Expression of Fap amyloids in *Pseudomonas aeruginosa*, *P. fluorescens*, and *P. putida* results in aggregation and increased biofilm formation

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Abstract
The *fap* operon, encoding functional amyloids in *Pseudomonas* (Fap), is present in most pseudomonads, but so far the expression and importance for biofilm formation has only been investigated for *P. fluorescens* strain UK4. In this study, we demonstrate the capacity of *P. aeruginosa* PAO1, *P. fluorescens* Pf-5, and *P. putida* F1 to express Fap fibrils, and investigated the effect of Fap expression on aggregation and biofilm formation. The *fap* operon in all three *Pseudomonas* species conferred the ability to express Fap fibrils as shown using a recombinant approach. This Fap overexpression consistently resulted in highly aggregative phenotypes and in increased biofilm formation. Detailed biophysical investigations of purified fibrils confirmed FapC as the main fibril monomer and supported the role of FapB as a minor, nucleating constituent as also indicated by bioinformatic analysis. Bioinformatics analysis suggested FapF and FapD as a potential β-barrel membrane pore and protease, respectively. Manipulation of the *fap* operon showed that FapA affects monomer composition of the final amyloid fibril, and that FapB is an amyloid protein, probably a nucleator for FapC polymerization. Our study highlights the *fap* operon as a molecular machine for functional amyloid formation.

Introduction

Pseudomonads are, like most other bacteria, able to adhere to surfaces and form biofilms allowing the bacteria to stay in a protected environment. Biofilms formed by pseudomonads are of medical and technical importance, and substantial research has been carried out to elucidate the molecular mechanisms involved in *Pseudomonas* biofilm formation (Klausen et al. 2006). Many different extracellular components such as polysaccharides, proteins, and extracellular DNA (eDNA) are involved in adhesion and biofilm formation by the pseudomonads (Pamp et al. 2007). These components are generally well studied and their specific function fairly well understood (Jahn et al. 1999; Whitchurch et al. 2002; Matsukawa and Greenberg 2004; Allesen-Holm et al. 2006; Ma et al. 2009). Recently, however, we demonstrated that functional bacterial amyloids (FuBA) may play a role in *Pseudomonas* biofilm formation (Dueholm et al. 2010). FuBA are known to be of key importance in adhesion, biofilm formation, and virulence in *Escherichia coli* and *Salmonella*, but remain poorly investigated in the pseudomonads.
Amyloids consist of protein monomers, which upon self-assembly stack as β-strands perpendicular to the fibril axis in the so-called cross-β structure (Tycko 2004; Nelson et al. 2005). FuBA fibrillation is under close temporal and spatial control, avoiding potentially cytotoxic effects of prefibrillar intermediates common to unregulated amyloid formation (Gebbink et al. 2005; Krishnan and Lindquist 2005; Epstein and Chapman 2008; Dueholm et al. 2011). Despite the common ability to form amyloid fibrils, FuBA monomers from different organisms share little to no amino acid sequence similarity (Shewmaker et al. 2011). Another common property of FuBA is their insolubility and extreme mechanical and chemical stability, resisting dissolution by urea and boiling in sodium dodecyl sulfate (SDS). Consistent with a biologically optimized system, FuBA formation occurs over a wide range of aggregation conditions (Collinson et al. 1991, 1992; Wessels et al. 1991; Alteri et al. 2007; Dueholm et al. 2011). Noticeable features of FuBA also include auxiliary proteins insuring, for example, targeted deposition of monomers and the regulation of fibrillation (Gebbink et al. 2005; Epstein and Chapman 2008). FuBA may serve as surfactants, adhesins, biofilm structural components, spore coating and host cytotoxic compounds as well as combinations thereof (Olsen et al. 1989; Donlan 2001; Gebbink et al. 2005; Nielsen et al. 2011).

Several studies have shed more light on the molecular machineries required for FuBA expression (White et al. 2001; Elliot and Talbot 2004; Epstein and Chapman 2008). Curli fibrils from Escherichia coli and Salmonella remain the most well-characterized system, demonstrating the concerted expression of transport proteins, transcription factors, and fibrillation nucleators to produce the final amyloid fibril (Barnhart and Chapman 2006; Epstein and Chapman 2008; Otzen et al. 2011). Other well-studied examples of FuBA include chaplins from Streptomyces coelicolor, the Bacillus subtilis protein TasA, and harpins of Xanthomonas axonopodia (Claessen et al. 2003; Oh et al. 2007; Romero et al. 2010).

In a recent study, the presence of a six-gene operon (fapΔ-F) responsible for expression of functional amyloid in Pseudomonas (Fap) in P. fluorescens UK4 (UK4) was discovered. Bioinformatics was used to demonstrate that homologous operons are present within several Pseudomonas species, including P. aeruginosa, P. fluorescens, and P. putida (Dueholm et al. 2010). Pseudomonas aeruginosa is an opportunistic human pathogen notoriously responsible for infectious biofilm in cystic fibrosis patients, chronic wounds, and on medical devices (Donlan 2001; Høiby 2006). Strains of P. fluorescens and P. putida are known plant growth-promoting bacteria, interacting with plant roots through, among other factors, secreted proteins and biofilm formation (Espinosa-Urgel et al. 2000; Haas and Défago 2005). Furthermore, strains of P. putida are prime candidates for bioremediation as they metabolize organic solvents and environmental toxins (Parales et al. 2000; Attaway and Schmidt 2002). The function of Fap and extent of expression in these pseudomonads remain currently unknown. The fap operon of UK4 enabled a laboratory E. coli strain to form biofilm, but other specific functions of fap and the individual Fap proteins remain unclear (Dueholm et al. 2010).

Given the medical and technical relevance of biofilm formation by the fap containing Pseudomonas species, we investigated the capacity of the three different strains, P. aeruginosa PAO1 (PAO1), P. fluorescens Pf-5 (Pf-5), and P. putida F1 (F1), to express amyloids, and the effect of amyloid expression on aggregation and biofilm formation. These strains do not express amyloids in a detectable quantity under typical laboratory growth conditions, which motivated our use of recombinant Pseudomonas cell lines to probe the effect of fap expression. We investigated the phenotypic effects on aggregation and biofilm formation to assay for functional importance, and analyzed the biophysical properties of the fibrils. As the fap operon seemingly encompassed all the genes necessary to form FuBA, we also used a bioinformatic approach to investigate the individual gene functions and thus uncover similarities between fap and the E. coli curli system (Barnhart and Chapman 2006).

**Experimental Procedures**

**Bacteria and media**

Growth medium for shake flask cultures was colony factor antigen (CFA) medium (10 g/L hydrolyzed casein, 50 mg/L MgSO₄, 5 mg/L MnCl₂, 1.5 g/L yeast extract, pH 7.4 in double distilled water). 40 mg/L tetracycline added for recombinant organisms. Incubation was at 25–37°C and 200 rpm. Bacteria strains and primers for PCR amplification of the fap operons were as listed in Table 1. Cloned plasmids (see below) contained the Pseudomonas fap operons controlled by an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lacUV5 promoter. The lactopromoters, however, are known to be leaky, but approximately threefold higher expression levels were obtained with IPTG induction.

