Microbiological Research 207 (2018) 53–64

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Stress response of a clinical *Enterococcus faecalis* isolate subjected to a novel antimicrobial surface coating

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ARTICLE INFO

Keywords:
Antimicrobial
Silver
Stress
RNA sequencing
Enterococcus

ABSTRACT

Emerging antibiotic resistance among pathogenic bacteria, paired with their ability to form biofilms on medical and technical devices, represents a serious problem for effective and long-term decontamination in health-care environments and gives rise to an urgent need for new antimicrobial materials. Here we present the impact of AGXX®, a novel broad-spectrum antimicrobial surface coating consisting of micro-galvanic elements formed by silver and ruthenium, on the transcriptome of *Enterococcus faecalis*. A clinical *E. faecalis* isolate was subjected to metal stress by growing it for different periods in presence of the antimicrobial coating or silver-coated steel meshes. Subsequently, total RNA was isolated and next-generation RNA sequencing was performed to analyze variations in gene expression in presence of the antimicrobial materials with focus on known stress genes. Exposure to the antimicrobial coating had a large impact on the transcriptome of *E. faecalis*. After 24 min almost 1/5 of the *E. faecalis* genome displayed differential expression. At each time-point the *cop* operon was strongly up-regulated, providing indirect evidence for the presence of free Ag^+-ions. Moreover, exposure to the antimicrobial coating induced a broad general stress response in *E. faecalis*. Genes coding for the chaperones GroEL and GroES and the Clp proteases, ClpE and ClpB, were among the top up-regulated heat shock genes. Differential expression of thioredoxin, superoxide dismutase and glutathione synthetase genes indicates a high level of oxidative stress. We postulate a mechanism of action where the combination of Ag^+-ions and reactive oxygen species generated by AGXX® results in a synergistic antimicrobial effect, superior to that of conventional silver coatings.

1. Introduction

Enterococci are Gram-positive bacteria with a two-sided nature. As harmless commensals they are present in the gastrointestinal tract of animals ranging from insects to humans (Gilmore et al., 2014). However, Enterococci are also known as opportunistic nosocomial pathogens that can cause endocarditis, sepsis and wound as well as urinary tract infections (Theilacker et al., 2012). Actually, *Enterococcus* species are the second most common pathogens in healthcare-associated infections; among enterococcal hospital-acquired infections approx. 60% are assigned to *E. faecalis* (Hidron et al., 2008). This species is able to form biofilms on medical devices such as catheters and orthopedic implants (Mohamed and Huang, 2007). In these biofilms the bacteria are protected from a variety of physical as well as chemical stresses making them hard to eradicate and thus medically important (Costerton et al., 1999; Lewis, 2001). Moreover, Enterococci are naturally resistant to many antibiotics and able to acquire and exchange antibiotic resistance determinants giving them a selective advantage in environments with heavy antibiotic usage such as hospitals (Gilmore et al., 2014). An infamous example is *E. faecalis* V583, the first clinical isolate resistant to vancomycin (Sahm and Olsen, 1990).

Silver was the most important antimicrobial compound before antibiotics were introduced in the 1940s (Alexander, 2009; Mijnendonckx et al., 2013). Since ancient times, silver and copper have been used for medical applications. Silver is receiving renewed attention as antimicrobial surface material and is becoming increasingly prevalent in clinics and general healthcare (Lansdown, 2006) as a broad-spectrum agent with activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and certain viruses but low toxicity to human cells (Maillard and Hartemann, 2012; Mijnendonckx et al., 2013). The most

