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Short Communication

Penicillin G increases the synthesis of a suicidal marker (CidC) and virulence (HlgBC) proteins in *Staphylococcus aureus* biofilm cells

Kirsi Savijoki[a,b,c,*], Malena Skogman[d], Adyary Fallarero[d], Tuula A. Nyman[b], Antti Sukura[c], Pia Vuorela[d], Pekka Varmanen[a]

[a] Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki, Finland
[b] Institute of Biotechnology, University of Helsinki, Finland
[c] Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland
[d] Pharmaceutical Biology, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland

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ABSTRACT

The present study reports the effect of Penicillin G (PenG) on the proteome dynamics of the *Staphylococcus aureus* strain Newman during biofilm mode of growth. The viability of the 18-h-old biofilm cells challenged with PenG at the concentration of 1 mg mL^{-1} was first assessed by plate counting, resazurin and LIVE/DEAD fluorescence staining, which indicated that the viability was reduced by ~35% and ~90% at 2 h and 24 h, respectively, after the addition of PenG. Subsequent two-dimensional difference gel electrophoresis (2D DIGE) assay of the treated and non-treated biofilm cells at the indicated time points revealed 45 proteins showing time- and treatment-specific change (1.5-fold, $p < 0.01$). The 2D DIGE results suggested that the PenG-induced decrease in viability was accompanied by an increased synthesis of pyruvate oxidase (CidC), a suicidal marker known to potentiate acetate-dependent cell death in *S. aureus*. Increased abundance was also found for the TCA cycle associated malate–quinone oxidoreductase (Mqo), the ClpC ATPase, the HlgBC toxin and phage-associated proteins, which suggests that surviving cells have induced these activities as a last effort to overcome lethal doses of PenG. Proteomic results also revealed that the surviving cells were likely to strengthen their peptidoglycan due to the increased abundance of cell-wall biogenesis associated proteins, FemA and Pbp2; a phenomenon associated with dormancy in *S. aureus*.

1. Introduction

Staphylococcus aureus (SA) causes a variety of acute and chronic infections (toxic shock syndrome, osteomyelitis, and infective endocarditis), and is recognized as one of the most frequent causes of biofilm-based medical, especially nosocomial and device-related, infections (Otto, 2013). It has been estimated that over 65% of all bacterial infections tolerant to chemotherapy are biofilm-related and this number will continue rising due to the increasing use of indwelling medical devices (Lebeaux et al., 2013). Biofilms are multicellular three-dimensional communities of bacteria that are enclosed by a self-produced extracellular polymeric substance, involving proteins, polysaccharides, and DNA (Flemming and Wingender, 2010). Once developed, their eradication is challenging, because unlike planktonic cells, the biofilm cells display enhanced resilience to antimicrobial treatments and host immune defenses (Hall-Stoodley et al., 2004). One of the treatment choices for SA infections is β-lactams whose main mode of action is by the inhibition of the peptidoglycan biosynthesis. These bactericidal drugs have also been shown to trigger the generation of radical oxygen species (ROS) resulting from transient depletion of NADH during the tricarboxylic acid (TCA) cycle, which eventually leads to cell death (Kohanski et al., 2007). There is also evidence that several bacteria aim to combat β-lactams by inducing cellular pathways, including the SOS response, that remediate hydroxyl radical damage (Kohanski et al., 2007). At sub-lethal concentrations, these drugs can even enhance the virulence, survival and biofilm formation as previously demonstrated for some other SA strains (Worlitzsch et al., 2001; Rosato et al., 2014; Kaplan et al., 2012). In the present study, pre-formed SA biofilm cells were challenged with lethal doses of Penicillin G (PenG), a β-lactam group antibiotic, for different time periods and cellular responses were examined

* Corresponding author at: Department of Food and Environmental Sciences P.O. Box 66, 00014 University of Helsinki, Finland. Tel.: +358 2 941 58309; fax: +358 2 941 58460.
E-mail address: kirsi.savijoki@helsinki.fi (K. Savijoki).

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by using three complementary viability tests and by quantitative proteomics to reveal the functionality of the cells under the given conditions. The Newman strain used in this study originates from a case of secondarily infected tubercular osteomyelitis; it is susceptible to methicillin and other β-lactams and it is a widely used model in infection assays (Baba et al., 2008; Johnson et al., 2008; Kaplan et al., 2012; Supersac et al., 1998). To the best of our knowledge, proteome level analyses of antibiotic-challenged biofilms have not previously been reported for *Staphylococcus* species.

2. Materials and methods

2.1. Biofilm formation assays

The SA Newman strain was cultured in tryptic soy (TS) broth (Fluka Biochemika) to the exponential phase and was diluted to obtain 10^6 colony forming units per millilitre (CFU mL^{-1}), from which 200 μL was added per well to flat-bottomed 96-well plates (Nunclon™ Δ surface, Nunc). The plates were incubated at 37 °C, 200 rpm for 18 h. The non-adherent cells were removed gently by washing once with sterile PBS, and fresh TS broth with or without 1 mg mL^{-1} PenG (potassium salt, Fluka Biochemika) was added to the formed biofilms. After antibiotic treatment for 2 or 24 h (at 37 °C, 200 rpm), the biofilms were subjected to resazurin staining, and the fluorescence emitted by the resorufin was measured using a Varioskan reader (Thermo Fisher Scientific) (Skogman et al., 2012). The density of the non-challenged (NC) and PenG-challenged (PC) biofilms left in the wells was measured using the standard plate count method (Pitts et al., 2003). At the end of the exposure period to PenG, planktonic cells were removed, while biofilms were gently washed once with PBS, before being scraped off the wells in 100 μL TSB using sterile plastic sticks and rinsed with an additional 100 μL of TSB. To disperse the bacterial aggregates, samples were immersed in a high power ultrasonic bath (Bandelin Sonorex Digitec, Zurich, Switzerland) using an in-house built-in device that allowed them to be in full contact with the water. Sonication was performed at RT (5 min, 35 kHz). The disaggregated biofilms were serially diluted, spread onto TS agar plates and incubated at 37 °C overnight. The log 10 density of viable cells (CFU/mL) in treated and control wells were determined, and log *R* was calculated from the difference of the log 10 density on the untreated biofilms (NC) and the log 10 density on the biofilms treated with PenG (PC). The LIVE/DEAD viability staining of the NC and PC biofilms was conducted with 5 μM of Syto9 and 30 μM of propidium iodide, according to the manufacturer's instructions (LIVE/DEAD® *Bac*Light™, Molecular Probes, Life Technologies). FM images were captured using an AxioVert 200 M fluorescence microscope (Carl Zeiss MicroImaging GmbH) and a FITC filter (Syto 9) or a TRITC filter (propidium iodide).