**Cloning of a tetracycline selective broad host expression vector**

At the time this study was initiated, all public available broad host expression vectors used the ampicillin-resistant gene (bla) as selective marker. The strains investigated in this study, however, have natural resistance against this...
Table 1. Bacteria, plasmids, and primers used in this study. The underlined primer sequences included restriction enzyme cleavage sites.

| Species and strain | Characteristics/Sequence | Reference |
|--------------------|-------------------------|-----------|
| **Escherichia coli** |                         |           |
| Mach1              | Used for routine subcloning | Invitrogen |
| INV110             | Nonmethylating plasmid host | Invitrogen |
| S17-1              | pro thi recA hsdR (r’-r”) Tp’ Sm’ Km’ Ω RP4-2-Tc::Mu-Km::Tn7 | Simon et al. (1983) |
| DH5x               | F-, φ80d lacZΔ M15, (A lacZYA-argF) U169, deoR, recA1, endA1, hsdR17(k-, m-, ϕ80Ac, supE4, ϕ80, thi-1, gyrA96, relA1 | Invitrogen |
| HB101/pRK600       | Sm’, recA thi pro leu hsdRM’ with pRK600 | Klausen et al. (2003) |
| HB101/pUX-BF13     | Sm’, recA thi pro leu hsdRM’ with pUX-BF13 | Klausen et al. (2003) |
| HB101/pBK-mini-Tn7(Sm’)-gfp | Sm’, recA thi pro leu hsdRM’ with pBK-mini-Tn7(Sm’)-gfp | Klausen et al. (2003) |
| **Pseudomonas**    |                         |           |
| P. fluorescens UK4 | Wild type | Dueholm et al. (2010) |
| P. fluorescens UK4 pFap | UK4 with pMMB190Tc-UK4fap | This study |
| P. aeruginosa PA01 | Wild type | Jacobs et al. (2003) |
| P. aeruginosa PA01 pFap | PA01 with pMMB190Tc-PA01fap | This study |
| P. aeruginosa PA01 GFP | PA01 tagged with eGfp in a mini-Tn7 construct; Sm’ | This study |
| P. aeruginosa PA01 Δfap | fapA-fapF inactivated in PA01 by allelic displacement with a gentamicin resistance cassette using pEX18Ap fapA-fapF, Gm’ | This study |
| P. aeruginosa PA01 Δfap GFP | fapA-fapF inactivated in PA01 by allelic displacement with a gentamicin resistance cassette using pEX18Ap fapA-fapF, Gm’; tagged with eGfp in a mini-Tn7 construct; Sm’ | This study |
| P. aeruginosa PA01 pV GFP | PA01 with pMM190TcPA01fap, tagged with eGfp in a mini-Tn7 construct; Sm’ | This study |
| P. aeruginosa PA01 fapA::lacZ | F2417, transposon mutant of PA01 with lacZ introduced in-frame into fapA. | Jacobs et al. (2003) |
| P. putida F1       | Wild type | ATCC 700007 |
| P. putida F1 pFap  | F1 with pMMB190Tc-F1fap | This study |
| P. putida F1 pFap (ΔfapA) | F1 with pMMB190Tc-F1fapA | This study |
| P. fluorescens F1-5 | Wild type | ATCC BAA-477 |
| P. fluorescens F1-5 pFap | F1-5 with pMMB190Tc-F1fap | This study |
| Plasmids           |                         |           |
| pMMB190            | IncQ lacZ βlA(Am’)+ Ptaclac lacZs | Morales et al. (1991) |
| pEX18Tc            | Source of tet(Tc) | Hoang et al. (1998) |
| pMMB190Tc          | pMMB190 Δbla tet(Tc) | This study |
| pMMB190Tc-UK4fap   | pMMB190Tc with UK4 fap-A-F | This study |
| pMMB190Tc-PA01fap  | pMMB190Tc with PA01 fap-A-F | This study |
| pMMB190Tc-Flfap    | pMMB190Tc with F1 fap-A-F | This study |
| pMMB190Tc-FlfapAA  | pMMB190Tc with F1 fap-A-F with premature stop codon in fapA | This study |
| pMMB190Tc-Pf-5fap  | pMMB190Tc with Pf-5 fap-A-F | This study |
| pUX-BF13           | mob*ori-R6K; helper plasmid providing the Tn7 transposition functions in trans; Amp’ | Bao et al. (1991) |
| pBK-mini-Tn7(Sm’)-gfp | Delivery plasmid for mini-Tn7-PΔA40ΔD-gfp; Amp’, Sm’ | Koch et al. (2001) |
| pRK600             | ori-ColE1 RK2-mob*RK2-tra+ helper plasmid for conjugation; Cm’ | Kessler et al. (1992) |
| pDONR221           | Gateway donor vector; Km’ | Invitrogen |
| pEX18ApGW          | Gateway compatible gene replacement vector; Suc’, Amp’ | Choi and Schweizer (2005) |
| pPS856             | 0.83 kb blunt-ended SacI fragment from pUCGM ligated into the EcoRV site of pPS854; Amp’, Gm’ | Hoang et al. (1998) |
| pDONR221 ΔfapA-fapF | fapA-fapF entry clone; Km’, Gm’ | This study |
| pEX18Ap ΔfapA-fapF | fapA-fapF knockout vector; Suc’, Amp’, Gm’ | This study |
| **Primers**        |                         |           |
| TetR-Fw            | 5’-CTCCTGTAGATACGCCCTATT | This study |
| TetR-Rw            | 5’-GGCGGTTTCTTGGTTTGGTGTT | This study |
| EcoRI-UK4fapFw     | 5’-CAGCTGAATCCGCGCCGAGGTTTTAGAAGT | This study |
| HindIII-UK4fapRw   | 5’-CAGCTGAATCCGCGCCGAGGTTTTAGAAGT | This study |
| EcoRI-PA01fapFw    | 5’-CAGCTGAATCCGCGCCGAGGTTTTAGAAGT | This study |
| HindIII-PA01fapRw  | 5’-CAGCTGAATCCGCGCCGAGGTTTTAGAAGT | This study |
antibiotic. A modified version of the broad host expression vector pMMB190, in which a tetracycline resistance gene replaced the bla gene, was therefore constructed (pMMB190Tc). This was achieved by amplifying the tetracycline resistance gene from pEX18Tc by PCR using the TetR-Fw and TetR-Rw primer pair (Table 1). The PCR was performed using the Pfu DNA polymerase (Life technologies, Paisley, UK) in a standard reaction mixture as suggested by the manufacturer and the following PCR settings: Initial activation (95°C, 180 sec), 25 cycles of denaturation (94°C, 30 sec), annealing (56°C, 60 sec), and extension (72°C, 180 sec) followed by a final extension (72°C, 10 min). pMMB190 was transformed into the nonmethylating E. coli strain INV110 (Life technologies) and purified using the Fast Purification kit (5 Prime, Hamburg, Germany). Purified plasmid was digested with FastDigest BsaI (Life technologies), trimmed with Klenow fragment (Life technologies), and dephosphorylated with shrimp alkaline phosphatase (Life technologies) according to the manufacturers’ recommendations. The tetracycline fragment was ligated into linearized plasmid using T4 DNA ligase (Life technologies) according to the manufacturers’ recommendations. The resulting vector was confirmed by shotgun sequencing of the whole plasmid (Macrogen, Seoul, South Korea).

Cloning the Pseudomonas fap operons into pMMB190Tc

Whole fap operons from UK4, PAO1, Pf-5, and F1 were amplified by PCR using the primer sets shown in Table 1. PCR was carried out using AccuPrime Pfx polymerase (Life technologies) in a standard reaction mixture as suggested by the manufacturer with the following PCR settings: Initial activation (95°C, 120 sec), 25 cycles of denaturation (95°C, 30 sec), annealing (60°C, 60 sec), and extension (68°C, 6 min) followed by a final extension (68°C, 10 min). Following PCR, A-overhangs were added using a taq polymerase (Life technologies) according to the manufacturers’ recommendations. The resulting fragments were gel purified and subcloned into pCR4-TOPO vectors (Life technologies). Fragments containing fap operons were obtained by digestion of the subcloned vectors with FastDigest EcoRI/BamHI (F1 and Pf-5) or FastDigest EcoRI/HindIII, (UK4 and PAO1) followed by gel purification using the UltraClean 15 DNA kit (MO-BIO, Carlsbad, CA). The pMMB190Tc was prepared for ligation using the same restriction enzyme combinations and purified as above. Ligations were done using T4 DNA ligase (Life technologies) according to the manufacturers’ recommendation and the resulting vectors (pFap) were confirmed by shotgun sequencing of the whole plasmids (Macrogen).

Transformation of pFap plasmids into homologous hosts

Electrocompetent Pseudomonas cells were prepared from overnight cultures grown in LB medium at 37°C (PAO1) or 30°C (UK4, Pf-5, and F1). For each transformation, bacteria were harvested from 6 mL culture (7500g, 10 min). The cells were washed twice in 4 mL of 300 mM room temperature sucrose before suspension in 100 μL of 300 mM sucrose. 100 μL of each strain was mixed with 5 μL of purified vector and 40 μL of the suspension was transferred to a 1-mm gap electroporation cuvette. Electroporation was performed with a Micro Pulser electroporator (Biorad, Hercules, CA) using 1.80 kV (PAO1) or 1.25 kV (UK4, Pf-5, and F1) pulses. 1 mL super optimal broth (SOC) medium was applied directly after electroporation and the sample was transferred to a 15-mL tube and incubated (28°C, 200 rpm, 2 h). 100 μL of the transformations were plated on LB agar plates containing 50 μg/mL gentamicin and the plates were incubated at 37°C (PAO1) or 30°C (UK4, Pf-5, and F1) until visible colonies appeared (1–3 days).

