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Reprint of "Inhibition of biofilm formation by Camelid single-domain antibodies against the flagellum of *Pseudomonas aeruginosa*"☆

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ABSTRACT

Pseudomonas aeruginosa is a leading cause of hospital-acquired infections in patients with compromised host defense mechanisms, including burn wound victims. In addition to its intrinsic resistance against most antibiotics, *P. aeruginosa* has the ability to form biofilms adhering to biotic or abiotic surfaces. These factors make treatment of *P. aeruginosa* infections complicated and demand new therapies and drugs. The flagellum of *P. aeruginosa* plays an important role in cell–cell and cell–surface interactions during the first stage of biofilm formation. In this study, we describe the selection of monoclonal anti-flagellin single-domain antibodies (VHHs) derived from the Camelid heavy-chain antibody repertoire of a llama immunized with *P. aeruginosa* antigens. The anti-flagellin VHHs could be produced efficiently in *Saccharomyces cerevisiae*, and surface plasmon resonance experiments demonstrated that they have apparent affinities in the nanomolar range. Functional screens showed that the anti-flagellin VHHs are capable of inhibiting *P. aeruginosa* from swimming and that they prevent biofilm formation in an in vitro assay. These data open doors for the development of novel methods for the prevention of *P. aeruginosa*-related infections.

1. Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen able to infect plants, nematodes, insects and mammals. In humans, it can cause persistent infections in cystic fibrosis patients and in burn wound victims, as well as in other immuno-compromised individuals. It is regarded as one of the most important causes of hospital-acquired infections. In general, treatment of the infections is difficult due to the high intrinsic antibiotic resistance of the bacterium and the organism's capability to exist in the host in biofilms. These biofilms are composed of cells, embedded in an extracellular matrix consisting of proteins, DNA and polysaccharides, and can be formed on biotic and abiotic surfaces (Joo and Otto, 2012). *P. aeruginosa* cells existing in such biofilms have a higher resistance against antibiotics and are less conspicuous to the immune system than free-living cells.

P. aeruginosa is motile via its single polar flagellum that is anchored to the cell envelope through the basal body and extends to the flagellar filament via the hook (Guttenplan and Kearns, 2013). The filament itself is formed by the polymerization of a single protein called flagellin. In addition to its contribution to motility, flagellin can also elicit the activation of the host inflammatory response via Toll-like receptor 5 (Feuillet et al., 2006). In view of these properties, flagella or the flagellin monomers are regarded as useful components in vaccines for active immunizations. Previous experiments with *P. aeruginosa* mutants defective in different stages of biofilm formation led to the characterization of various determinants responsible for this process. Type IV pili and the flagellum, which are both involved in motility, have been demonstrated to be important for biofilm development (Klausen et al., 2003). It appeared that the flagellum particularly acts during the attachment phase, whereas Type IV pili are needed for the formation of the typical mushroom-like structures in the mature biofilm. In addition, it has been found that the flagellum can bind to asialo GM1 and mucin (Feldman et al., 1998; Ramphal et al., 1996).

Prevention of early biofilm formation might be possible by inhibiting the function of the *P. aeruginosa* flagellum using targeted

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agents, such as antibodies. The use of these molecules might be very effective, as antibodies are highly specific and can have high affinity for their target. This is exemplified by the generation of conventional antibodies against flagella, which were proven to be effective in passive immunization experiments (Landsperger et al., 1994). However, the production of conventional antibodies in mammalian cell expression systems is time-consuming and expensive. A solution to this problem might be the use of antibody fragments derived from the llama immune repertoire of fully functional antibodies lacking light chains and the entire CH1 domain (Hamers-Casterman et al., 1993). The monoclonal variable domain of llama heavy-chain antibody (VHH) is distinct from the antigen-binding domain of the heavy-chain of conventional antibodies (VH) and has been recently reviewed (Muyldermans, 2013). These Camelid heavy-chain antibodies are applied in various fields of biotechnology, such as affinity purification of proteins and therapeutic applications (de Marco, 2011). The success of VHHs is supported by their relatively easy production in microorganisms, their excellent refolding features and their good stability compared to conventional antibodies. Furthermore, the interaction with their cognate antigens is with affinities and specificities that resemble those of conventional antibodies. In this study, we set out to discover Camelid antibody fragments capable of preventing the function of the *P. aeruginosa* flagellum during swimming and biofilm formation.

2. Material and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain TG1 {*supEthi*-1 Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 ($r_k^-m_k^-$) [F′ *traD*36 *proABlacI*^{q}*Z*ΔM15]; Agilent Technologies} was used as host for M13 bacteriophages and propagation of phagemids, and XL1-Blue MR [Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mrr*)173 *endA*1 *supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac*; Agilent Technologies] was used for cloning experiments. The following *P. aeruginosa* strains were used in this study: PAO1, wild type strain (Holloway, 1969), PW3631, *ISphoA* insertion in *flhB* (PAO1 ΔFlg), PW8626, *ISphoA* insertion in *pilC* (PAO1 ΔT4P) (Jacobs et al., 2003) and strain PAK (Totten and Lory, 1990). Bacterial cells were propagated at 37 °C in lysogeny broth (LB) and on LB-agar plates, supplemented with 2% (v/v) glucose for repression of the *lac* promoter when required. Plasmids and phagemids were maintained in *E. coli* by the addition of the appropriate antibiotic to the media (100 μg/mL of ampicillin, 50 μg/mL of kanamycin, or 25 μg/mL of chloramphenicol).

