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# Engineering *Escherichia coli* to Disrupt Poly-N-Acetylglucosamine Containing Bacterial Biofilms

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## Abstract

Biofilms are surface-associated structures formed by bacteria embedded in a self-produced matrix. Biofilms are exceptionally resistant to environmental stress, antimicrobial agents and host immune defense, and combating biofilms has recently become an important research topic. In this work, we present a biologically engineered system that can be applied against a wide range of biofilms formed by pathogenic and non-pathogenic bacteria. This system relies on *Escherichia coli* (disrupter) strain that was engineered to synthesize and secrete Dispersin B, an enzyme that can hydrolyze poly-N-acetyl glucosamine (PGA), a polymer found in the matrix of various bacterial biofilms. We show that the degradation of PGA by the disruptor strain results in the dispersion of the target biofilm. We propose that in the future this simple disrupter module can be combined with other biofilm detection and targeting systems aimed towards the destruction of an existing biofilm.

**Keywords**: Bacterial biofilms; Dispersin B; poly-N-acetyl glucosamine, Biofilm matrix; Confocal microscopy; Engineering bacteria; OmpA signal peptide; Heterologous protein secretion in bacteria; *E. coli csrA* mutant

## Introduction

Many bacteria in nature exist in the form of biofilms, where surface-attached bacteria are embedded in a self-produced matrix and form complex three-dimensional structures. Such sessile lifestyle in structured communities potentially offers several advantages, such as protecting bacteria from starvation, desiccation, antimicrobial compounds or host immune system. With the help of their extracellular polymeric substances, bacteria build complex three-dimensional structures within biofilms. Unsurprisingly, biofilms are associated with infections by many well-known bacterial pathogens, such as *Pseudomonas aeruginosa or Staphylococcus epidermidis.*

Various strategies have been explored to combat bacterial biofilms [1]. Generally, these strategies can be broadly divided into physicochemical, biochemical and biological methods. Physicochemical methods involve eradicating biofilms with detergents or disinfectants. Detergent treatment has been tested for removing biofilms from endoscopes [2]. Biochemical methods are exemplified by interfering with bacterial signaling networks [3] and using surfactants [4] or degrading extracellular matrix [5] to induce dispersion. Finally, biological methods that involve engineering phages [6] or bacteria [7] for biofilm disruption have recently gained a lot of attention.

The aim of this work was to expand the repertoire of biological methods of biofilm dispersion to a diverse range of bacterial biofilms, which contain poly-N-acetyl glucosamine (PGA) in their matrix. For that, we have engineered *E. coli* strain (termed as disrupter) that synthesizes and secretes the enzyme Dispersin B, which degrades PGA and disrupts the target biofilm (Figure 1). We propose that this module of secretion and disruption can in future be easily combined with other modules for biofilm sensing and/or dispersal.

## Methods

### Bacterial strains and growth conditions

*E. coli* K12 W3110 (F-λ-ilvG rib-50 rph-1) [8] was used to engineer the disrupter strain. *E. coli* TRMG1655 (*E. coli* K12 MG1655 *csrA::kan*) [9] was used as a target biofilm-forming strain. *E. coli* DH5α (Invitrogen)

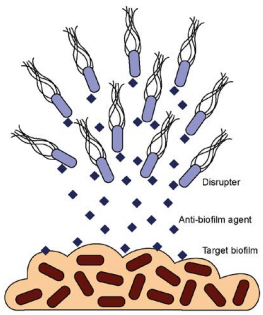


**Figure 1:** The concept of biofilm disruption, using a strain that synthesizes and secretes an anti-biofilm agent.

was used for plasmid construction, while *E. coli* M15 (Qiagen) was used for protein expression and purification. All bacterial cells were routinely grown in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Ampicillin (100 μg/ml) and/or Kanamycin (50 μg/ml) were added to the culture media whenever required. Overnight cultures were grown at 37°C for 12 to 16 h with rotary shaking. For enzyme-secretion assay, M9 minimal medium supplemented with 0.2% glycerol and 0.01% casein hydrolysate was used.