Construction of a Pseudomonas PAO1 Δfap mutant

A knockout fragment containing a gentamycin (Gm) resistance cassette was generated by PCR overlap extension essentially as described by Choi and Schweizer (2005). Primers (whose sequence will be supplied upon request) were used to amplify chromosomal regions upstream and downstream of PAO1 fapA-fapF, and to amplify a Gm resistance cassette from plasmid pPS856 (Hoang et al. 1998). The PCR fragments were fused together and amplified with primers GW-attB1 and GW-attB2 incorporating the attB1 and attB2 recombination sites at either end of the knockout cassette. Using the Gateway cloning system (Life technologies), the resulting knockout fragment was first

| Species and strain | Characteristics/Sequence | Reference |
|--------------------|--------------------------|-----------|
| EcoRI-F1fapFw      | 5’-CACTGAAATCTCTGCCTCTGGTTCGC | This study |
| BamHI-F1fapRw      | 5’-CACTGGATCCGATGGCACTATCGAAGTA | This study |
| EcoRI-Pf-5fapFw    | 5’-CACTGAATTCGAAACAGTCCCGAAAGCC | This study |
| BamHI-Pf-5fapRw    | 5’-CACTGGATCCGGGTGGGTCAGAAGTAGT | This study |

(Continued)
transferred by the BP reaction into pDONR221 generating entry plasmid pDONR221 fapA-fapF, and subsequently transferred by the LR reaction into pEX18ApGW generating the knockout plasmid pEX18A::fapA-fapF.

A PAO1 ΔfapA-fapF mutant (PAO1 Δfap) was constructed as follows: The pEX18ApA::fapF knockout plasmid was transferred into PAO1 by two-parental mating using the donor strain E. coli S17-1 with selection on Pseudomonas Isolation agar plates supplemented with Gm. Resolution of single crossover events was achieved by streaking on 5% sucrose plates via the counter-selectable sacB marker on the knockout plasmid. The mutant construction was confirmed by PCR analysis.

**Green fluorescent protein tagging of PAO1 strains**

The PAO1 wild type and derivatives were green fluorescent protein (GFP) tagged by inserting a mini-Tn7-gfp cassette into a neutral site of the genome, using four-parental mating, essentially as described previously by (Klausen et al. 2003).

**Pseudomonas aeruginosa PAO1 fap promoter activity**

Activity of the P. aeruginosa PAO1 fap promoter was determined using the FluoReporter lacZ/galactosidase quantification kit (F-2905, Life technologies) and the fap promoter reporter mutant PAO1 fapa::lacZ. Optical density at 600 nm (OD600 nm) was measured during growth and 1 mL culture samples were collected in triplicate. Bacteria was pelleted by centrifugation (1 000g, 1 min) and resuspended in 1 mL of enzymatic lysis buffer (10 mM Tris-HCl, 0.1 g/L DNase I (#DN25, Sigma-Aldrich, St. Louis, MO), 0.1 g/L RNase A (#83833, Sigma-Aldrich) 0.1 g/L alginate lyase (#A1603, Sigma-Aldrich), 1 g/L lysozyme (#L6876, Sigma-Aldrich), 1 mM MgSO4, 0.1% (V/V) triton-X100). The samples were subjected to three freeze-thaw cycles using a water bath at 37°C and a −80°C freezer. Between each cycle the samples were mixed using a vortex mixer and samples were kept in the freezer after the final cycle. β-galactosidase activity was measured in each sample according to the FluoReporter lacZ/galactosidase quantification kit recommendations and the results were normalized according to OD600 nm.

**Shake flask culturing and sampling**

A volume of 5 mL CFA media in 50 mL centrifuge tubes were seeded with frozen glycerol stocks and grown overnight. A volume of 50 mL CFA medium in 250 mL shake flasks were inoculated to an initial OD600 nm of 0.075 and grown until 0.5 before induction with a final concentration of 1 mM IPTG. One culture flask for each culture was used as reference, with the remaining incubated undisturbed. At an OD600 nm of 1.5, cultures were harvested for biofilm assays, bright-field microscopy, and transmission electron microscopy (TEM). For purification of fibrils in bulk, expression was done in 400 mL cultures and 2.5L shake flasks. Harvest was done by centrifugation at 28 000g for 30 min. Pellets were resuspended in 8.75% (volume/culture volume) buffer (10 mM Tris-HCl at pH 8) and homogenized using a power drill mounted tissue grinder. A proportion of 1.25% (volume/culture volume) enzyme mix (0.4 g/L RNase A, 0.4 g/L DNase 1, 4 g/L lysozyme, 4 mM MgCl2, and 0.4% (V/V) Triton X-100) was added and following 0.5 h incubation at 37°C, harvests were frozen at −80°C.

**Crystal violet biofilm assay**

Biofilm formation in microtiter plates was quantified by crystal violet staining essentially as described by O’Toole and Kolter (1998). A volume of 100 µL of LB, containing 1000-fold diluted overnight culture, was added to the wells of a 96-well microtitre plate (#92096, TPP, Trasadingen, Switzerland) #92096), which was subsequently incubated (37°C, 18 h, 175 rpm). The wells were aspirated and remaining planktonic bacteria were removed by addition and removal of 120 µL 0.9% saline. The biofilms were then stained for 15 min with 120 µL 0.1% crystal violet (Sigma-Aldrich) in 0.9% saline. Crystal violet quantification was done by washing the wells twice with 150 µL 0.9% saline, solubilizing the crystal violet with 96% ethanol for 30 min, and measuring the absorbance at 590 nm. For this assay, leaky expression of cloned fap operons was sufficient and no IPTG was used.

**Cultivation of flow-chamber biofilms**

Biofilms were cultivated at 37°C in flow chambers which were assembled and prepared as described previously (Sternberg and Tolker-Nielsen 2006). Flow chambers were inoculated with P. aeruginosa overnight cultures diluted to an OD600 nm of 0.01 in FAB-glucose medium as described by Pamp et al. (2008). Replicate experiments were done with the velocity of the laminar flow in the flow-chamber channels at 0.2 mm/sec, using a Watson Marlow 205S peristaltic pump (Watson Marlow, Falmouth, U.K.). For this cultivation, leaky expression of cloned fap operons was sufficient and no IPTG was used.

**Microscopy and image analysis**

Microscopy observation and image acquisition of biofilms were performed with a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).
equipped with an argon laser and detectors and filter sets for simultaneous monitoring of GFP (excitation, 488 nm; emission, 517 nm). Images were obtained using a 63x/1.4 objective. Simulated fluorescence projections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

**Amyloid fibril purification**

Harvests were subjected to three cycles of thaw–freeze using a 37°C water bath (0.5 h) and a −80°C freezer (>1.5 h) followed by another 2 h incubation at 37°C. Preparations were boiled three times in 2% (W/V) SDS and subsequently washed two times in buffer (10 mM Tris-HCl at pH 8) before final resuspension. Insoluble material was collected by centrifugation 0.5 h at 28 000 × g. When washing away SDS during the last purification steps, the purified, insoluble materials became very sticky and adhered to laboratory plastics.

**Amyloid SDS-PAGE**

A volume of 2 × 25–250 μL samples from each of the purified preparations were frozen in liquid nitrogen and lyophilized overnight. Samples were then resuspended in 50 μL formic acid (FA) or milliQ water, respectively, lyophilized again. Samples were finally resuspended in 50 μL reducing SDS-PAGE loading buffer (75 mM Tris, 6.6% (W/W) SDS, 15% (V/V) glycerol, 7.5% (V/V) β-mercaptoethanol, 0.9 mg/L bromophenol blue, 8 M urea at pH 6.8). Urea was required to avoid precipitation of amyloid proteins. A volume of 4–15 μL was loaded to an AnyKD gel (Biorad) and electrophoresis done at 200 V for 0.5 h. Gels were stained with Coomassie Brilliant Blue G250 and digitally scanned.

