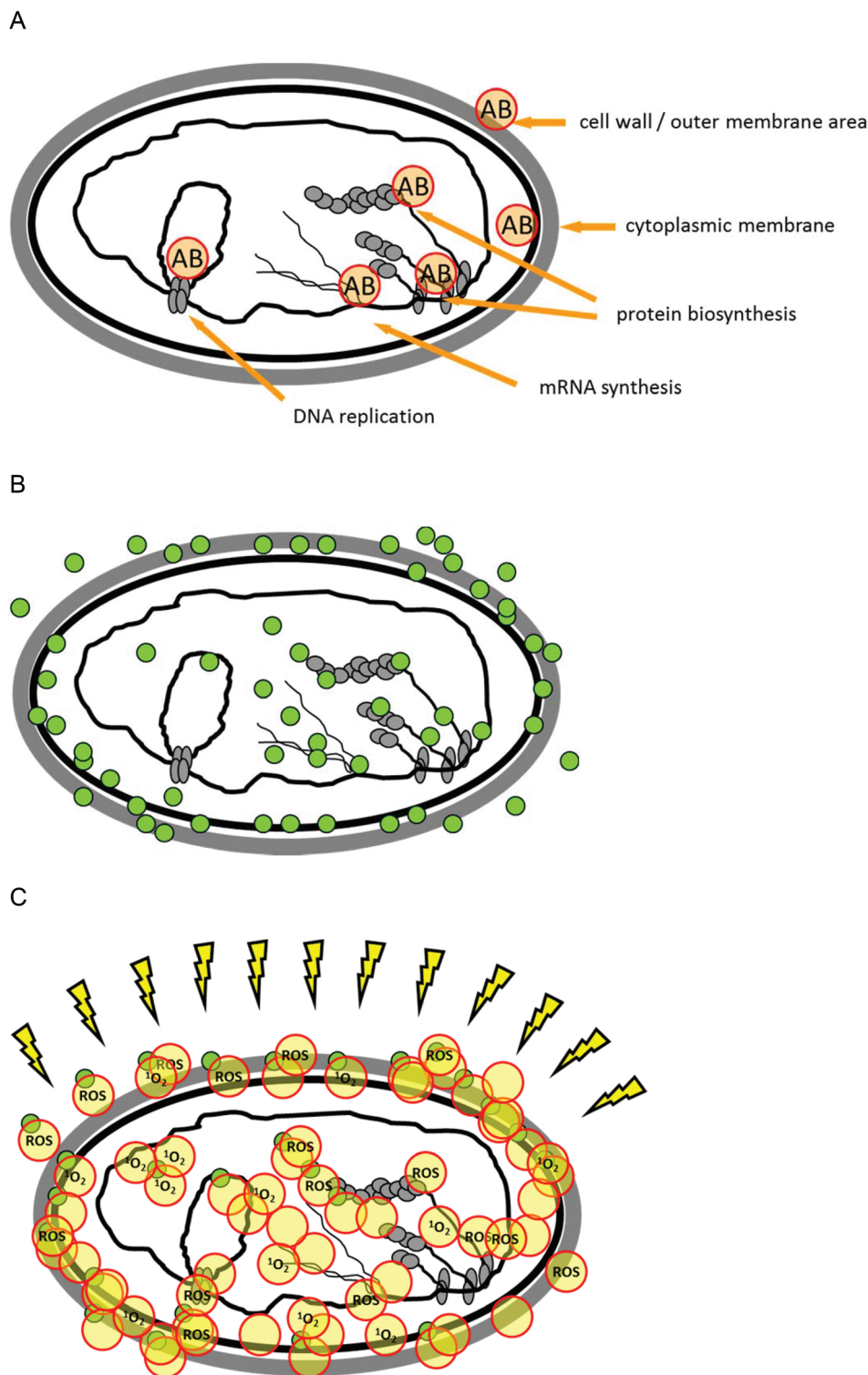


Fig. 2 Modes of action: antibiotic vs. PACT. (A) Different antibiotics (AB) react selectively with different molecules on certain specific organelles/structures, like cell wall components/outer membrane areas, cytoplasmic membrane and nucleic acid, or the presence of antibiotic interrupt pathways, mRNA and protein synthesis or DNA replication. (B) Unspecific localization of a given photosensitizer prior to illumination of the bacteria. (C) Generation of ROS, especially singlet oxygen (1O_2), after light activation of the photosensitizer. The photodestructive oxidative burst occurs proximately at the site of the photosensitizer localization. ROS riddle the bacteria with oxidation processes in such a way that survival is not possible. (Adopted and modified from Maisch *et al.*,²³ reprinted by permission of Eureka Science Ltd).