### Plasmid construction

Plasmids constructed in this study are listed in (Table 1). The CDS of *dspB* was amplified by PCR from the plasmid pJK618 [10]. The secretion signal sequence of *ompA* was amplified from *E. coli* K12 W3110 genome. The amplified fragments were digested and ligated into

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| Construct name | Fragment cloned | Cloning vector | Antibiotic resistance |
|---|---|---|---|
| pVG22 | dspB::10X Gly::6X His | pQE60 (Qiagen) | Ampicillin |
| pVG29 | ompA::dspB | ptrc99a [11] | Ampicillin |
| pVG30 | dspB | ptrc99a [11] | Ampicillin |
| pOB2 | mCherry | ptrc99a [11] | Ampicillin |
| pVG18 | gfp | pUA66 [12] | Kanamycin |

**Table 1:** List of plasmids constructed in this study.

suitable vectors, and constructed plasmids were propagated in *E. coli* DH5α cells. The sequences of constructed plasmids were verified by DNA sequencing.

## Protein purification

An overnight culture of *E. coli* M15 harboring the construct pVG22 was diluted 1:100 in one liter of LB medium and grown in a rotary shaker at 37°C, 110 rpm until $OD_{600}$ ~0.6. The expression of Dispersin B was then induced by adding 100 µM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h [11]. The cells were harvested by centrifugation at 6000 rpm for 20 minutes at 4°C and homogenized by sonication. Lysate was cleared by centrifugation at 10000 rpm for 25 min at 4°C. The purification of His-tagged Dispersin B was done using Ni-IDA 2000 columns (Machery Nagel) according to the manufacturer's instructions. The eluted fractions were pooled together and concentrated using Amicon ultra centrifugal filter units (Ultra 15, MWCO 10 kDa, Sigma-Aldrich) at 4000 rpm for 15 min at 4°C [12].

## Biofilm formation assay

The biofilm assay was adapted from the protocol described previously [13]. An overnight culture of *E. coli* TRMG1655 was diluted 1:1000 in LB medium, dispensed as 200 µl per well in Costar 96-well microplate (Sigma-Aldrich) and incubated at 26°C for indicated time. The planktonic cells were removed from the wells by gentle pipetting, and the wells were washed thrice with phosphate buffer. Attached cells were then stained with 1% Crystal Violet (CV) for 30 min, washed with distilled water, allowed to dry and then de-stained using 70% ethanol for 30 min at room temperature. The intensity of the dissolved stain was measured by determining the $OD_{590}$ using Tecan Infinite M1000 Pro microplate reader.

## Enzyme secretion assay

Overnight cultures of *E. coli* W3110 harboring constructs pVG29 and pVG30 were diluted 1:100 in M9 minimal medium containing 1 mM of 4-nitrophenyl N-acetyl-ß-D-glucosaminide (NP-GlcNAc) (Sigma-Aldrich). The cells were grown in a 96-well microplate at 37°C for 24 h without shaking. Protein expression was induced by adding IPTG at the indicated concentration. After incubation the reaction was stopped by adding 2 µl of 40% NaOH, and the absorbance of the reaction mixture was measured at 400 nm.

## Disruption of the target biofilm by purified Dispersin B or by the disrupter strain

The target biofilm was grown in Costar 96-well microplate for 24 hours at 26°C. The planktonic cells were gently removed and purified Dispersin B diluted in phosphate buffer (20 µg/ml) was added on the attached cells. After incubation for 1 h at room temperature, the wells were washed thoroughly with phosphate buffer, stained with 1% CV and de-stained to quantify the biofilm formation as described above. To test the activity of the disrupter strain, the target biofilm was grown as above and subsequently treated with the diluted (1:100) overnight culture of the disrupter strain induced by indicated levels of IPTG. The plate was further incubated at 26°C for indicated time, washed, stained and de-stained to quantify biofilm formation.