**Tandem MS peptide sequencing**

SDS-PAGE gel protein bands were excised, split in two, and in-gel digested using trypsin or chymotrypsin with ProteaseMax detergent (Promega, Madison, WI). In situ digestion was done according to modified protocol of Shevchenko et al. (1996) including reduction and alkylation of Cys. The peptides were analyzed on a nanoflow UPLC ( Dionex Ultimate3000/RSLC, ThermoFisher Scientific, Waltham, MA) system coupled online by a nanospray ion source (Proxeon, ThermoFisher Scientific) to an Orbitrap Q-Exact mass spectrometer (Thermo-Fisher Scientific). The peptides were separated on two successive reverse phase columns (Acclaim PepMap100 C18 Nano-Trap, and Column Acclaim PepMap300 C18, ThermoFisher Scientific) using a linear gradient (10–35% acetonitrile in 35 min) and a constant flow rate of 300 nL/min. The mass spectrometer was operated in a data-dependent mode to switch between full MS scans and tandem MS/MS. Fragmentation was performed using higher energy collision induced dissociation (HCD) and sequenced precursor ions were dynamically excluded for 30 sec. The raw mass spectrometry files were analyzed and exported as mgf using Thermo Proteome Discover (version 1.3.0.339). MS/MS spectra were searched (Matrixscience Ltd., London, U.K., in-house Mascot server version 2.3) against Uniprot database (complete proteomes UK4; PAO1; Pf-5; F1) choosing carboxymethyl (C), propionamide (C), and oxidation (M) as variable modifications and allowing enzyme specificity at one terminus only (Perkins et al. 1999). Peptide and fragment tolerances were set at 10 ppm and 20 mmu, respectively.

**Fourier transformed infrared spectroscopy**

Fourier transformed infrared (FTIR) was carried out using a Tensor 27 FTIR spectrophotometer (Bruker, The woodlands, TX) equipped with a deuterated tri-glycine sulfate Mid-infrared detector and a Golden Gate single reflection diamond attenuated total reflectance (ATR) cell (Specac, Kent, U.K.). Purified fibers from cultures were dried on the ATR crystal using dry nitrogen. ATR spectra were recorded from 4000–1000 cm⁻¹ using a nominal resolution of 2 cm⁻¹ and 64 accumulations. Resulting spectra were baseline corrected and interfering signals from H₂O and CO₂ were removed using the atmospheric compensation filter in the OPUS 5.5 system (Bruker). Different components of the amide I region were identified by second derivative analysis in the OPUS 5.5 system.

**Transmission electron microscopy**

Suspensions culture samples and purified amyloid fibrils were mounted on 400 mesh carbon coated, glow-discharged nickel grids for 30 sec. Grids were washed with one drop of double distilled water and stained with three drops of 1% (W/V) phosphotungstic acid at pH 7.2. Samples were inspected in a transmission electron microscope (JEM-1010, JEOL, Eching, Germany) at 60 kV. Images were obtained using an electron sensitive CCD camera (KeenView, Olympus, Center Valley, PA). For size determination, a standard grid size 264 nickel plate (462-nm grid) was used.

**Bioinformatics**

Searching the NCBI Nucleotide collection (restricted to Pseudomonas, taxid:286) with the UK4 fapC sequence using BLASTn with NCBI default settings identified
**Results**

**Assaying laboratory strains for fap expression**

*Pseudomonas fluorescens* UK4 was originally identified as a Fap-producing strain through its ability to form deep-red colonies when grown on agar plates containing the amyloid-binding dye Congo red (Dueholm et al. 2010). However, despite extensive efforts, we have not been able to identify laboratory growth conditions under which PAO1, Pf-5, and F1 produce detectable amounts of Fap fibrils. For all organisms, shake flask growth in LB, tryptic soy broth, and CFA media at temperatures ranging from 25–37°C were assayed including variations in NaCl concentrations and culturing times (12–72 h) prior to purification. Finally, cultures spread on CFA and LB agar plates with harvest of culture layers were assayed. Presence of amyloid fibrils was investigated using TEM, Congo red, and amyloid conformational specific antibody (WO1) staining of bacteria cultures and amyloid purification (O’Nuallain and Wetzel 2002). To further investigate the expression of fap in wild type *Pseudomonas*, we employed a *P. aeruginosa* PAO1 strain with the β-galactosidase gene (lacZ) cloned chromosomally in-frame into fapA (PAO1 fapA::lacZ). In this setting, PAO1 fapA::lacZ culturing allowed for measuring fap promoter activity at various growth conditions with a standard β-galactosidase activity assay. The activity measurements demonstrated that the fap promoter was constitutively active in shake flask cultures of PAO1 fapA::lacZ with a peak activity during the exponential growth phase (Fig. 1A). Furthermore, results also indicated that lower temperature and high salt concentrations

![Figure 1](image-url)
favored promoter activity (Fig. 1B and C). No noteworthy phenotypical effects accompanied the promoter activity, however, an active promoter implied Fap expression in the wild type PAO1.

Construction of homologous recombinant Fap model systems

In order to study the functional features of Fap expression, a recombinant approach was chosen where the fap operons from each strain, including UK4, were cloned into an inducible broad host expression vector, and reintroduced into the original strains for homologous Fap overexpression. This approach yielded four recombinant strains, termed UK4 pFap, PAO1 pFap, Pf-5 pFap, and F1 pFap. Fap fibrils can be purified from the UK4 wild type growing on agar plates, albeit not from suspension cultures (Dueholm et al. 2010). Unfortunately, suspension culturing for propagation of UK4 stocks reduces the yield of purified fibrils from agar plate culturing approximately 40-fold. However, the ability of UK4 to produce detectable amounts of Fap fibrils allows for comparisons of native and plasmid-encoded fibrils. In the case of PAO1, we also constructed a defined fap operon knockout mutant, and furthermore the PAO1 wild type, PAO1 pFap, and PAO1 Δfap strains were chromosomally tagged with GFP. The more extensive experiments with PAO1 were motivated by its importance as a biofilm model system and a human pathogen (Harmsen et al. 2010).

Expression of the fap operons results in aggregated growth and biofilm formation

The phenotypic effect of fap expression was first assayed in shake flask cultures, where the recombinant derivatives formed biofilm at the air-medium interface on flask sides. Cultures of the wild type strains and the recombinant counterparts were compared using confocal laser scanning microscopy (CLSM) (Fig. 2A). PAO1 pFap formed large clumps in shake flask cultures, whereas the PAO1 wild type and the vector control strain, PAO1 pVC, as well as the fap deletion mutant PAO1 Δfap mainly, were present as planktonic cells. PAO1 pFap bacterial aggregates were also readily visible in the culture flasks and quickly settled from suspension. Expression of the fap operon thus led to a highly aggregative and adherent phenotype in liquid PAO1 cultures. Highly similar results were obtained with Pf-5, F1, UK4 wild type and their recombinant derivatives (data not shown).

Subsequently, the ability of the PAO1 strains to form biofilm in microtiter trays was investigated. The amount of biofilm formed after 24 h of incubation was quantified by the use of a crystal violet-based staining assay. PAO1 pFap formed five- to sixfold more biofilm than the PAO1 wild type and PAO1 pVC strain (Fig. 2B). Moreover, we found that PAO1 Δfap formed biofilm to the same extent as the wild type under these conditions. The ability of fap overexpressing strains to form excessive amounts of biofilm was also demonstrated for Pf-5, F1, and UK4 (data not shown). We have made similar observations for E. coli overexpressing fap (Dueholm et al. 2010).

The ability of the PAO1 strains to form biofilms was furthermore investigated using flow chambers. The PAO1 wild type, pVC, and Δfap strains all attached to the glass surface (Day 1) and initially formed a flat biofilm (Day 2), followed by small microcolonies (Day 3), and eventually formed mushroom-shaped microcolonies (Day 4) (Fig. 3). The fact that the PAO1 Δfap mutant formed biofilms similar to PAO1 wild type biofilms shows that Fap is not an absolute requirement for biofilm formation under these experimental conditions. The PAO1 pFap strain showed clear differences in biofilm formation and structure. Notably, PAO1 pFap initiated microcolony formation much earlier than the PAO1 wild type and
pVC (Day 1), and after 4 days of cultivation the microcolonies had an elongated unusual appearance, presumably caused by the effect of shear force on these unusually large microcolonies (Fig. 3).

