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For Publication

Biofilm formation and cellulose expression by *Bordetella avium* 197N, the causative agent of bordetellosis in birds and an opportunistic respiratory pathogen in humans

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Abstract

Although bacterial cellulose synthase (*bcs*) operons are widespread within the *Proteobacteria* phylum, subunits required for the partial-acetylation of the polymer appear to be restricted to a few γ-group soil, plant-associated and phytopathogenic pseudomonads, including *Pseudomonas fluorescens* SBW25 and several *Pseudomonas syringae* pathovars. However, a *bcs* operon with acetylation subunits has also been annotated in the unrelated β-group respiratory pathogen, *Bordetella avium* 197N. Our comparison of subunit protein sequences and GC content analyses confirms the close similarity between the *B. avium* 197N and pseudomonad operons and suggests that, in both cases, the cellulose synthase and acetylation subunits were acquired as a single unit. Using static liquid microcosms, we can confirm that *B. avium* 197N expresses low levels of cellulose in air-liquid interface biofilms and that biofilm strength and attachment levels could be increased by elevating c-*di*-GMP levels like the pseudomonads, but cellulose was not required for biofilm formation itself. The finding that *B. avium* 197N is capable of producing cellulose from a highly-conserved, but relatively uncommon *bcs* operon raises the question of what functional role this modified polymer plays during the infection of the upper respiratory tract or survival between hosts, and what environmental signals control its production.

Keywords: Air-liquid interface; Biofilm; *Bordetella avium*;c-*di*-GMP; Cellulose; Microcosm

1. Introduction

Bordetella avium is the causative agent of bordetellosis (tracheobronchitis), a highly contagious upper respiratory disease of domesticated and wild birds [1 - 3], as well an opportunistic human pathogen that may also be associated with cystic fibrosis [4 - 6]. It is phylogenetically distinct from *B. bronchiseptica*, *B. parapertussis,* and *B. pertussis,* which are respiratory pathogens of mammals, though *B. avium* expresses similar virulence factors and also produces biofilms. However, whilst *Bordetella* polysaccharide (Bps) has been identified as the primary matrix component in *B. bronchiseptica* RB50 and *B. pertussis* 536 biofilms [7, 8], it has not been reported for *B. avium* biofilms, nor has a *bps*-like operon been found in the genome of the virulent turkey isolate *B. avium* 197N [9 - 11]. This raises the question of what extracellular polymeric substance (EPS) or substances are used as the biofilm matrix during host infection or colonisation of other environments during transmission between hosts.

We have noted that the *B. avium* 197N genome [11] has been annotated with a potential bacterial cellulose synthase (*bcs*) operon (genes BAV2632 – 2623) (Fig. 1). *Bcs* operons have been identified in the genomes of a wide range of *Proteobacteria*, but cellulose expression per se has been reported for relatively few strains, including human gastrointestinal commensals and pathogens, and soil and plant-associated pseudomonads [12 - 14]. Although the functional role and fitness value of cellulose is poorly understood [13 - 15], it is associated with biofilm formation in the intestinal epithelium and invasion of epithelial cells and macrophages [14], and provides a fitness advantage in soils and plant environments where it may help reduce water stress [16 - 19]. More generally, cellulose may promote survival in natural environments during transmission between hosts [12].

We are interested in determining whether *B. avium* 197N is capable of expressing cellulose as part of a greater understanding of the functional role of cellulose in a range of environments and life strategies. Unusually, the *B. avium* 197N *bcs* operon subunits show close amino acid sequence homology with those found in pseudomonads, particularly the soil and plant-associated *P. fluorescens* SBW25 (annotated as *wssA-J*, PFLU0300 – 0309 [20], Fig. 1), despite the fact that the *Bordetellae* and *Pseudomonas* are distantly related genera.

The *B. avium* 197N and *P.fluorescens* SBW25 *bcs* operons are further linked by the inclusion of cellulose acetylation-associated subunits originally described for *P.fluorescens* SBW25 (WssF-I), which affects colony morphology and air-liquid (A-L) interface biofilms that develop in static microcosms [20, 21]. These subunits are rarely seen, but are present in a number of plant pathogens, including *P. syringae* pv. *tomato* DC3000, but not, for example, the closely-related soil-associated *P. putida* KT2440 [13, 21, 22] (Fig. 1). Cellulose is also expressed by *P.putida* KT2400 and *P.syringae* DC3000 in similar conditions [19, 23], and static microcosms have been used to investigate biofilm-formation more widely amongst the pseudomonads [23, 24] where biofilms can be quantitatively measured by combined growth, strength and attachment assays [24, 25].

