Biochimica et Biophysica Acta 1864 (2016) 835–839

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

The role of the globin-coupled sensor YddV in a mature *E. coli* biofilm population

Joke Donné [a], Marian Van kerckhoven [b], Louis Maes [b], Paul Cos [b], Sylvia Dewilde [a,*]

[a] Protein Chemistry, Proteomics and Epigenetic Signalling (PPES), Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium
[b] Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Antwerp, Belgium

ARTICLE INFO

Article history:
Received 13 January 2016
Received in revised form 30 March 2016
Accepted 7 April 2016
Available online 12 April 2016

Keywords:
Globin-coupled sensor
Biofilm
E. coli
Persister cells
Diguanylate cyclase

ABSTRACT

Biofilm-associated infections are hard to treat because of their high antibiotic resistance and the presence of a very persistent subpopulation of bacteria. The second messenger molecule cyclic di-guanosine monophosphate (c-di-GMP) plays a very important role in this biofilm physiology. Here, we evaluated the role of YddV, an enzyme with a c-di-GMP synthesis function, in the formation and maturation of *Escherichia coli* biofilms. Our results suggest that YddV stimulates biofilm growth via its role in the production of c-di-GMP and this likely by influencing the production of matrix (e.g. poly-N-acetylglucosamine (PGA)). However, lowering the YddV expression did not alter the biofilm formation since there was no significant difference between the biofilm phenotypes of WT *E. coli* and YddV-knockout bacteria. Additionally, YddV expression had no significant influence on the amount of persister cells within the biofilm population, questioning the use of YddV as therapeutic target.

1. Introduction

Most of the bacterial infections are caused by their biofilm growth mode. A biofilm is a population of microorganisms that adhere to a surface and are surrounded by a self-produced polymeric matrix. Bacteria in a biofilm are physiologically different from the planktonic cells of the same species. They show an increased resistance to antibiotics and the host immune system. Consequently, biofilm-associated infections are difficult to treat and often result in persistent and chronic diseases [1].

Because of the close proximity of the bacteria in a biofilm population, the responsiveness on secreted signalling molecules happens fast and results in a very quick adaptation of the biofilm bacteria to environmental changes. An important intracellular regulator molecule in this signalling network is c-di-GMP, a second messenger molecule widely used among bacteria. C-di-GMP regulates several pathways within the process of biofilm formation, such as (i) the production of extracellular polymeric matrix, (ii) adhesion, (iii) stress resistance, (iv) motility, (v) virulence, etc. [2,3] The levels of c-di-GMP are mainly regulated by diguanylate cyclases (DGC) and phosphodiesterases (PDE). The first group of enzymes is characterized by a GGDEF (Gly-Gly-Asp-Glu-Phe) or a GGEEF (Gly-Gly-Glu-Glu-Phe) motif and is responsible for the production of c-di-GMP from two molecules of GTP. The phosphodiesterases are degrading c-di-GMP and possess an EAL (Glu-Ala-Leu) or a HD-GYP domain. Bacteria mostly contain several c-di-GMP metabolizing proteins [4,5]. The genome of *Escherichia coli*, for example, encodes for 29 c-di-GMP metabolizing enzymes: 12 DGCs, 10 PDEs and 7 hybrid proteins (both EAL and GGDEF motifs). Additionally to this repertory of 29 enzymes, fusions of the enzymatic domain with different N-terminal sensory input domains extend the c-di-GMP regulatory network. Two *E. coli* c-di-GMP metabolizing enzymes, namely YddV (also referred to as EcDosC) and EcDosP (also referred to as YddU or Dos), possess a heme-based sensor domain and are subsequently characterized as heme-based oxygen sensors. The heme-based domain in YddV has a globin fold and belongs as such to the family of globin-coupled sensors (GCS). However, the EcDosP protein detects its signal by a PAS-domain which is also associated with a heme cofactor [6]. Structural information of both full-length YddV and EcDosP are characterized [7–9]. Both proteins are expressed together, translated from the polycistronic *dosCP* operon, with YddV having a DGC function and EcDosP operating as a PDE. The YddV protein seems to be one of the most strongly expressed DGCs in *E. coli* bacteria [10]. Sommerfeldt et al. evaluated the expression level of all DGCs as a function of growth phase and concluded that YddV is predominantly expressed during the entry into the stationary phase [10]. Moreover, overexpression of YddV results in an enhanced intracellular c-di-GMP concentration [11,12]. Gene-expression studies of bacteria overexpressing *YddV* displayed an altered transcriptional profile of for instance genes encoding cell wall-modifying proteins and genes involved in motility, cell division, adhesion and matrix production [11,12].