**Expression of the fap operons yield amyloid fibrils**

The presence of amyloid fibrils in the fap overexpressing cultures was confirmed by TEM (Fig. 4). Copious amounts of fibrils were present both adjacent to bacterial cells and free in suspension. The fibrils varied slightly in morphology. PAO1 pFap and Pf-5 pFap fibrils were generally thicker and slightly more curved than the more straight and staggered ones of UK4 pFap. Fibrils from F1 pFap were somewhat intermediate between these two categories. The fibrils expressed by UK4 pFap were highly similar in morphology to those previously observed for UK4 wild type (Dueholm et al. 2010). As expected from efforts with Fap purification from wild type strains, no fibrils were seen in samples from the PAO1, Pf-5, F1, and UK4 wild type cultures. All the fibrils displayed a tendency to aggregate, and for UK4 pFap and F1 pFap the association was generally parallel with the length axis of the fibrils. Although the TEM sample preparation may influence the fibril association, this is unlikely to account for the consistent differences among the species.

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*Figure 3. PAO1 biofilm formation in flow chambers. Confocal laser scanning micrographs of GFP-tagged PAO1 wild type and derivative cultures and biofilms formed in flow chambers. Images were captured after 1, 2, 3, and 4 days of cultivation. Each image contains top view (upper left) and two side view panes (bottom and right linings) with focus depth indicated (white markers). Bars, 50 µm.*
Apart from the fibril shape, the hallmark of FuBA fibrils is their cross-\(\beta\) structure, aqueous insolubility, and stability, and UK4 Fap fibrils were previously confirmed to possess all these characteristics (Dueholm et al. 2010). Purification by repeated boiling in SDS demonstrated the fibrils’ resistance to denaturing conditions. However, fibril purification for the F1 pFap culture failed, most likely because these fibrils were less stable than their counterparts and could not be efficiently separated from contaminants. The successfully purified preparations of PAO1, Pf-5, and UK4 Fap were also analyzed by TEM (Fig. 5A) and shown to contain fibrils with an identical appearance to those observed in the unpurified culture samples.

To demonstrate the presence of amyloid structures, at the level of protein secondary structure, purified preparations were subjected to FTIR spectroscopy (Fig. 5B). The purified preparations all showed characteristic FTIR spectrum peaks (1617–1625 cm\(^{-1}\)) well within the interval consistent with a cross-\(\beta\) amyloid structure (1615–1630 cm\(^{-1}\)) (Zandomeneghi et al. 2004). The peaks at 1660–1662 cm\(^{-1}\) may indicate an antiparallel arrangement of the \(\beta\)-sheets, although this is not a definitive marker (López De La Paz et al. 2002; Zandomeneghi et al. 2004). Even more significant, the 2nd derivative FTIR spectra of PAO1 pFap and Pf-5 pFap fibrils were identical in shape with each other and with that of UK4 pFap fibrils. Furthermore, the three FTIR spectra of recombinant-expressed fibrils were identical in shape to that of Fap purified from the UK4 wild type in an earlier study (Dueholm et al. 2010), indicating the same structural constraints on their peptide backbone across all fibril species.

To determine the Fap fibrils monomer composition, purified preparations were subjected to reducing SDS-PAGE analysis with prior solubilization using pure FA

![Figure 4](image1.png)

**Figure 4.** In situ imaging of Fap fibrils in recombinant cultures. Transmission electron microscopy (TEM) images of PAO1 pFap, Pf-5 pFap, UK4 pFap, and F1 pFap shake flask cultures at different magnifications. No fibrils were found in the corresponding wild type cultures (no images). Bars 0.5 \(\mu\)m.

![Figure 5](image2.png)

**Figure 5.** Analysis of purified fibrils. (A) TEM images showing fibrils identical to those in culture. (B) FTIR spectra (top) and with the calculated 2nd derivative spectra (bottom) for peak and shape identification. (C) SDS-PAGE gel of preparations resuspended in water (\(\dagger\)) or FA (\(\ddagger\)) prior to lyophilization and reconstitution in loading buffer. Calculated M\(_{\text{w}}\) of FapC is 33.0, 34.0 and 25.1 kDa for Pf-5, PAO1, and Pf-5, respectively.
The SDS-PAGE analysis demonstrated that the proteinaceous contents of the preparations were composed of primarily one constituent each. Protein bands from the UK4, Pf-5, and PAO1 Fap preparations corresponded to the expected molecular weight (Mw) of the FapC proteins from the respective organisms (UK4 ~22.6, Pf-5 ~30.7, and PAO1 ~31.5 kDa). Gel bands (Fig. 5C) were confirmed to be mainly FapC by tryptic in-gel digestion followed by tandem mass spectrometry peptide sequencing. Interestingly, the excised gel bands were also all found to contain FapB (~17 kDa) and FapE (~23 kDa) although not consistent with the molecular weight expected from gel electrophoresis. However, given the sensitivity of the applied mass spectrometry instrument, any imperfections in the gel separation and contaminating proteins were detected. Notably, few high abundance membrane proteins, for example, OmpF- and LemA-like, were also identified, however, with significantly lower protein scores than the Fap proteins and also varying among samples. Protein bands corresponding to FapB and FapE were not detectable in the gels with intense bands of FapC (Fig. 5C), indicating that FapB and FapE only constitute a very small mass percentage of the purified fibrils.

**Manipulation of fap genes probes individual gene functions**

The function of the protein FapA, the product of the first gene in the fap operon, remains currently unknown. However, a point mutation fortuitously created a stop codon in the middle of the fapA gene in a F1 pFap plasmid, and the phenotype of the resulting mutant (F1 pFap [ΔfapA]) indicated a potential function for FapA. F1 pFap[ΔfapA] showed extensive biofilm formation in shake flask cultures (Fig. 6A) albeit only minor aggregation of cells in suspension, while purification of the expected fibrils yielded short, fibril-like aggregates (Fig. 6B). The purified fibril-like aggregates produced a FTIR spectrum highly similar to that of the fibrils from an intact fap operon (compare Figs. 6C and 5B). SDS-PAGE analysis with subsequent mass spectrometry peptide sequencing (Fig. 6D) showed the copious amounts of insoluble, extremely stable aggregates produced by F1 pFap[ΔfapA] to consist of mainly FapB protein (~18-kDa band, Fig. 6D) with small amounts of F1 FapC (~38 kDa band, Fig. 6D). Apparently, FapB readily formed amyloid fibrils with only small amounts of FapC as the result of fapA deletion, and these also resulted in an aggregative and biofilm adhering phenotype. These results imply that FapA has a regulatory or facilitating function affecting the distribution of FapC and FapB in the final amyloid fibril.

**The Fap proteins are not equally conserved**

The FapC protein of the UK4 fap operon was prior to this study the only verified *Pseudomonas* amyloid fibril monomer. An updated BLASTn search using the UK4 fapC to query the NCBI database identified 18 *Pseudomonas* strains that all harbored fap homologous operons. These
strains were all found within the species *P. aeruginosa*, *P. brassicacearum*, *P. entomophila*, *P. fluorescens*, and *P. putida*. In all species, *fapC* was part of the six-gene *fap* operon (Fig. 7A). Examining the phylogeny of the strains using *FapA-F* showed these sequences had different degrees of conservation (Fig. 7B). *FapA* and *FapC* appeared less evolutionarily conserved than the most conserved proteins (*FapD* and *FapF*), while *FapE* and *FapB* showed an intermediate degree of conservation. An equivalent examination of the *CsgBAC* and *CsgDEFG* proteins from 88 *csg* curli operons from various *Enterobacteriales* (see methods) also demonstrated different degrees of conservation (Fig. 7C). In this case, *CsgDEFG* was more conserved than *CsgA* and *CsgC* with *CsgB* showing an intermediate degree of conservation.