## Microscopy of biofilms

For microscopy, the target strain *E. coli* TRMG1655 was transformed with the plasmid pOB2 encoding mCherry. Biofilms were grown in LB medium in Ibidi 96-well uncoated microplate (Ibidi) for 24 h at 26°C. The expression of mCherry was induced by adding 10 µM IPTG. To assay the activity of Dispersin B, the planktonic cells were gently removed, around 20 µg/ml of the enzyme was added on the attached cells and the plate was incubated for 1 h at room temperature. After incubation the wells were gently washed with phosphate buffer and 100 µl of phosphate buffer was added to the wells to keep the attached cells hydrated. The target biofilm was then observed using 40 X water immersion objectives (1.2 NA) on Zeiss LSM 880 confocal microscope. To analyze the activity of the disrupter strain on the target biofilm, the disrupter strain was transformed with pVG18, which expresses GFP constitutively. The 24 h old pre-formed mCherry-labeled target biofilm was treated with the disrupter strain induced with 100 µM IPTG and then imaged as above.

# Results

## Purified Dispersin B disrupts pre-formed target biofilms

Dispersin B is synthesized by a periodontal pathogen *Actinobacillus actinomycetecomitans* [10], and it degrades PGA found in biofilms of many Gram-positive and Gram-negative bacteria, such as *Staphylococcus* spp, *Yersinia* spp, *Actinobacillus* spp, etc [14]. *E. coli* biofilms also contain PGA, which is involved in the transition from temporary to permanent attachment [15]. By degrading PGA, Dispersin B efficiently disrupts *E. coli* MG1655 WT biofilms [14]. Here we used *E. coli* TRMG1655, which possesses a transposon insertion mutation in the gene csrA [9] that results in depressed PGA production and allows increased biofilm formation under standard laboratory conditions [16]. We first tested the effect of Dispersin B on *E. coli* TRMG1655 biofilms using the purified His-tagged enzyme. The addition of purified enzyme completely disrupted the pre-formed 24 h old target biofilm within one hour, whereas 48 h old biofilm was disrupted by ~40% (Figure 2), as estimated using the standard assay based on CV staining (see Methods).

Since CV staining mostly follows the biomass of attached cells and

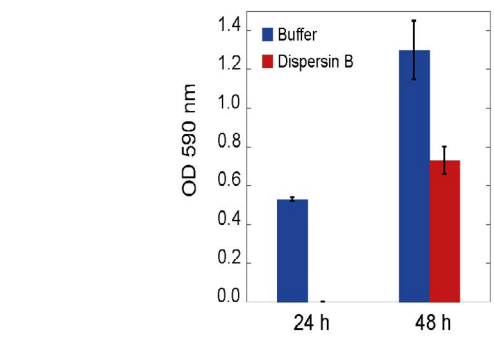



**Figure 2:** Purified Dispersin B disrupts pre-formed target biofilm. A 24 h old pre-formed *E. coli* TRMG1655 biofilm was treated with 60 µg/ml of purified Dispersin B for 1 h at room temperature and biofilm formation was quantified by CV staining. This experiment was performed 3 times with 4 technical replicates in each experiment and similar results were obtained. The error bars represent standard deviation from the mean calculated from a single experiment.

does not provide any information about the structure of the biofilm, we further investigated how the target biofilm was affected by the treatment with Dispersin B using confocal microscopy. We observed that the mCherry-labeled pre-formed target biofilm possessed a thick three-dimensional structure (Figure 3a). This structure was apparently destroyed by Dispersin B reducing attached cells to a sparse thin layer (Figure 3b). Moreover, while cells within the target biofilm did not show any significant movement (S1 Movie), upon Dispersin B treatment