ones (SAPyR) have different quantum yields of singlet oxygen: MB: 0.52,³⁶ TMPyP: 0.77³⁷ and SAPyR: 0.99.³⁸ Therefore MB and TMPyP generate different amounts of type-I related reactive oxygen species. In contrast, SAPyR, a new recently developed photosensitizer, generates singlet oxygen only.^{38,39} So far the PACT induced damage of bacteria is specific in terms of the localization of a given photosensitizer. That means, when a photosensitizer is only attached to the surface of a bacterium, oxidative damage of proteins and fatty acids appears only at the site of localization due to the high reactivity, short lifetime and limited diffusion of the generated ROS.^{18,20,34,40} Therefore the distance might be too long between the oxidative burst at the outer membrane area of bacteria and the intracellular remaining defense enzymes. Therefore intracellular localized defense systems like superoxide dismutases and catalases might not help bacteria to survive. So far a direct distinct localization of photosensitizers by fluorescence microscopy is not possible due to the limit of resolution.^{41–43} Nevertheless scavenging of ROS induced not only by PACT, but also under ambient air is important for aerobic survival. Besides enzymatic quenching, e.g. hydrogen peroxide is detoxified by catalase KatA,⁴⁴ quencher molecules can physically/chemically scavenge ROS. One class of these anti-oxidative molecules are carotenoids which are able to quench the triplet state of chlorophyll (photosensitizer) as well as of singlet oxygen in photosynthetically active microorganisms.⁴⁵ Krieger-Liszkay, A. *et al.* showed that singlet oxygen is efficiently quenched by β -carotene, tocopherol or plastoquinone.⁴⁵ Photosynthetically active bacteria need a very effective defense system to protect their photosystem II (PSII) against photo-oxidative damage. Photo-oxidative damage as a result of the light-induced reactions of photosynthesis is caused not only by singlet oxygen but also by ROS like hydrogen peroxide, superoxide and hydroxyl radicals.^{45,46} As a consequence singlet oxygen was identified as a direct inducer of the RpoH_{II}-type sigma factor in photosynthetic active bacteria.⁴⁷ The RpoH_{II}-type sigma factor is required for the inducible expression of several defense factors in these bacteria. The authors could demonstrate that singlet oxygen affected the RpoH_{II} dependent downstream expression cascade, and thereby singlet oxygen was generated by light activated methylene blue. Activation of the RpoH_{II} gene cluster directly promotes the expression of proteins involved in the quenching and detoxification of ROS.⁴⁷ Until now a specific bacteria defense system against singlet oxygen itself is not present in pathogenic bacteria. Therefore it is questionable if bacteria can develop a defense system against singlet oxygen directly, because singlet oxygen is the excited state of oxygen (+0.98 eV). Along with catalase KatA, the superoxide dismutase is part of the oxidative stress defense system.⁴⁸ This enzyme metabolizes superoxide anions to hydrogen peroxide and oxygen. In *E. coli*, the presence of superoxide anions can damage the enzymes responsible for the synthesis of amino acids, because Δ sodA sodB double mutants are not able to grow aerobically on a minimal medium.⁴⁹ In *S. aureus* likewise, the inactivation of sodA resulted in a reduced ability to survive superoxide anions generated intracellularly by a paraquat

stress.³¹ Furthermore a Δ ahpC Δ katA double mutant showed an additional increase in sensitivity to paraquat compared to the isogenic wild type, probably due to an increased sensitivity to hydrogen peroxide, which is generated intracellularly by enzymatic dismutation of superoxide anions to H₂O₂ and O₂. Furthermore hydrogen peroxide itself serves as a sensor molecule activating the OxyR transcription factor which regulates the expression of catalase and peroxidase enzymes coding on the oxyR gene regulon. Thereby hydrogen peroxide is quenched to water and oxygen.^{33,50} Not only H₂O₂ activates the OxyR gene regulon, but also singlet oxygen is able to do this job. Kim, S.Y. investigated the control mechanism of singlet oxygen induced oxidative damage in *E. coli*.⁵¹ Again in this study singlet oxygen was generated by light-activated methylene blue. The study showed that overexpression of the *E. coli* OxyR regulon decreased the bactericidal effect of ¹O₂ by both an increased catalase and SOD activity which reduced the strength of protein oxidation.⁵¹ Furthermore a Δ oxyR mutant was hypersensitive to singlet oxygen generated by MB and light, and this mutant exhibited increased protein damage compared to the wildtype.⁵¹ These results emphasize the potential mechanism in which the activation of the OxyR regulon protects bacteria from photo-oxidative damage by overexpression of relevant antioxidant enzymes.