**Structural and functional predictions of the Fap proteins**

Assigning functions of the individual Fap proteins based on BLAST searches for homologs and motifs was unsuccessful. Protein tertiary structure prediction and homology analysis using the protein homology/analogy recognition engine (Phyre2), however, identified *FapF* as a β-barrel membrane pore and *FapD* as homologous with peptidase C39-like domains of various ABC transporters, that is, cysteine proteases. Purifications showed that *FapB*, *FapC*, and *FapE* were part of the extracellular amyloid fibrils, and a bioinformatic investigation found Type-I N-terminal secretion signal peptides in 104 of the 108 (6 × 18) Fap proteins and their homologs. The exceptions were *P. fluorescens* F113 *FapA*, *P. putida* S16 *FapC* and *FapF*, and *P. putida* W619 *FapA*. These exceptions are likely attributed to annotation errors. A separate study has previously found secretion signal peptides present in all proteins today known as Fap proteins from *P. aeruginosa* PAO1 (Lewenza et al. 2005). Amino acid sequence alignments of Fap proteins, not including secretion signal peptides, identified characteristic 100% conserved Cys residues. *FapC* from *P. brassicacearum*, *P. fluorescens* and *P. aeruginosa* organisms contained a C-terminal Cys-X-X-Cys motif and their *FapE* also had a Cys residue at the C-terminal. In contrast, *P. entomophila* and *P. putida* sequences did not contain these Cys motifs. All 18 *Pseudomonas* FapD sequences contained a Cys residue at 25 residues from their mature N-terminal. *Pseudomonas* FapA, FapB, and FapF sequences did not contain Cys residues outside the secretion signal peptides.

We previously reported *FapC* to contain three imperfect repeat regions (R1-3) interspaced by so-called linker regions (L1-2), and also *FapB* was found to contain three similar repeat sequences (Fig. 8). An updated amino acid sequence alignment of all FapC and FapB homologs, respectively, identified novel properties of these repeats. Immediately N-terminal to the previously established *FapC* R1 and R2 sequences, we observed repeating sequences where Asn and Gln residues were 100% conserved across the 18 strains, indicating *FapC* R1 and R2 to be expanded versions of R3 (Fig. 8B). Repeat regions in *FapB* were all highly similar and truncated versions of those found in *FapC* (Fig. 8B). All the *FapB* and *FapC* repeat motifs showed characteristic conservation of Gln,
Asn, and Ser residue rich stretches interrupted by Gly and/or Ala residues. A similar pattern was observed in the CsgA amyloid monomer of *E. coli* curli fibrils and shown to be important for amyloid fibril formation and stability (Wang and Chapman 2008). Despite sharing motif characteristics, FapB and FapC repeats were not homologous to those of CsgA.

Repeat motifs are expected to make up the β-strands of the amyloid fibril cross-β structure. The length and key conservation of Gly residues implied each repeat folded to form several β-strands. To probe FapB and FapC folding, protein sequences were submitted to Phyre2 for secondary and tertiary structure predictions. In general, tertiary structure predictions were limited due to the lack of determined structures for any homologs. FapB secondary structure predictions, however, indicated R1-3 consisted of two β-strands each with a possible turn centered on the 100% conserved Ala–Gly residues roughly in the middle of the repeat regions. Predictions also indicated that FapB contained α-helical segments outside the repeat regions. The tertiary structure predictions could not fully accommodate the predicted strands, although FapB R1-2 showed a tendency to fold in a strand-loop-strand-like motif. Secondary structure predictions for FapC showed extensive β-strands throughout the sequence. Given that the FapB repeats were truncated versions of the FapC R1-3, the results suggested FapC R1 and R2 could each form approximately four β-strands and R3 three β-strands (Fig. 9).

**Discussion**

Fap fibrils enhance cell aggregation, attachment, and provide strength to biofilms

Recombinant expression of the *fap* operons in *P. aeruginosa*, *P. fluorescens*, and *P. putida* suspension cultures had a dramatic effect on their mode of growth. The otherwise planktonic cells turned highly aggregative and formed extensive biofilms in solution, on the culturing flask at the air-medium interface, and in the microtiter assay. Furthermore, very early microcolony formation and extre-
mely large biofilm structures were observed for PAO1 pFap in the flow chamber. To our knowledge, such unusually large structures have never been observed before in PAO1 flow-chamber biofilms, and it highlights the potent effect of Fap on PAO1 biofilm formation. Collectively these results show that the fap formed fibrils likely function as an adhesin for the attachment to an abiotic surface, and also as a stabilizing structural component providing strength to the mature biofilms. Interestingly, the purified fibril material also adhered vigorously to laboratory plastic and glass surfaces (see Methods), supporting an adhesive function. The results, however, also showed that the Fap fibrils were not an absolute requirement for adhesion and biofilm formation under the tested culturing conditions tested. The PAO1 Δfap mutant did not show any biofilm deficiency compared with PAO1 wild type, likely because biofilm formation in microtiter and flow-chamber studies also involves other components, for example, pili, eDNA, and polysaccharides (Harmsen et al. 2010). In this context, Fap expression greatly added to the PAO1 biofilm-forming capacity. The question remains, why it was not possible to show any significant expression of the amyloids in the wild type strains at the protein level in spite of the fap promoter activity, except for the agar plate grown UK4 (Dueholm et al. 2010). A likely explanation is that the PAO1 cultures do not express fap in significant amounts. A low expression level may be a laboratory artifact and if so, would also explain the lack of differences in biofilm properties of the Δfap mutants and the wild types.

**Laboratory growth conditions apply selection pressure against Fap expression**

It is likely that wild type strains lose the ability to express amyloids as laboratory growth conditions favor a planktonic lifestyle. This hypothesis is further supported by the significant decrease in the yield of Fap purified from our current stocks of UK4 wild type compared to the original isolate. Loss/modification of genes is not uncommon in lab cultures and even culture collection stocks of *P. aeruginosa* PAO1 undergo genomic diversification (Klockgether et al. 2010). Rainey et al. demonstrated the adaptive radiations of *P. fluorescens* SBW25 as a response to the heterogeneous growth conditions obtained in static cultures. This yielded three distinct morphotypes, the smooth morphotype (SM), the winky-spreader (WS), and the fuzzy-spreader (FS). The SM is associated with planktonic growth, whereas the WS forms biofilm with cells adhering to surfaces and each other (Rainey and Travassano 1998). The FS is not very well described, but it also forms aggregates, albeit in the low oxygen zone near the bottom of the static culture. It was furthermore shown that homologous culturing applied a strong selection pressure for the SM. If Fap expression is associated with either the WS or the FS, this explains why the wild type bacteria did not express detectable amounts of Fap. Conversely, the domesticated, nonbiofilm forming *E. coli* K-12 has been shown to produce an OmpR-mutated variant, capable of curli expression, surface adherence, and aggregation, using atypical culturing conditions (Vidal et al. 1998).

**Pseudomonas aeruginosa and *P. fluorescens***

**Fap fibrils are FuBA composed of FapC with some FapB and FapE**

The high thermal and chemical stabilities of the Pf-5, PAO1, and UK4 fibrils were demonstrated. The fibrils survived repeated boiling in SDS and required FA pretreatment to dissolve in loading buffer with 8 M urea. Protein assays showed fibrils from UK4 pFap, Pf-5 pFap, and PAO1 pFap consisted of mainly FapC protein, although with very small amounts of FapB and FapE included. This supported the hypothesis of FapB as an amyloid fibril component and potentially a fibrillation nucleator, also in agreement with the repeat homology of FapB and FapC. The role of FapE in the amyloid fibrils is more uncertain, although it may be speculated that the C-terminal conserved Cys residues of *P. fluorescens* and *P. aeruginosa* FapC and FapE, respectively, could be a site of protein–protein interaction. The FTIR spectra of purified fibrils were highly similar and consistent with recombinant fibrils having the amyloid cross-β structure. The FTIR spectra of the recombinant produced UK4 Fap were identical to those from the UK4 wild type, and the UK4 wild type fibrils were previously assayed by x-ray diffraction for confirmation of cross-β structure (Dueholm et al. 2010). Consequently, the FTIR data in this study indicated that fibrils from all species formed cross-β structures with similar constraints on the protein backbone, which also fits well with their similar repeat sequences forming the β-strands.

