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Global analysis of the impact of linezolid onto virulence factor production in *S. aureus* USA300

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ABSTRACT

The translation inhibitor linezolid is an antibiotic of last resort against *Gram-positive* pathogens including methicillin resistant strains of the nosocomial pathogen *Staphylococcus aureus*. Linezolid is reported to inhibit production of extracellular virulence factors, but the molecular cause is unknown. To elucidate the physiological response of *S. aureus* to linezolid in general and the inhibition of virulence factor synthesis in particular a holistic study was performed.

Linezolid was added to exponentially growing *S. aureus* cells and the linezolid stress response was analyzed with transcriptomics and quantitative proteomics methods. In addition, scanning and transmission electron microscopy experiments as well as fluorescence microscopy analyses of the cellular DNA and membrane were performed.

As previously observed in studies on other translation inhibitors, *S. aureus* adapts its protein biosynthesis machinery to the reduced translation efficiency. For example the synthesis of ribosomal proteins was induced. Also unexpected results like a decline in the amount of extracellular and membrane proteins were obtained. In addition, cell shape and size changed after linezolid stress and cell division was diminished. Finally, the chromosome was condensed after linezolid stress and lost contact to the membrane. These morphological changes cannot be explained by established theories. A new hypothesis is discussed, which suggests that the reduced amount of membrane and extracellular proteins and observed defects in cell division are due to the disintegration of transertion complexes by linezolid.

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1. Introduction

Methicillin resistant strains of the nosocomial pathogen *Staphylococcus aureus* (MRSA) are a major problem in global healthcare. Healthcare-associated (HA)-MRSA strains, for whose acquisition prior illness is a predisposing risk factor, infect primarily immune-suppressed, frail and elderly people. In contrast, community-associated (CA)-MRSA also infect healthy individuals, suggesting a greater virulence of CA-MRSA strains (DeLeo et al., 2010). Linezolid (LZD) is one of few remaining treatment options for infections caused by these important pathogens. LZD was the first antibiotic of the oxazolidinone translation inhibitor class approved for clinical use in the year 2000. Oxazolidinone translation inhibitors represented the first truly new class of antibiotics after nearly 20 years that act via a unique mechanism of action. Oxazolidinones prevent protein biosynthesis by binding to the peptidyl transferase center at the ribosomal A site. LZD stabilizes a distinct conformation of the conserved 23S rRNA nucleotide U2585. In this conformation the second tRNA cannot reach the correct position for formation of the first peptide bond. After GTP hydrolysis and

Abbreviations: LZD, linezolid; GeLC-MS, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by liquid chromatography mass spectrometry; EMF, enriched membrane fraction; TEAB, triethylammonium bicarbonate buffer; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TBS, tris-buffered saline; Hi3 quantification, absolute protein quantification by the average signal intensity of the three most intense peptides per protein compared to an internally digested standard protein; PVL, Panton-Valentine toxin.

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release of EF-Tu the second tRNA dislocates from the ribosome, which remains locked (Wilson et al., 2008).

As with all antibiotics, development of bacterial resistance against LZD is of greatest concern. More than 99.5% of all *S. aureus* isolates tested in several studies were susceptible to LZD and all resistant Staphylococci were obtained from patients who had previously been treated with LZD (Gu et al., 2012). Resistance phenotypes identified so far are based on mutations in the 23S rRNA gene (Besier et al., 2008) and the genes coding for ribosomal proteins L3 and L4 (Locke et al., 2010). Other mechanisms of resistance in *S. aureus* include methylation of 23S rRNA by the plasmid-coded chloramphenicol florfenicol resistance (*cfr*) gene product (Morales et al., 2010). In addition, a mutation in the *relA* gene switching on the stringent response and reducing LZD susceptibility has been reported (Gao et al., 2010).

The effects of LZD on bacterial cells have been studied by several research groups. In a first study a decreased amount of staphylococcal and streptococcal exotoxins was detected after the cells were grown in presence of subinhibitory concentrations of LZD (Gemmell and Ford, 2002). A series of similar studies examining the expression and/or amount of certain exotoxins in presence of LZD and other antibiotics has been performed since then (Coyle et al., 2003; Dumitrescu et al., 2007; Otto et al., 2013). These conclusively show that LZD might inhibit toxin production (Diep et al., 2012). However, these studies focused only on specific virulence factors and the general impact of LZD onto bacterial physiology was not studied. Functional genomics studies can provide a detailed picture of the physiological adaptation responses of *S. aureus* to antibiotic treatment (Wecke and Mascher, 2011; Wenzel and Bandow, 2011). For instance, Bernardo et al. analyzed the exoproteome of LZD stressed *S. aureus* and detected a specific reduction in amount of extracellular virulence factors (Bernardo et al., 2004). Furthermore LZD was shown to inhibit accumulation but not the transcription of the exotoxin Panton-Valentine leukocidin, when added to exponentially growing cells at a concentration of five times the MIC (Stevens et al., 2007). However, none of these studies revealed the mechanism of the initially observed inhibition of virulence factors, which was also observed in in vivo studies with animal models (Yoshizawa et al., 2012; Diep et al., 2013).