Abbreviations: AB, antibiotics; c-di-GMP, cyclic di-guanosine monophosphate; CV, crystal violet; DGC, diguanylate cyclase; GCS, globin-coupled sensor; IPTG, isopropyl β-D-1-thiogalactopyranoside; PDE, phosphodiesterase; PGA, poly-N-acetylglucosamine.

* Corresponding author at: Protein Chemistry, Proteomics and Epigenetic Signalling (PPES), Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Antwerp, Belgium.
E-mail address: sylvia.dewilde@uantwerp.be (S. Dewilde).

http://dx.doi.org/10.1016/j.bbapap.2016.04.005
1570-9639/

This all suggests an important role of YddV in the formation of *E. coli* biofilms. However, little is known about the effect on the maturation stage of the biofilm and the highly resistant property of the biofilm population. Here, we investigated the role of this GCS YddV in the process of biofilm formation and maturation by comparing the biofilm phenotype of overexpression strains and knockout strains, focusing on the biofilm mass, the cell viability and the persistent property of the biofilm population.

2. Materials and methods

2.1. Cloning, overexpression and knockout of YddV

Genomic DNA was isolated from *E. coli* ATCC8739 using a QIAEX II Gel Extraction Kit (Qiagen, Venlo, Netherlands). The *YddV* gene (GenBank ID: NC_010468.1) was amplified by PCR using the forward primer (sequence 5′-GGAATTCCATATGGAGATGTATTTTAAAAG-3′) and reverse primer (sequence 5′-CCGCTCGAGAAGACTGGCTTTCCAGAGT TC-3′) on the isolated genomic DNA. After the initial incubation at 95 °C for 4 min, the *YddV* cDNA was first amplified using only the reversed primer during 10 cycles of 30 s at 95 °C (melting phase), 1 min at 55 °C (annealing phase) and 1 min at 72 °C (amplification phase). The addition of the forward primers during the melting phase of cycle 11 provided further amplification of the *YddV* gene for 25 similar cycles with 48 °C set as annealing temperature. Next, the PCR-fragment was cloned into the pET23-a vector, based on NdeI and XhoI restriction sites. Finally, the plasmid was transformed in *E. coli* BL21(DE3)pLysS cells, creating an YddV-overexpression strain (BL21-YddV(+)). Transformants were screened on LB agar plates with 50 μg/ml ampicillin and verified with both PCR using the grown colonies as templates and Sanger sequencing of the isolated plasmids (data not shown). To create an YddV knockout BL21(DE3)pLysS strain (BL21-YddV(−)), the *YddV* chromosomal deletion was performed using the phage λ Red system, as described by Datsenko and Wanner [13]. In short, a linear kanamycin resistance cassette flanked with *YddV* homology sequences is transformed in a BL21(DE3)pLysS strain, which expresses λ Red recombinase via a pKD46 plasmid system. Positive chromosomal knockouts were selected as kanamycin-resistant transformants. Knockouts were verified by PCR, using previous mentioned forward and reverse primers, followed by Sanger sequencing of the amplified gene fragment (data not shown). The *E. coli* BW25113 strain was a kind gift from Jakob Møller-Jensen (University of Southern Denmark). The *E. coli* JW5241 strain, an *YddV* knockout strain constructed from the BW25113 strain, was purchased from the Coli Genetic Stock Center (CGSC, Yale University, VS). All *E. coli* strains were subcultured in RPMI 1640 (Life technologies Europe, Gent, Belgium) at 37 °C and stored at −80 °C in aliquots containing 10^9 CFU/ml.