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https://doi.org/10.1016/j.micres.2017.11.006
Received 20 May 2016; Received in revised form 21 April 2017; Accepted 7 November 2017
Available online 12 November 2017
0944-5013/

prominent forms are silver ions (mostly as $AgNO_3$) and silver nanoparticles (Prabhu and Poulose, 2012). However, extensive use of silver has raised questions and concerns about its safety and toxicity for the human body and the environment, as well as the risk associated with the reported increase in microbial resistance (Lansdown, 2010; Maillard and Hartemann, 2012; Mijnendonckx et al., 2013). Biofilm production, in combination with the huge problem of emerging antibiotic resistance among clinically relevant bacteria, gives rise to an urgent need for new antimicrobial materials. One of these is AGXX®, a novel broad-spectrum antimicrobial surface coating (Guridi et al., 2015) consisting of silver (Ag) and ruthenium (Ru) conditioned with ascorbic acid. The antimicrobial surface coating can be electroplated homogenously on various carrier materials such as steel, glass, ceramics and organic polymers like polydimethylsiloxane. In contrast to conventional silver nanotechnology, the electroplating process results in a surface structured by micro-galvanic elements of Ag and Ru. The antimicrobial coating is durable, recyclable and thus environmentally friendly. It has already been successfully applied in industrial water disinfection (Landau, 2013).

Although the mechanism of antimicrobial action of copper and silver is not yet fully understood, it is known that free bioavailable ions of these metals and the generation of reactive oxygen species (ROS) such as the highly reactive hydroxyl radicals (OH·) play important roles. Cu^+- and Ag^+-ions are isovalent electronic and have the same d^{10} electron configuration. Moreover, they show a similar protein coordination chemistry (Loftin et al., 2007). Therefore, it is not surprising that some proteins can bind and transport both metal ions, e.g. the *E. coli* binding protein CusF (Kittleson et al., 2006) and the exporter CusCBA encoded by the *cus* (Cu *s*ensitivity) operon (Franke et al., 2003). Silver ions interact with thiol groups of proteins, block respiration and electron transfer and promote the production of ROS (Gordon et al., 2010; Park et al., 2009). Upon excess of intracellular copper, an important trace element and cofactor for many indispensable enzymes, hydroxyl radicals can be generated in a Fenton-type reaction (Grass et al., 2011) that participate in the oxidation of proteins, lipids and DNA (Imlay, 2003; Yoshida et al., 1993). Therefore, metal ion homeostasis plays a critical role in the defense against oxidative stress and is even key to successful host colonization, infection and survival (Agranoff and Krishna, 1998), making it an important modulator of bacterial pathogenicity (Abrantes et al., 2013, 2011; Wang et al., 2014). The best understood prokaryotic metal homeostasis system is specified by the extensively studied *cop* operon of *E. hirae*. It regulates the uptake, availability and export of copper (Odermatt and Solioz, 1995; Odermatt et al., 1994, 1993; Solioz and Stoyanov, 2003; Wunderli-Ye and Solioz, 1999). The *cop* operon is inducible by Cu^{2+}, Cd^{2+} and Ag^+ (Odermatt et al., 1993) and consists of four genes: *copY* codes for a copper-responsive repressor, *copZ* encodes a copper chaperone/transport protein, and *copA* and *copB* specify copper transporting ATPases (Solioz and Stoyanov, 2003).