Regardless of multiple attempts we were not able to purify fibrils from F1 pFap, although Fap fibrils clearly were present in the cultures and conferred the same phenotypic effects as seen, for example, PAO1 pFap. Furthermore, the repeat sequences are highly similar to those of the other pseudomonads, that is, F1 fibrils may be an example of FuBA in terms of cross-β structure, while not displaying their classical high stability. The *B. subtilis* TasA protein also forms functional amyloids of lower stability. TasA fibrils will dissolve in 10% (vol/vol) FA or SDS-PAGE loading buffer, while fibrils purified for this study required > 90% (vol/vol) FA and withstood boiling in 2% (weight/vol) SDS (Romero et al. 2010). Compared to the homologs, F1 FapC lacks the C-terminal...
Cys-X-X-Cys motif. These Cys residues may engage in intermolecular disulfide bonds, and their absence may explain the putative decreased stability of the F1 Fap fibrils. In addition, F1 FapC also has an enlarged, hydrophilic L2 (~260 aa), compared to the other strains (~100 aa), and this could also affect stability.

Operon manipulation provides information on the individual Fap proteins

The serendipitous stop codon in the F1 pFap(DfapA) allowed us to manipulate components of the vector-based fap operons to probe single gene functions and properties. Culturing and purification results from the F1 pFap (DfapA) showed that F1 FapB, with a small amount of F1 FapC, formed amyloid fibrils with a phenotypic effect similar to the overexpression of the intact fap operons. These observations further support the role of FapB as an amyloid protein and potential nucleator. The effect of interrupting fapA suggests that FapA is a chaperone for the amyloid monomers affecting the ratio of FapB to FapC in the final fibril. However, the specific interaction between FapC, FapB, and FapA remains unclear.

The Fap operon shares common properties with the curli system

The homologous UK4, PAO1, Pf-5, and F1 fap operons conferred the ability to form FuBA, implying that the fap operon encompasses the necessary components for producing functional amyloids. In this sense, the fapA-F function is analogous to the E. coli csg operons, csgBAC and csgDEFG (Barnhart and Chapman 2006). The seven genes of the csg operons include a primary and nucleating amyloid monomer, CsgA and CsgB, respectively, a membrane pore (CsgG) and regulatory proteins (CsgC, CsgE, and CsgF). The combined results from culturing, biophysical and bioinformatic investigations of the fapA-F expression suggested parallels in FapA-F. A model sketching out a possible fibrillation machinery is provided in Figure 9.

FapA is potentially a chaperone for the amyloid monomers, while FapC and FapB are the main and nucleating amyloid monomers, respectively. The FapF β-barrel is a likely candidate for an outer membrane pore for FapB and FapC secretion to the extracellular environment. In keeping with this hypothesis, FapF is shown to be membrane associated in a proteome study of P. putida-Cd001 isolated from Arabidopsis halleri rhizosphere (Manara et al. 2012). FapD could have proteolytic activity relevant for processing the Fap proteins during protein secretion, potentially functioning in combination with FapF. Further operon similarities include the targeting of FapA-F for Sec-dependent secretion to the periplasm, equivalent to the secretion of CsgE-F and CsgA-C (Barnhart and Chapman 2006). In addition, the FapC and FapB repeat regions’ variation over the same motif of conserved Gln, Asn, and Ser residues is similar to that of CsgA. Phyre2 analysis indicated that repeats fold to multiple β-sheets in a strand-loop-strand-like motif also similar to the models proposed for the CsgA repeats in the mature fibril (Barnhart and Chapman 2006) (Fig. 9). The fap operons do not include a transcription factor equivalent to CsgD. However, organization of the fap genes into a single operon removes the need for an internal transcription factor.

Differences in FapA-F evolutionary divergence may support the functions suggested for Fap (compare Fig. 7B and C). Regulation of FuBA fibrillation, for example, to avoid cytotoxic intermediates, is a common feature across Pseudomonas strains, that is, regulatory proteins are subject to similar selection pressure. As an example, the function and outer membrane interaction of the FapF membrane is the same in the strains. Conversely, the extracellular fibril components are expected to be optimized for different habitats and growth conditions of the strains and thus subject to less stringent selection pressure. This hypothesis is further supported by the equivalent pattern observed for the Csg-DEFG fibrillation regulating proteins relative to the CsgBA amyloid fibril monomers (Fig. 7C).

Biological significance of the Fap operon

As fap is directly involved in Pseudomonas biofilm formation, Fap is potentially a virulence factor for P. aeruginosa. This is supported by the identification of a fapC deletion mutant of P. aeruginosa TBCF10839 as one of the most attenuated mutants, among 480 random transposon deletion mutants, in a Caenorhabditis elegans infection model and in a polymorphonuclear neutrophil leukocytes phagocytosis assay (Wiehlmann et al. 2007). Furthermore, investigations of the E. coli equivalent curli fibrils have also implicated these as a virulence factor (Collinson et al. 1992; Wang and Chapman 2008). In the case of P. fluorescens and P. putida, Fap fibrils are likely to be important in colonization of the rhizosphere. P. fluorescens forms biofilm-like colonies in grooves between plant root epidermal cells, while P. putida extracellular proteins are known to be important for adhesion to plant seeds in plant growth-promoting bacteria formulations (Espinosa-Urgel et al. 2000; Haas and Défago 2005). This is also consistent with P. putida biofilm, in general, having a high content of extracellular protein and FapF found in environmental rhizosphere samples (Jahn et al. 1999; Manara et al. 2012).

Based on the results of this study, we propose that Fap are extracellular biofilm components of equal importance to polysaccharides, other proteins, and eDNA. This
hypothesis is also in agreement with the widespread presence of FuBA in nature (Larsen et al. 2007, 2008; Jordal et al. 2009). We further suggest that the fap operon comprises a molecular machinery for the spatially and temporally regulated formation of FuBA.

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Conflict of Interest

None declared.

References

Allesen-Holm, M., K. B. Barken, L. Yang, M. Klausen, J. S. Webb, S. Kjelleberg, et al. 2006. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol. Microbiol. 59:1114–1128.

Alteri, C. J., J. Xicohténcatl-Cortes, S. Hess, G. Caballero-Olín, J. A. Giron, and R. L. Friedman. 2007. Mycobacterium tuberculosis produces pili during human infection. Proc. Natl. Acad. Sci. USA 104:5145–5150.

Attaway, H. H., and M. G. Schmidt. 2002. Tandem biodegradation of BTEX components by two Pseudomonas sp. Curr. Microbiol. 45:30–36.

Bao, Y., D. P. Lies, H. Fu, and G. P. Roberts. 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109:167–168.

Barnhart, M. M., and M. R. Chapman. 2006. Curli biogenesis and function. Annu. Rev. Microbiol. 60:131–147.

Choi, K.-H., and H. P. Schweizer. 2005. An improved method for rapid generation of unmarked Pseudomonas aeruginosa deletion mutants. BMC Microbiol. 5:20.

Claessen, D., R. Rink, W. de Jong, J. Siebring, P. de Vreugd, F. G. H. Boersma, et al. 2003. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in Streptomyces coelicolor by forming amyloid-like fibrils. Genes Dev. 17:1714–1726.

Collinson, S. K., L. Emödy, K. H. Müller, T. J. Trust, and W. W. Kay. 1991. Purification and characterization of thin, aggregative fimbriae from Salmonella enteritidis. J. Bacteriol. 173:4773–4781.

Collinson, S. K., L. Emödy, T. J. Trust, and W. W. Kay. 1992. Thin aggregative fimbriae from diarrheagenic Escherichia coli. J. Bacteriol. 174:4490–4495.

Donlan, R. M. 2001. Biofilms and device-associated infections. Emerg. Infect. Dis. 7:277–281.

Dueholm, M. S., S. V. Petersen, M. Sønderkær, P. Larsen, G. Christiansen, K. L. Hein, et al. 2010. Functional amyloid in Pseudomonas. Mol. Microbiol. 77:1009–1020.

Dueholm, M. S., S. B. Nielsen, K. L. Hein, P. Nissen, M. Chapman, G. Christiansen, et al. 2011. Fibrillation of the major curli subunit CsgA under a wide range of conditions implies a robust design of aggregation. Biochemistry 50:8281–8290.

Elliot, M., and N. Talbot. 2004. Building filaments in the air: aerial morphogenesis in bacteria and fungi. Curr. Opin. Microbiol. 7:594–601.

Epstein, E. A., and M. R. Chapman. 2008. Polymerizing the fibre between bacteria and host cells: the biogenesis of functional amyloid fibres. Cell. Microbiol. 10:1413–1420.

Espinosa-Urgel, M., A. Salido, and J. L. Ramos. 2000. Genetic analysis of functions involved in adhesion of Pseudomonas putida to seeds. J. Bacteriol. 182:2363–2369.