2.2. Isolation of flagella and outer membranes

PAO1 cells were grown on LB-agar plates, and after collection of the cells, flagella were isolated by shearing them from the cells by passing the cell suspension through a needle as described (Montie et al., 1982). For the isolation of outer membrane vesicles (OMVs), PAO1 cells were grown overnight in 1 L LB. After collection of the cells by centrifugation at 5000 × *g* for 20 min, the cell pellet was suspended in 30 mL of 20 mM Tris–HCl (pH 7.5), 20% (v/v) sucrose, supplemented with Complete protease inhibitor (Roche). After addition of 3 mL of 1 mg/mL chicken egg white lysozyme (Sigma), cells were disrupted in a French press twice at 15,000 lb/in^2. The intact cells were removed by centrifugation at 5000 × *g*, and a membrane fraction enriched in OMVs was obtained by centrifugation at 113,600 × *g* for 10 min. The membrane pellet was resuspended in 20 mM Tris–HCl (pH 7.5) and used for the immunization experiments. It should be noted that the OMV fraction obtained is contaminated with inner membranes.

2.3. Construction of an immune VHH phage display library

Immunization and the construction of a VHH library were performed as described before (Adams et al., 2009). In brief, a llama was immunized three times with a 3-week interval using 400 μg of isolated flagella and 5 mg of PAO1 OMVs injected intramuscularly. The co-immunization with OMVs will allow us to screen the same library for VHHs against other outer membrane components in future experiments. A 150-mL blood sample containing 10^8 peripheral blood lymphocytes (PBLs) was taken at 35 and at 55 days after the priming injection, and total RNA was extracted from the PBLs of both bleeds. Preparation of cDNA using the SuperScript® II reverse transcriptase kit (Life Technologies) was done according to the manufacturer's instructions. The cDNAs encoding the VHHs were amplified by PCR using primers LAM16/LAM17 and Lam-07 or Lam-08 annealing to the short and the long hinge-encoding regions, respectively (Frenken et al., 2000). Upon digestion with *Pst*I and *Not*I, the DNA fragments with a length between about 300 and 450 bp were purified via agarose gel electrophoresis and subsequently ligated into the phagemid pUR8100 as described (Adams et al., 2009). The ligation mixture was used for the transformation of TG1 cells.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were conducted at room temperature (RT) in 96-well Maxisorb™ immunosorbent plates (Nunc), precoated overnight with 100 μL isolated flagella at a concentration of 1 μg/mL in phosphate-buffered saline (PBS). Excess of antigen was removed by washing the plate once with PBS supplemented with 0.05% Tween 20 (PBS-0.05%T), and the plate was tapped vigorously on a stack of paper towels to rid the plate of all excess fluid. Unoccupied binding sites were blocked for 1 h using 200 μL 2% (w/v) non-fat dried milk (Protifar; Nutricia) in PBS (2%P-PBS) per well. After discarding the blocking buffer, the primary antibodies (diluted llama serum samples or purified VHH) were added to the wells in a 100 μL volume. After 1 h of incubation, unbound antibodies were removed by five consecutive washes with PBS-0.05%T. As secondary antibody was added 100 μL of affinity-purified rabbit anti-VHH IgG (1:1000 diluted in 1%P-PBS). After 1 h incubation, microtiter plates were washed as before, and swine anti-rabbit IgG conjugated with horseradish peroxidase (SWARPO; Dako) was added as tertiary antibody. After 1 h incubation, microtiter plates were washed as before, rinsed with demineralized water, and plates were developed using a 1:1 mixture (100 μL per well) of tetramethylbenzidine and urea peroxide (both from Organon Teknika). The reaction was stopped with 0.33 M H_2SO_4 (50 μL of 1 M H_2SO_4 per well), and the OD_{450} of the wells was determined using a SpectraMax M5 microtiter plate reader (Molecular Devices).

2.5. Selection and screening of VHHs directed to flagellum

Phage display was utilized to select phages that specifically bind PAO1 flagella. For packaging of phagemids into M13 phages, TG1 cells containing the VHH phagemid libraries were infected with VCSM13 helper phage, which contains a kanamycin-resistance marker (Stratagene). Phages were produced overnight on 2xTY medium (Tritium Microbiologie) supplemented with ampicillin and kanamycin, and the next day phages were purified using polyethylene glycol precipitation (Verheesen et al., 2006). For the selection of anti-flagellin VHH carrying phages, the following panning method was used. A Maxisorp plate was coated with different amounts of flagella per well (2, 1 or 0.5 μg) at 4 °C overnight, followed by blocking with 2%P-PBS for 1 h. The phages, preincubated for 30 min on a rotating wheel in 1%P-PBS, were then added to the wells and incubated for 1 h at RT. Non-binding phages were