The evolution of specific and adaptive responses is crucial for survival in habitats with varying conditions. Stress response pathways, such as the heat shock and SOS response pathways, as well as the response to oxidative stress, are widely conserved and exhibit regulatory connections (Derré et al., 1999; Layton and Foster, 2003; Neher et al., 2006). Upon exposure to various stress conditions such as heat shock, but also during chemical and oxidative stresses, a signaling pathway known as the heat shock response is turned on; it is characterized by a markedly increased expression of genes coding for molecular chaperones and Clp proteases (Hartl et al., 2011; Mattoo and Goloubinoff, 2014). Mis- or unfolded proteins and polypeptide chains are assisted in (re-) folding to their native conformation by these chaperones (Hartl et al., 2011) or degraded by the Clp proteases (Neher et al., 2006) to prevent their aggregation. Oxidative stress can be generated by oxygen and various ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). ROS can cause serious damage to nucleic acids, proteins and lipids via oxidation (Imlay, 2003), which may ultimately lead to cell death (Gordon et al., 2010). They are usually inactivated quickly by protective enzymes such as superoxide dismutase and catalase. The thioredoxin system and glutaredoxins keep proteins in their reduced state (Arnér and Holmgren, 2000) and are therefore important additional players in the response to oxidative stress. DNA damage or the collapse of DNA replication forks as a consequence of ROS results in the exposure of vulnerable single stranded DNA, inducing the bacterial SOS response (Lusetti and Cox, 2002; Michel, 2005). It generally consists of the induction of genes coding for DNA repair proteins such as exonucleases, helicases and recombinases as well as translesion DNA polymerases (van der Veen and Abee, 2011) and is primarily regulated by the repressor LexA (Butala et al., 2009) and the activator RecA (Cox, 2007). All these conserved rescue pathways enable bacteria to tolerate and survive various forms of stress.

In this study, we subjected the clinical *E. faecalis* 12030 isolate to metal stress by exposing it to AGXX® or Ag-coated V2A steel meshes, examined the transcriptome responses by next-generation RNA sequencing and analyzed variations in gene expression to elucidate the mechanism of action of the antimicrobial AGXX® at the molecular level.

2. Material and methods

2.1. Preparation and testing of antimicrobial metal meshes

The metal meshes were essentially prepared as previously described (Guridi et al., 2015). Stainless steel gauze (V2A: DIN ISO 1.4301), 50 µm mesh width, was used as base material for Ag and AGXX® coatings as well as reference material. The silver and AGXX® coatings were electroplated on the stainless steel carrier meshes with the same thickness of 3–5 µm. The resulting AGXX® coating is structured such that many micro-galvanic cells are formed on the surface layer consisting of Ag micro anodes and Ru micro cathodes. The antimicrobial activity of AGXX® meshes was routinely checked by incubation with *Escherichia coli* DSM 498 at 37 °C for 18 h.

2.2. Media and growth conditions

E. faecalis 12030 (Huebner et al., 1999) was grown at 37 °C in Brain Heart Infusion (BHI, Oxoid Deutschland GmbH, Wesel, Germany) medium with constant agitation at 150 rpm or on BHI agar (Oxoid Deutschland GmbH). For generation of growth curves, bacteria were pre-cultured overnight, diluted in BHI medium to an optical density at 600 nm (OD_{600}) of 0.05 and incubated for 8 h either in the presence of an uncoated V2A mesh (abbreviated as V2A) or a V2A mesh coated with AGXX® (AGXX®) or silver (Ag) (12 cm^2 each in 30 ml medium to obtain a mesh-surface to medium-volume ratio of 0.4); cultures grown in the absence of a metal mesh served as controls. The OD_{600} of the cultures was measured using a Genesys™ 10 UV–vis spectrophotometer (Thermo Spectronic, Rochester, USA). Colony Forming Units (CFU) ml^{-1} were determined hourly from 0 to 8 h post inoculation. Growth experiments were performed in triplicate with independent biological replicates.

2.3. Metal stress and RNA isolation

Overnight cultures of *E. faecalis* 12030 were diluted as described above and grown until mid-exponential growth phase ($OD_{600} \approx 0.6$). The cultures were then either subjected to metal stress by exposure to an AGXX®- or Ag-coated metal mesh or exposed to an uncoated V2A steel mesh (mesh-surface to medium-volume ratio of 0.4) followed by further incubation for 3, 6, 12, 24, 60 and 90 min at 37 °C with constant agitation of 150 rpm. As a control, no metal mesh was added.

Cells from 30 ml culture were harvested by centrifugation for 1 min at 10 000 rpm and 4 °C in a Sorvall RC6+ centrifuge (Thermo Scientific GmbH, Langenselbold, Germany). Cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C or directly used for RNA