Gebbink, M. F. B. G., D. Claessen, B. Bouma, L. Dijkhuizen, and H. A. B. Wösten. 2005. Amyloids—a functional coat for microorganisms. Nat. Rev. Microbiol. 3:333–341.

Haas, D., and G. Defago. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3:307–319.

Harmsen, M., L. Yang, S. J. Pamp, and T. Tolker-Nielsen. 2010. An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. FEMS Immunol. Med. Microbiol. 59:253–268.

Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. Gene 212:77–86.

Hoiby, N. 2006. Pseudomonas aeruginosa in cystic fibrosis patients resists host defenses, antibiotics. Microbe 1: 571–577.

Jacobs, M. A., A. Alwood, I. Thaipsuttikul, D. Spencer, E. Haugen, S. Ernst, et al. 2003. Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA 100:14339–14344.

Jahn, A., T. Griebel, and P. H. Nielsen. 1999. Composition of Pseudomonas putida biofilms: accumulation of protein in the biofilm matrix. Biofouling 14:49–57.

Jordal, P. B., M. S. Dueholm, P. Larsen, S. V. Petersen, J. J. Enghild, G. Christiansen, et al. 2009. Widespread abundance of functional bacterial amyloid in mycolata and other Gram-positive bacteria. Appl. Environ. Microbiol. 75:4101–4110.

Kessler, B., V. de Lorenzo, and K. N. Timmis. 1992. A general system to integrate lacZ fusions into the chromosomes of Gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. 233:293–301.
Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jørgensen, S. Molin, et al. 2003. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol. Microbiol. 48:1511–1524.

Klausen, M., M. Gjermansen, J.-U. Kreft, and T. Tolker-Nielsen. 2006. Dynamics of development and dispersal in sessile microbial communities: examples from Pseudomonas aeruginosa and Pseudomonas putida model biofilms. FEMS Microbiol. Lett. 261:1–11.

Klockgether, J., A. Munder, J. Neugebauer, C. F. Davenport, Koch, B., L. E. Jensen, and O. Nybroe. 2001. A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods 45:187–195.

Krishnan, R., and S. L. Lindquist. 2005. Structural insights into a yeast prion illuminate nucleation and strain diversity. Nature 435:765–772.

Larsen, P., J. L. Nielsen, M. S. Dueholm, R. Wetzel, D. Otzen, and P. H. Nielsen. 2007. Amyloid adhesins are abundant in natural biofilms. Environ. Microbiol. 9:3077–3090.

Larsen, P., J. L. Nielsen, D. Otzen, and P. H. Nielsen. 2008. Amyloid-like adhesins produced by floe-forming and filamentous bacteria in activated sludge. Appl. Environ. Microbiol. 74:1517–1526.

Lewenza, S., J. L. Gardy, F. S. L. Brinkman, and R. E. W. Hancock. 2005. Genome-wide identification of Pseudomonas aeruginosa exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen. Genome Res. 15:321–329.

López De La Paz, M., K. Goldie, J. Zurdo, E. Lacroix, C. M. Dobson, A. Hoenger, et al. 2002. De novo designed peptide-based amyloid fibrils. Proc. Natl. Acad. Sci. USA 99:16052–16057.

Ma, L., M. Conover, H. Lu, M. R. Parsek, K. Bayles, and D. J. Wozniak. 2009. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. PLoS Pathog. 5: e1000354. doi: 10.1371/journal.ppat.1000354.

Manara, A., G. DalCorso, C. Ballardini, S. Farinati, D. Cecconi, and A. Furini. 2012. Pseudomonas putida response to cadmium: changes in membrane and cytosolic proteomes. J. Proteome Res. 11:4169–4179.

Matsukawa, M., and E. P. Greenberg. 2004. Putative exopolysaccharide synthesis genes influence Pseudomonas aeruginosa biofilm development. J. Bacteriol. 186: 4449–4456.

Morales, V. M., A. Bäckman, and M. Bagdasarian. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97:39–47.

Nelson, R., M. R. Sawaya, M. Balbirnie, A. Ø. Madsen, C. Rickel, R. Grotte, et al. 2005. Structure of the cross-β spine of amyloid-like fibrils. Nature 435:773–778.

Nielsen, P. H., M. S. Dueholm, T. R. Thomsen, J. L. Nielsen, and D. E. Otzen. 2011. Functional bacterial amyloids in biofilms. Pp. 41–62 in H.-C. Flemming, U. Szwazyk and J. Wingender, eds. Biofilm highlights, 5th ed. Springer-Verlag, Berlin Heidelberg.

Oh, J., J.-G. Kim, E. Jeon, C.-H. Yoo, J. S. Moon, S. Rhee, et al. 2007. Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. J. Biol. Chem. 282:13601–13609.

Olsen, A., A. Jonsson, and S. Normark. 1989. Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. Nature 338:652–655.

O’Neillain, B., and R. Wetzel. 2002. Conformational Abs recognizing a generic amyloid fibril epitope. Proc. Natl. Acad. Sci. USA 99:1485–1490.

O’Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol. Microbiol. 30:295–304.

Otzen, D. E. 2011. Assembling good amyloid: some structures at last. Structure 19:1207–1209.

Pamp, S. J., M. Gjermansen, and T. Tolker-Nielsen. 2007. The biofilm matrix – a sticky framework. Pp. 37–69 in S. Kjelleberg and M. Givskov, eds. The biofilm mode of life. Horizon Bioscience, Norfolk.

Pamp, S. J., M. Gjermansen, H. K. Johansen, and T. Tolker-Nielsen. 2008. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol. Microbiol. 68:223–240.

Parales, R. E., J. L. Ditty, and C. S. Harwood. 2000. Toluene-degrading bacteria are chemotactic towards the environmental pollutants benzene, toluene, and trichloroethylene. Appl. Environ. Microbiol. 66:4098–4104.

Perkins, D. N., D. J. Pappin, D. M. Creasy, and J. S. Cottrell. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551–3567.

Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8:785–786.

Rainey, P. B., and M. Travisano. 1998. Adaptive radiation in a heterogeneous environment. Nature 394:69–72.

Romero, D., C. Aguilar, R. Losick, and R. Kolter. 2010. Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. Proc. Natl. Acad. Sci. USA 107:2230–2234.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68:850–858.

Shewmaker, F., R. P. McGlinchey, and R. B. Wickner. 2011. Structural insights into functional and pathological amyloid. J. Biol. Chem. 286:16533–16540.

Simon, R., U. Priefert, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat. Biotechnol. 1:784–791.
Sternberg, C., and T. Tolker-Nielsen. 2006. Growing and analyzing biofilms in flow cells. Curr. Protoc. Microbiol. John Wiley & Sons, New York. 1B.2.1–1B.2.15.

Taylor, J. D., Y. Zhou, P. S. Salgado, A. Patwardhan, M. McGuffie, T. Pape, et al. 2011. Atomic resolution insights into curli fiber biogenesis. Structure 19:1307–1316.

Tycko, R. 2004. Progress towards a molecular-level structural understanding of amyloid fibrils. Curr. Opin. Struct. Biol. 14:96–103.

Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. J. Bacteriol. 180:2442–2449.

Wang, X., and M. Chapman. 2008. Sequence determinants of bacterial amyloid formation. J. Mol. Biol. 380:570–580.

Wessels, J., O. De Vries, S. A. Asgeirsdottir, and F. Schuren. 1991. Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in Schizophyllum. Plant Cell 3: 793–799.

Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. Science 295:1487.

White, A. P., S. K. Collinson, P. A. Banser, D. L. Gibson, M. Paetzel, N. C. Strynadka, et al. 2001. Structure and characterization of AgfB from Salmonella enteritidis thin aggregative fimbriae. J. Mol. Biol. 311:735–749.

Wiehlmann, L., A. Munder, T. Adams, M. Juhas, H. Kolmar, P. Salunkhe, et al. 2007. Functional genomics of Pseudomonas aeruginosa to identify habitat-specific determinants of pathogenicity. Int. J. Med. Microbiol. 297:615–623.

Zandomeneghi, G., M. R. H. Krebs, M. G. McCammon, and M. Fändrich. 2004. FTIR reveals structural differences between native β-sheet proteins and amyloid fibrils. Protein Sci. 13:3314–3321.