Heat shock protein expression induced by PACT

Besides the expression of enzymes to catalyze the dismutation and/or partitioning of reactive oxygen species to molecular oxygen, bacteria are able to express heat shock proteins (chaperones) after photo-oxidative stress induction.²⁴ Ziegelhoffer, E. C. and Donohue, T. J. demonstrated that singlet oxygen can activate a cascade of transcriptionally relevant genes in bacteria, especially in *Rhodobacter sphaeroides*.²⁴ The generation of singlet oxygen increases the activity of the group IV alternative σ -factor, σ^E . This σ^E factor is responsible for the transcription activation of genes that are predicted to protect cells from ROS. As an example, induction of σ^E factor expression leads to the transcription of RpoH_{II} (a homologue protein to the alternative σ^{32} factors, responsible for recognizing heat shock gene promoters).⁵² RpoH_{II} can transcribe genes like *ecfE*. EcfE is a membrane-bound zinc metalloprotease involved in intramembrane proteolysis.⁵³ Such proteolytic reactions contribute to the maintenance of bacterial integrity by eliminating misfolded proteins. Therefore such a redundant system of alternative and homologous σ -factors helps bacteria, especially photosynthetically active bacteria, to respond quickly toward photo-oxidative stress. Such a system might also make it possible for human pathogenic bacteria to increase the power of PIB induced oxidative stress responses. Analysis of the heat shock protein response of *E. faecalis* and *E. coli* have shown that both Gram-positive and Gram-negative bacteria responded to PACT mediated oxidative stress at sublethal PACT conditions.⁵⁴ An enhanced overexpression of GroEL and DnaK was observed indicating the fact that bacteria might be able to become less susceptible to PACT. GroEL and DnaK are two relevant heat shock proteins protecting bacteria against un-



specific stress conditions from the environment.^{28,29} Furthermore St. Denis and colleagues demonstrated that a heat shock pretreatment of bacteria prior to lethal PACT treatment limits the effectiveness of PACT in inactivation of bacteria. Using TBO as the respective photosensitizer, 2–4 log₁₀ units less killing were achieved after light activation of heat pretreated bacteria, demonstrating a protective effect of HSP against PACT.⁵⁴ Overall upregulation of HSPs by bacteria induced by a different stressor may contribute to stress tolerance of bacteria in the face of a lethal PACT treatment.

Differences in the susceptibility of bacteria to PACT

Inactivation of antibiotic resistant bacteria by PACT is practicable using the same photodynamic conditions, like PS concentration, incubation time or applied light dose as compared to an antibiotic sensitive strain of the same species.^{55,56} Certainly a strain-dependent difference in susceptibility to the PACT effect was observed.^{57,58} Grinholm *et al.*, showed that 4 out of 80 clinical *S. aureus* isolates (40 MSSA and 40 MRSA) were less susceptible to PACT.⁵⁷ The log₁₀ unit reduction ranged from >0.03 (resistant) to 3 log₁₀ units (sensitive). In this study only one specific photosensitizer concentration and light dose were used. Therefore it is unclear whether “stronger” photodynamic conditions (higher concentrations of PS and/or light doses) are able to achieve more efficient photodynamic inactivation of these resistant MRSA strains. Until now the principal mechanism of different susceptibilities of distinctive isolates of the same species to the bactericidal effect of PACT has not been fully understood. However the diminished PACT sensitivity could not be correlated with an enhanced biofilm formation of these four strains.⁵⁷ This is surprising, because biofilm formation (mainly extracellular matrix protein secretion) is known to be part of an unspecific defense of bacteria to survive adverse environmental conditions. In a further study Grinholm *et al.* investigated the overall PACT killing efficacy of 424 MRSA and MSSA isolates.⁵⁹ Again the differences detected in the antibacterial PACT efficiency could not be correlated with both the antibiotic effectiveness and the resistance pattern of the investigated strains.⁵⁹ Overall the genes that determine the methicillin resistance (*mec* gene cassette) do not explain the observed differences between MRSA and MSSA strains. That means, regardless of the antimicrobial resistance mechanism, the difference in response to PACT between MRSA and MSSA exists.⁵⁹