2.2. Growth of E. coli biofilms

Biofilms were grown in RPMI 1640 medium in 24-well plates (Greiner Bio-one, Frickenhausen, Germany), starting from an inoculum of 10^5 CFU/ml. The well plates were incubated on a horizontal shaking plate at 25 rpm at 37 °C for 72 h to reach mature biofilms. Every 24 h, the medium was refreshed. Non-inoculated RPMI 1640 was included as a negative control sample. YddV overexpression in BL21-YddV(+) bacteria was induced with 1 mM IPTG after 24 h of growth, keeping this inducer present in every following medium-refreshing step. After a total growth period of 72 h, the biofilm mass (matrix and cells) was quantified using the crystal violet (CV) staining assay [14] and the viable cell density was determined by the viable plate count method.

2.3. Persister growth

Biofilms of 72 h old were washed two times with PBS and then exposed for 24 h to different norfloxacine concentrations (0–1–2-4-8-16-32 μM), dissolved immediately in RPMI 1640 medium. Next, the 24-well plates were washed again two times with PBS and the surviving attached biofilm cells were analysed by viable plate counting. The surviving cells were demonstrated to be persister cells by re-culturing them, growing biofilms and re-exposing them again to the same AB concentrations.

2.4. Biofilm quantification by crystal violet assay

To confirm and quantify biofilm formation in 24-well plates, the growth medium was discarded and planktonic cells were washed away. First, the attached cells were fixed with 100% methanol for 15 min. After discarding the methanol, the plates were air-dried and stained for 5 min with 0.1% (w/v) crystal violet (CV) (Sigma-Aldrich, St. Louis, MO). Next, the plates were washed with tap water to remove unabsorbed staining colour and air-dried overnight. Finally, the absorbed stain into the biofilm cells was dissolved by adding 33% glacial acetic acid to each well for 15 min and subsequently the OD at 570 nm of solubilized CV was measured with a plate reader (Titertek Multiskan MCC/340, Pforzheim, Germany).

2.5. Biofilm viability assay

After 72 h or 96 h incubation, the biofilms were washed twice with PBS to discard the free-floating planktonic cells. Next, the biofilm cells, which were attached to the bottom and the walls of the wells, were collected by scraping off de bacterial cells with mini cell-scrapers (Leap Bioscience Corp, Palo Alto, CA) in 200 μl PBS. Decimal series of dilutions were spread on LB agar plates with selective antibiotics (ampicillin and chloramphenicol for BL21-YddV(+);kanamycin and chloramphenicol for BL21-YddV(−)) to count the colony forming units after overnight growth at 37 °C.

2.6. Statistical analysis

The number of experimental replicates was indicated in the figure legends. The statistical significance was assessed by 2way-ANOVA, followed by a Bonferroni test in the GraphPad Prism software package (version 6). A p-value of 0.05 was considered significant.

3. Results

In order to study the specific effects of YddV on the process of biofilm formation, we compared the biofilm phenotypes of wildtype (WT) *E. coli* (BL21(DE3)pLysS) with its in-house created YddV knockout strain (KO) (BL21-YddV(−)) and YddV overexpression strain (BL21-YddV(+)). By creating our own genetic manipulated strains, we were able to compare the gene expression modulation in the same *E. coli* strain. The *E. coli* BW25113 and its purchased YddV knockout strain JW5241 were compared as control strains.

3.1. Comparison based on mature biofilm mass

Based on the CV staining, the plasmid-driven overexpression of *YddV* resulted in a significant increase in biofilm mass when *E. coli* BL21-YddV(+) biofilms were grown for 72 h in the presence of the IPTG inducer (Fig. 1). To rule out the effect of IPTG on the biofilm growth itself, IPTG-supplemented medium was used to grow WT BL21(DE3)pLysS biofilms. As shown in Fig. 1, this had no effect on the WT biofilm phenotype. We can conclude that the increase in BL21-YddV(+) biofilm mass is due to the overexpression of *YddV* caused by IPTG, and not due to the IPTG compound itself. In addition, WT and BL21-YddV(+) biofilms were grown without inducer, showing comparable amounts of biofilm mass. This expected result can be explained by the similar endogenous YddV gene expression levels in both strains.