To resolve the question if (and how) LZD reduces the synthesis of virulence factors, we designed an approach integrating complementing omics approaches with microscopy. An *S. aureus* USA300 derivative was used as a biological model. We performed absolute, label-free quantification of cytosolic and extracellular proteins to monitor the effect of translation inhibition on protein levels. For exact protein quantification we performed a metabolic labeling and analyzed the cytosolic and membrane proteome. In addition, we performed a DNA microarray analysis and microscopy experiments. Using this integrated approach we provide a global view of the physiological effects of LZD onto a clinically highly relevant pathogen and reveal that diminished protein translocation potential might result in decreased levels of extracellular toxins and reduced virulence observed after LZD treatment.

2. Material and methods

2.1. Bacterial growth conditions

Cells were grown aerobically at 37 °C in LB medium (Invitrogen), or for creation of the labeled standard in ^{15}N-enriched BioExpress 1000 medium (Cambridge Isotopes). All transcriptomics and proteomics experiments were performed in triplicates. The minimal inhibitory concentration (MIC) of LZD in *S. aureus* USA300 was determined by broth microdilution. To analyze the LZD response of growing cells LZD was added at different concentrations (0.5–8× MIC) at OD_{540} of 0.5 (Fig. 1). For proteomic, transcriptomic and microscopy experiments LZD was added to a final concentration of 2× MIC (5 mg l^{-1}). Samples were harvested 1 h after addition of LZD (III), at the beginning of the interim stationary phase (IV) and after the cells had resumed growth (V). As control samples exponentially growing cells and early transient cells control samples were harvested at OD_{540} of 0.9 (I) and 3.4 (II), respectively (Fig. 1). LZD concentrations in the culture media were assayed with an agar-diffusion test.

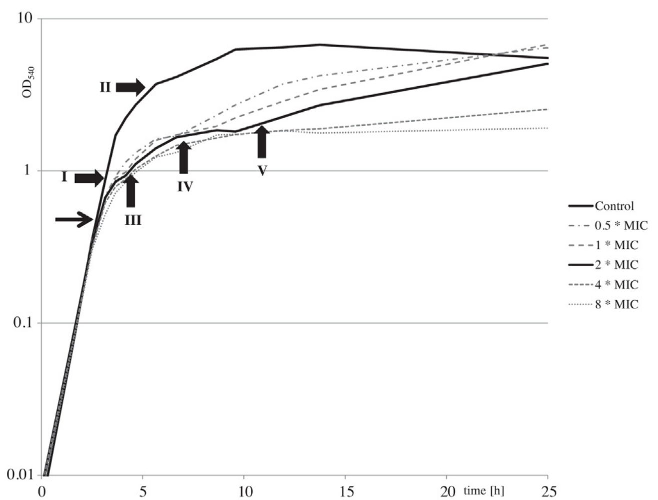

Fig. 1. Different doses of LZD (0.5–8× MIC) were added to exponentially growing cultures of *S. aureus* USA300. LZD addition is indicated by the thin arrow, sampling points by bold arrows.

2.2. DNA microarray analysis

Total RNA was prepared by acid-phenol extraction after mechanical cell disruption as described previously (Nicolas et al., 2012). Gene expression analysis was performed using an Agilent custom *S. aureus* microarray (Charbonnier et al., 2005). Fluorescently labeled cDNA was synthesized, purified and hybridized as described previously (Müller et al., 2014). In brief, 300 ng of Cy5-labeled sample cDNA and 300 ng Cy3-labeled reference pool cDNA were hybridized together to the microarray following Agilent's protocols for array processing and data extraction. The data-set has been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and is accessible through GEO Series accession number GSE65209 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65209).

2.3. Label-free quantification of protein levels

S. aureus cells were harvested by centrifugation (8000 × *g*, 8 min, 4 °C) and washed two times with 50 mM triethylammonium bicarbonate buffer (TEAB) (Fluka). Cell disruption was performed with a Precellys 24 homogenisator (PeqLab; 3 × 30 s at 6.5 m s^{-1}). Glass beads (0.1–0.11 mm diameter) and cell debris were removed by centrifugation (21,000 × *g*, 10 min, 4 °C). For analysis of extracellular proteins, 45 ml cell culture supernatant was sterile-filtered, 1 μg bovine serum albumin was spiked in for normalization, and extracellular proteins were enriched with StrataClean beads (Bonn et al., 2014).

The cell extract protein fraction was prepared and analyzed as described previously (Muntel et al., 2014) by ion mobility mass-spectrometry on a Synapt G2 mass spectrometer (Waters) with minor modifications as described in the Supplemental Text 1. The extracellular protein fraction was enriched via StrataClean

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