In addition Nakonieczna, J. *et al.* showed for *S. aureus* that a strain specific intrinsic dependence of increased activity of SOD could be correlated with a reduced susceptibility to PACT.⁶⁰ Moreover, a sublethal porphyrin-based photodynamic treatment of *S. aureus* leads to an enhanced enzymatic activity of SOD. However, a real resistance to PACT was not observed because the photodynamic conditions used were suitable in terms of applied light dose and PS incubation time for efficient inactivation of these strains, thus increased SOD activity was measured.⁶⁰ Therefore an increased SOD activity can lead to a

reduced susceptibility, but not to a complete resistance towards PACT. That means the SOD activity is not a direct factor causing the reduced susceptibility to PACT, but rather the phenotype of enhanced SOD activity seems to be already present in *S. aureus* and protecting these isolates from oxidative stress.

However after the exposure of bacteria to a given stress factor it takes a minimum of a few minutes (5–10 min) until the bacteria react with a higher synthesis rate of protecting proteins for surviving. The majority of protecting proteins are first induced within 30 min after a given stressor.²⁵ Chang, W. *et al.* demonstrated that mRNA levels of oxidative stress-induced genes were increased and decreased 10 min and 20 min respectively after hydrogen peroxide exposures.⁶¹ Therefore a minimum of a few minutes is necessary to activate the protein expression machinery of bacteria. Recently Maisch, T. *et al.* could show that PACT yielded a photodynamic killing efficacy of up to 6 log₁₀ (>99.99999%) of the viable number of bacteria *in vitro* when using very short IPL light pulses of 100 ms and a very short incubation of a few seconds. From this observation it seems unlikely that bacteria can react with the overexpression of protective proteins within such a short total treatment time to survive PACT. However these results are in accordance with already published data that a PACT induced resistance was not possible in any pathogen that has been investigated so far.⁶² Lauro, F. M. *et al.* demonstrated that repeated photosensitization of bacteria which survived a PACT treatment did not induce the selection of resistant bacteria strains.⁶² The photodynamic killing efficacy suffered no change during 10 cycles of repeated irradiation experiments. Furthermore Giuliani, F. *et al.* demonstrated that 20 consecutive PACT treatments with a tetracationic Zn(II) phthalocyanine did not result in any resistant mutants of *S. aureus*, *P. aeruginosa* or *C. albicans*.⁶³ No variations in MBC (minimal bactericidal concentration) or MFC (minimal fungicide concentration) were observed at the end of the multistep resistance selection. The authors concluded that the absence of any PACT resistance validates the multi-target nature of the antimicrobial photodynamic mechanism of action.

In addition bacteria colonies that recovered after each PACT treatment showed no difference in their sensitivity to different classes of antibiotics (*e.g.* β-lactams, aminoglycosides or tetracyclines) as compared with untreated controls.⁶² This observation highlights the practicability of PACT even under an antibiotic treatment.

Nevertheless different parameters of incubation time and/or applied light doses are necessary to achieve efficient PACT efficacy. This different susceptibility of PACT among various pathogens is due to the differences in the structure of the cell wall areas of Gram-positive, Gram-negative and fungi cells, and not to a possible existing resistance mechanism.^{64–67}

Biofilm resistance to PACT

Successful eradication of bacteria embedded in biofilms requires up to 100–1000 times higher concentrations of a par-



ticular disinfectant or antibiotic compared to the freely floating, planktonic counterparts.^{68,69} This reinforced strength of biofilm-growing bacteria against antimicrobials is attributed to the following points:

(i) *Genetic diversity within the biofilm*: genetic diversity can protect microorganisms from unstable environmental conditions.⁷⁰ That means that certain bacterial subpopulations within the biofilm exhibit an increased ability to disseminate or accelerate biofilm formation. The presence of functionally diverse bacteria (bystander cells, persister cells) within a biofilm helps to resist an environmental stress.