B. avium 197N has also been reported to produce limited biofilms at the meniscus of static liquids [26, 27]. We therefore decided to use a static microcosm approach to characterise *B. avium* 197N biofilm formation more fully under a range of growth conditions. Although low levels of cellulose expression was observed in wild-type *B. avium* 197N biofilm samples and colonies, we constructed a cellulose-deficient (CD) mutant to demonstrate that cellulose was not an essential matrix component for biofilm-formation, suggesting that *B. avium* 197N utilises another but unidentified EPS for this purpose.

2. Materials and methods

2.1 .Bioinformatics

Bacterial cellulose synthase subunit homologues were identified in *Proteobacteria* complete genomes using NCBI TBLASTN and *P.fluorescens* SBW25 WssA – J protein query sequences (Accession numbers AAL71841 – AAL71850) following the selection criteria described in the Supplementary Methods. A WspR homologue was identified in the *B. avium* 197N genome using *P.fluorescens* SBW25 WspR (AAL71852). Phylogenetic trees were produced using the NCBI COBALT multiple sequence alignment tool and the Neighbour Joining (NJ) method (see Suppl. Methods). Gene and whole genomic GC content data were obtained from the *Pseudomonas* Genome Database (www.pseudomonas.com) and NCBI Genbank. DNA dot plots were produced using the YASS Genomic Similarity Search Tool (bioinfo.lifl.fr).

2.2. Bacteria and plasmids

Bacteria and plasmids used in this work are listed in Table 1. All strains were kept at -80°C for long-term storage, and stocks produced by adding 15% (v/v) glycerol to over-night cultures. *B. avium* 197N was acquired directly from the American Type Culture Collection (ATCC BAA-1003). Antibiotic susceptibility was assessed using antibiotic disks (MAST, UK). Kanamycin (Km) sensitivity [10] was confirmed using LB plates containing 100 $\mu g.ml^{-1}$ Km incubated at 28°C for 48 hr.

2.3. Culturing conditions

Bacteria were cultured at 20, 28, 37, and 42°C using Brain Heart Infusion (BHI; Oxoid, UK), King's B (KB; 20 g proteose peptone No. 3 (BD Biosciences, UK), 10 g glycerol, 5 g K_2HPO_4, 1.5 g $MgSO_4$ per litre) and Luria-Bertani (LB; 10 g NaCl, 10 g

tryptone (Oxoid), and 5 g yeast extract (Merck, UK) per litre) media with 1.5% (w/v) Agar Technical (Oxoid) for plates and antibiotics added as required. Overnight shaken cultures were used to provide inocula for experiments. Microcosms were 30 ml glass tubes containing 6 ml growth media [23]. Swimming motility was assessed using soft-agar LB plates (see Suppl. Methods).

2.4. Biofilm formation in static microcosms

Microcosms inoculated with 100 µl aliquots of culture and incubated statically were inspected daily for up to 5 days for signs of A-L interface biofilm-formation, and then by pouring out into Petri dishes to assess strength and attachment [23]. Biofilms were quantitatively characterised using the combined biofilm assay in which replicate microcosms (n = 8) were tested for biofilm strength (grams) using small glass balls, biofilm attachment to the tube walls by staining with crystal violet (A_{570}) and total growth by OD_{600} measurements [24, 25]. These assays were conducted for a number of strains, media and temperatures, and data sets analysed using a General Linear Model (GLM) approach.

2.5. Cellulose expression

Cellulose was assessed using Congo red plates and by fluorescent microscopy. Congo red plates were BHI, KB, and LB (without NaCl) plus 0.001% (w/v) Congo red [21], and were drop-inoculated with 5 µl aliquots of culture and incubated for 2 – 3 days. Images were taken using a Nikon D3200 DSLR camera. Samples of colonies and biofilm material were stained with 10 $\mu g.ml^{-1}$ calcofluor for 1 h before viewing at 10x – 40x magnification using a Leica DMR fluorescent microscope and imaging with an AxioCam MRc digital camera [21, 28]. Semi-purified biofilm matrix samples were investigated by ELISA using a cellulose binding domain–containing (SPA-CBD) fusion protein [29] as described in the

Suppl. methods. Biofilm samples were also investigated by Fourier transform infrared spectroscopy (FTIR) and visualised by scanning electron microscopy (SEM) (see Suppl. methods).

2.6. Construction of pAS296 and the cellulose-deficient (CD) mutant

The suicide plasmid pAS296 was designed to disrupt *bcs* operons by homologous recombination of a *P.fluorescens* SBW25 mini-transposon *wssB*::IS-Ω-Km/hah (Km^R) cassette (the insertion is located 1,693 bp from the start of the 2,219 bp *wssB* gene). This was integrated into the *B. avium* 197N chromosome following kanamycin selection to produce the cellulose-deficient (CD) mutant (see Suppl. methods).