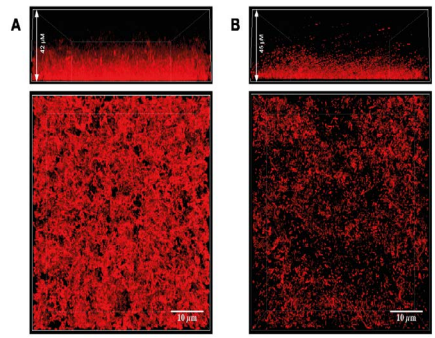
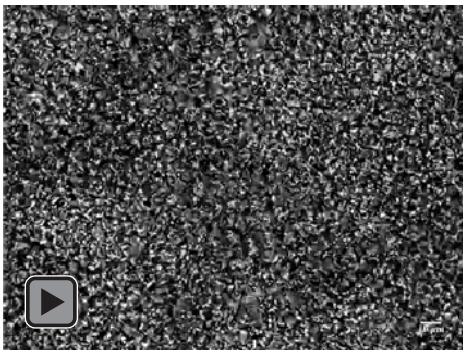
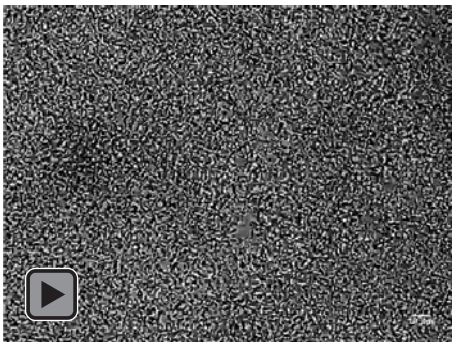



**Figure 3:** The effect of Dispersin B on the biofilm structure observed by confocal microscopy. A 24 h old pre-formed mCherry labeled *E. coli* TRMG1655 biofilm was treated with (a) phosphate buffer or (b) 20 µg/ml Dispersin B for 1 h at room temperature. This experiment was performed 2 times with 2 technical replicates in each experiment and similar results were obtained. The images in the figure are representative of all replicates.



**S1 Movie:** Bright field movie of untreated *E. coli* TRMG1655 biofilm (10 frames/s). A 24 h old *E. coli* TRMG1655 biofilm was treated with phosphate buffer for 1 h at room temperature, gently washed, and observed with 40X dry objective on Zeiss Axio Observer.Z1 microscope.



**S2 Movie:** Bright field movie of Dispersin B treated *E. coli* TRMG1655 biofilm (10 frames/s). A 24 h old *E. coli* TRMG1655 biofilm was treated with ~ 20 µg/ml Dispersin B for 1 h at room temperature, gently washed, and observed with 40X dry objective on Zeiss Axio Observer.Z1 microscope.

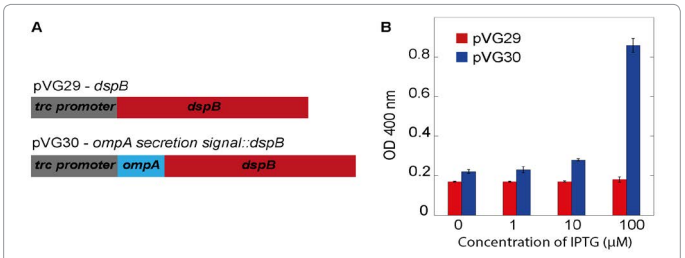



**Figure 4:** Disrupter cells secrete Dispersin B fused to OmpA signal peptide. (a) Design of the constructs pVG29 and pVG30, encoding Dispersin B without and with the OmpA secretion tag. (b) *E. coli* W3110 cells carrying the constructs pVG29 and pVG30 were grown in M9 minimal medium containing 1 mM NP-GlcNAC and indicated levels of IPTG for 24 h. This experiment was performed 3 times with 2 technical replicates in each experiment and similar results were obtained. The error bars in the figure represent standard deviation from the mean calculated from a single experiment.

many cells were found to be either swimming in the overlaying buffer or moving locally within the remaining biofilm (S2 Movie).

## Addition of the OmpA signal peptide can mediate the secretion of Dispersin B

In the proposed system, disrupter strain should both synthesize and secrete Dispersin B. To achieve the latter, we made use of OmpA (Outer Membrane Porin A) a non-specific diffusion channel located in the outer membrane of *E. coli* that allows various solutes to pass through [17]. The first 21 amino acids of OmpA are known to be sufficient to pass the protein through the inner membrane in a Sec-dependent manner and to subsequently translocate it through the outer membrane by autotransport [18, 19]. This sequence has been previously used to drive the secretion of heterologous proteins [20, 21]. In order to make the disrupter strain secrete Dispersin B, the OmpA secretion tag was fused to the N-terminus of Dispersin B (Figure 4a).