(ii) *Gene expression is altered between freely floating and sessile bacteria*: up to one-fifth of the genome is expressed definitely when bacteria are growing as a biofilm. Thereby the expression profile of genes is changed, predominantly for those involved in DNA replication, catabolism, and binding and transport.⁷¹

(iii) *Biofilm matrix*: the EPS (extrapolymetric substance) is a 3D structure surrounding the bacteria within the biofilm and acts as a physically rugged barrier to protect the biofilm. Drug diffusion is slowed down by the higher viscosity of the EPS network.⁷²

(iv) *Communication via quorum sensing*: the ability of bacteria to communicate within a biofilm has the advantage that bacteria can adapt to changing environmental conditions very fast. The mechanism to regulate concerted physiological activities is called “quorum sensing (QS)”.^{73,74}

So far the PACT efficacy of many photosensitizers, especially phenothiazinium dyes and porphyrins, has been already demonstrated for the inactivation of biofilms grown *in vitro* (review: ref. 75). In general longer preincubation times (up to 24 h), higher concentrations (up to 25 times) and light exposure times (up to 30 min) were required to reach a phototoxicity of 3 log₁₀ steps against biofilm growing bacteria compared to planktonically growing counterpart. However the presence of the negatively charged EPS may protect the bacteria from the action of positively charged photosensitizers (no sufficient attachment, no uptake to induce a photodynamic reaction after light activation). TMPyP (5,10,15,20-tetrakis(1-methyl-4-pyridino)-porphyrin tetra-(*p*-toluenesulfonate)), a four-fold positively charged dye, may cause stronger electrostatic interactions with the EPS as compared to SAPYR (2-((4-pyridinyl)methyl)-1*H*-phenalen-1-one chloride, one positive charge) which leads to a reduced biofilm inactivation.³⁸ Light-activated SAPYR exhibits *Enterococcus* biofilm inactivation efficacy, whereas TMPyP does not in spite of the absolute quantum yield for ¹O₂ generation being 4.8 times higher for light-activated TMPyP under the given conditions.³⁸ Furthermore TMPyP is a larger molecule compared to SAPYR (682.2 vs. 272.3 g M⁻¹ without counterions), thereby steric reasons may block its permeability through the EPS. The total biomass volume is a further factor influencing the divergent permeability of the EPS. Normally the EPS permeability decreases strongly with increasing biomass volume of the biofilm (mature biofilm vs. young biofilm).⁷⁶

Overall a specific biofilm tolerance to PACT is questionable. So far no specific regulatory process is known for evident toler-

ance to PACT like that accepted for UV radiation. Nosanchu, J. D. and Casadevall, A. showed that bacteria can secrete pyomelanin as a resistance factor for an increased tolerance to UV radiation.⁷⁷ However such a scenario is possible in case of PACT. Higher amounts of exogenous or endogenous pigments in biofilm growing bacteria may act as quenchers to ROS, thereby reducing the susceptibility to PACT.

Conclusion and outlook

The photodestructive effect of ROS at multiple bacterial sites by light-activated photosensitizers is one of the advantages of PACT compared to the key-hole principle of antibiotics. From that point of view resistance to PACT seems to be unlikely within the meaning of antibiotic resistance. However differences in the photodynamic susceptibility are known.⁵⁷ The strains with a lower PACT susceptibility were not selected by the photodynamic process itself, but these “stronger strains” already acquired their genetic background as an intrinsic resistance/susceptibility to survive oxidative stress by the environment. Whether bacteria can directly develop a resistance to ROS triggered by PACT, especially singlet oxygen, is questionable, because PACT treatment can be very fast and effective.^{20,78} Therefore bacteria do not have the time to react adequately by overexpression of a protective protein shield. Nevertheless when the amount of generated ROS is sufficiently high, the basal defense protein levels are insufficient to scavenge the ROS, so the bacteria can't survive. However bacteria have learnt by adaptive responses to scavenge ROS like hydrogen peroxide.³³ So far all these scavenging enzymes expressed by bacteria are not able to overcome the oxidative burst induced by PACT, because these ROS riddle the bacteria at multiple sites (where the photosensitizer is located) in such a way that survival is not observed. In view of the downward trend of the development of new classes of antibiotics, PACT can support antibacterial treatment options in such a way that the number of colony forming units is further reduced, and thus bacterial infections can be dominated by the immune system.

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