2.2. 2D DIGE and protein identification

The NC and PC biofilm cells in three biological replicates at the 2 h and 24 h time points, were scraped off of the wells in ice-cold 100 mM Tris-HCl (pH 8.0). Proteins were purified as previously described by Frees et al. (2012). Shortly, the cells harvested by centrifugation (5000×*g*, 5 min, 4 °C) were disrupted by bead-beating and proteins were purified using the 2D Clean Up according to the instructions provided by GE Healthcare. Proteins solubilised in 7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Trisma base were subjected to differential labelling with Cy2, Cy3, or Cy5 dyes (CyDye DIGE Fluor minimal dyes; GE Healthcare) (Table S1) and isoelectric focusing (IEF). The labelled proteins (150 μg in total) were separated using IEF with rehydrated IPG strips (24 cm, pH 3–10 nonlinear; Bio-Rad) in a Protean IEF Cell (Bio-Rad) using the linear ramping mode. Equilibrated strips after IEF were loaded onto 12% acrylamide gels, and the two-dimensional separation of proteins occurred using the Ettan DALTsix Electrophoresis Unit (GE Healthcare). The fluorescent proteomes were detected with FLA-5100 laser scanner (Fujifilm) at wavelengths of 473 nm (for Cy2), 532 nm (Cy3), and 635 nm (Cy5) using voltages of 420, 410, and 400 V, respectively (100-μm resolution). With the use of a batch processor function of the DeCyder software, the cropped gel images were first automatically analyzed in a differential in-gel analysis (DIA) module, which normalized the Cy2, Cy3, and Cy5 image from each gel. The spot boundaries were detected, and the spot volumes, analogous to the protein abundances, were calculated. The spot volumes of the Cy3 and Cy5 samples were compared with the spot volumes of the Cy2 sample, as an internal standard, to generate standard spot volumes for correcting inter-gel variations. In the biological variation analysis (BVA) module, the Cy2 images of three replicate gels were matched, and the standard spot volume ratios between all six of the gels were compared. ImageQuant TL 7.0 software (GE Healthcare and DeCyder 2D 7.0 software (GE Healthcare) were used to crop and quantitatively analyse the proteome changes. To test for significant differences in protein abundance between the different experimental groups, one-way analysis of variance (ANOVA) was performed at a significance level $p < 0.01$. Unsupervised principal component analysis (PCA) and *K*-means clustering using average linkage were performed using the DeCyder Extended Data Analysis (EDA) module on the group of spots identified as significantly changed to compare differential protein expression pattern between the NC and PC proteomes at indicated time points. Two-way ANOVA-treatment, 2-way ANOVA-time and 2-way-ANOVA-interaction were computed to assign time and treatment specific changes in individual protein abundances. The spots displaying ≥1.5-fold change ($p < 0.01$) in spot volume ratio in at least one of the conditions tested and appearing in at least five of the six analyzed gels (i.e., 15 of the 18 gel images) were picked for identification. Proteins were subjected to in-gel tryptic digestion and identification by peptide-mass fingerprinting (PMF) using an Ultraflex TOF/TOF instrument (Bruker Daltonik) or fragment ion analysis using an Ultimate 3000 nano-LC (Dionex, Sunnyvale, CA) and QSTAR Elite hybrid quadrupole TOF mass spectrometer (Applied Biosystems/MDS Sciex), as detailed by Frees et al. (2012).

2.3. 1DE immunoblotting

The proteins were precipitated from the biofilm culture supernatants (NC and PC biofilms at 2 h and 24 h) using 10% TCA/acetone. After centrifugation (16,000×*g*, 30 min, +4 °C) the pelleted proteins were solubilised in 100 mM Tris-HCl (pH 7.5) containing 1% SDS and the protein concentration was determined using a 2D Quant kit (GE Healthcare). After electrophoresis in a 12% TGX™ Gel (BioRad) using 1xTris-glycine-SDS as the running buffer, an equal amount of protein (10 μg from each sample) was transferred onto a PVDF membrane using the TransBlot Turbo™ Transfer System (BioRad), according to the manufacturer's instructions. After electroblotting, the membrane was probed using rabbit anti-Rabbit HlgC antibodies (1:5000)(IBT Bioservices) and then using IRDye® 800CW goat anti-Rabbit IgG (LI-Cor® Biosciences) (1:20,000). After probing, the membrane was blocked using Odyssey blocking buffer and was washed with PBS (phosphate buffered saline, pH 7.4), according to the instructions provided by LI-Cor® Biosciences. The cross-reacting antigens were detected using an Odyssey® infrared imaging system (LI-Cor® Biosciences) and quantitatively analyzed using AlphaView software version 3.4 (Thermo Scientific).