The secretion potential of the ompA::dspB construct was analyzed using the artificial substrate called 4-nitrophenyl N-acetyl-beta-D-glucosaminide (NP-GlcNAc). This substrate mimics the β 1-4 linkage found in PGA. The hydrolysis of NP-GlcNAc leads to the release of 4-nitrophenoxide, which can be quantified by measuring the absorbance at 400 nm [22]. Cells expressing the *ompA::dspB* construct induced with 100 µM IPTG showed 4-fold increase in $OD_{400}$ of the reaction mixture (Figure 4b) indicating that Dispersin B was successfully secreted into the medium in its active form. In contrast, no activity was observed for cells carrying the cytoplasmic dspB.

## *E. coli* cells secreting Dispersin B efficiently disrupt the target biofilm

To demonstrate the ability of Dispersin B secreting cells to disrupt the PGA-containing biofilms, these cells were first added to the target biofilm grown for 24 h in a 96-well microplate. Indeed, we observed ~90% reduction of the biomass of the target biofilm by the disrupter strain, as estimated by CV staining (Figure 5a). Interestingly, a modest disruption of the target biofilm was observed even in the absence of the secretion tag, but not with the control cells carrying the empty vector. This indicates a residual non-specific release of Dispersin B, probably due to lysis of expressing cells. This non-specific release of the enzyme was however not detectable in the enzyme secretion assay (Figure 4b). The observed biofilm disruption was time-and induction dependent, with 12 h being required for the 50% disruption and 24 h for the 90% disruption with induction by at least 100 µM IPTG (Figure 5b). Although the time required by the disrupter cells to disrupt the target

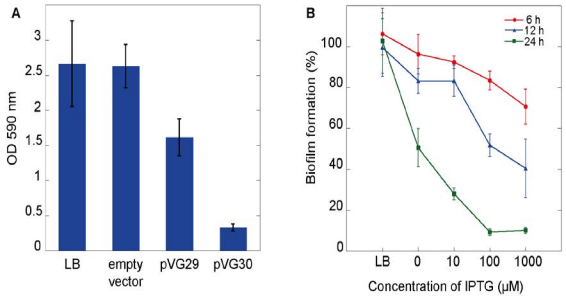



**Figure 5:** Cells that synthesize and secrete Dispersin B disrupt the target biofilm. (a) A 24 h old pre-formed *E. coli* TRMG1655 biofilm was incubated with disrupter cells induced with 100 μM IPTG for 24 h. (b) The target biofilm was formed as in (a) and incubated with disrupter cells induced with indicated concentrations of IPTG and incubated for indicated time intervals. Biofilm formation was quantified by CV staining as in Figure 2. This experiment was performed 3 times with 2 technical replicates in each experiment and similar results were obtained. The error bars in the figure represent standard deviation from the mean calculated from a single experiment.

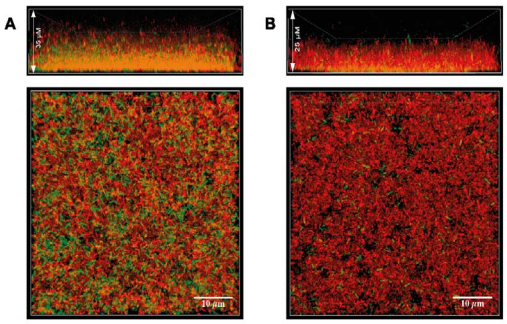



**Figure 6:** Disruption of the target biofilm by the disrupter cells- observed by confocal microscopy. A 24 h old pre-formed mCherry-labeled *E. coli* TRMG1655 biofilm was treated with GFP-labeled *E. coli* W3110 cells carrying (a) the empty vector ptrc99a (b) the construct pVG30 (ompA::dspB) induced with 100 μM IPTG for 24 h. This experiment was performed 2 times with 2 technical replicates in each experiment and similar results were obtained. The images in the figure are representative of all replicates.

biofilm was much longer than the treatment by the purified enzyme, Dispersin B secreted by the disrupter cells was able to successfully disrupt the target biofilm.

We also investigated structural changes in the target biofilm due to the action of the disrupter cells by imaging the mCherry-labeled pre-formed target biofilm treated with the GFP-labeled disrupter cells (Figure 6). The biofilm structure was indeed affected by the disrupter cells in a similar manner as observed for purified Dispersin B treatment. The cells carrying the empty vector became associated with the target biofilm settling primarily in the void spaces (Figure 6a) but did not apparently affect its structure (compare Figure 6A with Figure 3A). In contrast, the disrupter cells reduced the thickness of the target biofilm. Interestingly, the disrupter cells were not incorporated into the target biofilm and were removed during the wash step (Figure 6b). This observation indicates that effects of the secreted Dispersin B might be local, but also implies that even modest reduction of the structure of the target biofilm makes it vulnerable to complete detachment upon rigorous washing.

## Discussion

Many bacterial infections in humans, animals, and plants are associated with biofilm formation. Biofilms are exceptionally resistant to environmental stress and antimicrobial activity, and are able to evade the host immune system. Combating biofilms is thus an important part of eradication of bacterial infections, and engineering bacteria for this purpose has certain advantages over physical or chemical methods. An attractive target for biofilm disruption is the extracellular matrix. The biofilm matrix is a dominant component of biofilms, which provides structural scaffold, retains water and nutrients, and protects the cells from antimicrobials and host immune cells. Indeed, several matrix-degrading enzymes that can be exploited for therapeutic applications have been identified [23].

In this work, we have developed a biofilm disrupter system using *E. coli*, the most commonly used chassis organism for the design of novel biological functions. This disrupter strain was designed to target PGA, a major component of the biofilm matrix of *E. coli* and other bacteria such as *Staphylococcus epidermidis*, *Bordetella* spp, and *Yersinia* spp [14]. The anti-biofilm agent used in this study is Dispersin B, an enzyme with β-hexosaminidase activity found in *Actinobacillus actinomycetecomitans* biofilms. Purified Dispersin B has been previously shown to degrade PGA and disrupt bacterial biofilms [14]. To enable its secretion by the disrupter strain Dispersin B was fused N- terminally to the signal peptide of OmpA. We could show that the disrupter strain synthesized and secreted Dispersin B in its active form, and it efficiently disrupted the target PGA-containing biofilm formed by *E. coli* TRMG1655. When using the disrupter strain, biofilm disruption was slower in comparison to the treatment with the purified enzyme. The disruption kinetics might thus be limited by secretion of the enzyme, which relies on the Sec machinery [19]. Despite slow secretion, the disrupter cells could efficiently reduce the biomass of the target biofilm by 50% in 12 h of incubation. Confocal microscopy unraveled structural changes in the target biofilm due to Dispersin B action. The purified enzyme apparently destroyed the three-dimensional structure of the target biofilm reducing it to a thin layer of locally moving cells that could be easily detached from the surface upon rigorous washing. The disrupter strain expressing OmpA-DspB could also bring about a moderate change in the structure of the biofilm and rendered the cells vulnerable to complete detachment with higher shear force. Here the action of Dispersin B secreted by the disrupter strain was apparently local. While control *E. coli* cells carrying the empty vector were incorporated in the target biofilm, the disrupter strain cells were efficiently washed out suggesting that the matrix around these cells was degraded.

Previous attempts to design an engineered biological system for targeted biofilm disruption have been successful against only a few specific biofilms. For example, bacteriophage T7 was engineered to express a biofilm matrix-degrading enzyme that was released upon infection and cell lysis [6]. However, since bacteriophages are specific to their bacterial hosts, this approach cannot easily be expanded to other target biofilms. In another similar approach, *E. coli* was engineered to specifically target *P. aeruginosa* biofilm by secreting a nuclease and a bacteriocin [7]. Here we present a system that is applicable against a diverse range of PGA-containing biofilms and it can potentially be combined with other sensory or signaling networks.

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