2.7. Statistical analyses and modelling

Quantitative assays were undertaken with replicates, and means with standard errors (SE) are shown where appropriate. Data were analysed using JMP v12 (SAS Institute Inc., USA) statistical software. A general linear model (GLM) approach was used to investigate the significance of strain, test media, growth and attachment (effects) on biofilm strength (response) using the combined biofilm assay dataset. Attachment levels were also modelled as response. Significant effects were examined by LSMeans Differences Student's t-test and Tukey HSD tests, and associations examined by pairwise correlations. Differences between means were also tested by ANOVA, t-tests and Turkey-Kramer HSD tests.

3. Results and discussion

3.1. Bacterial cellulose synthase (bcs) operons within the Proteobacteria.

We identified over 100 bacterial whole genome sequences likely to contain fully functional *bcs* operons based on amino acid sequence homologies to the core cellulose synthase subunits of *P.fluorescens* SBW25 (Fig. 1 and data not shown), and it is noteworthy that such a poorly reported or tested phenotype such as the ability to express cellulose is so commonly annotated amongst the *Proteobacteria*. Within this phylum, species from one α-group, two β-group and ten γ-group genera were found to have convincing *bcs* operons (see Suppl. Table S1). As expected, the organisation of these operons, in terms of gene order and orientation, was highly variable [14], though phylogenetic-based clustering at the genus or family levels was seen in Neighbour-Joining (NJ) trees constructed using core subunit sequences. An example of one NJ tree generated using WssB homologues (see Suppl. Table S2) and rooted using α-group *Proteobacteria* is given in Fig. 2. The phylogenetic-based clustering of homologues seen here suggests that most sequence variation in core subunits is probably acquired through vertical transmission. However, the close branching of distantly related genera such as the *Bordetellae* and *Pseudomonas* in these trees suggests that *bcs* operons may have been horizontally transmitted at an early stage in the radiation of the *Proteobacteria*.

We note the increasing number of plant pathogens including *Burkholderia* (β-group), *Dickeya*, and *Pseudomonas* (γ-group) spp., and animal pathogens including *Bordetella* (β-group), *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella* (γ-group) spp., encode *bcs* operons and are likely to express cellulose (Suppl. Table S1). Although these pathogens have diverse hosts and survive in a wide range of environments, cellulose might play a common role in single cell attachment, microcolony and biofilm formation, and protection against predation, disturbance or stress [13, 14].

3.2. The bcs *operon in* B. avium *197N*

Following our survey of *bcs* operons within the *Proteobacteria*, we shifted our attention to the operon annotated in the *B. avium* 197N genome. The link between this avian and opportunistic human respiratory pathogen [9 - 11] and the soil- and plant-associated pseudomonads is intriguing, because of the high levels of *bcs* subunit homology with *P.fluorescens* SBW25 and the presence of the rarely seen cellulose acetylation-associated subunits apparently restricted to *P.fluorescens* SBW25 and a number of related plant pathogens, including *P.syringae* DC3000 [21, 22].

The *B. avium* 197N *bcs* operon is also notable, as in NJ trees, the WssBCDE homologues from *B. avium* 197N, *P.fluorescens* SBW25 and *P.syringae* DC3000 were always clustered together, but distant from the *P.putida KT2400* homologues (see also [22]). A representative NJ tree for WssB homologues is shown in Fig. 2 and a comparison of WssA – I homologues provided in Table 2.

However, the *B. avium* 197N *bcs* operon differs significantly from the *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 operons by the duplication of WssC (annotated as WssC1 and WssC2), which challenges our presumption that this bacterium could express cellulose. Although the duplication is apparent in an alignment of the *B. avium* 197N and *P.fluorescens* SBW25 genomic sequences, WssC1 and WssC2 are not direct copies of one another and share only 47% identity at the protein level (97% coverage, 0.0 E value) (see Suppl. Fig. S1). Furthermore, WssC1 and WssC2 branch separately in a NJ tree containing *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 WssC homologues (Suppl. Fig. S1). The alignment of *B. avium* 197N and *P.fluorescens* SBW25 sequences also indicates that the two genomes do not share significant levels of homology beyond the boundaries of the *bcs* operons, suggesting that one or the other acquired the operon by a limited lateral gene transfer event.

A comparison of the GC content of the *bcs* genes indicates that the *B. avium* 197N, *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 operons are more similar to one another than they are to the *P.putida KT2400* operon ($\alpha = 0.05$) (Fig. 3). The mean GC contents of the *bcs* genes of *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 were also significantly different from their whole genome GC content ($p \leq 0.0003$), suggesting that these bacteria had acquired the *bcs* operons relatively recently and before much amelioration of divergent sequences could occur. In contrast, no significant difference was observed between the *B. avium* 197N *bcs* genes and whole genome GC content ($p = 0.2033$), suggesting that the transfer event occurred much earlier in this bacterium or that the ancestral *bcs* donor was more similar to the *Bordetellae* than to the pseudomonads. As the GC content of intergenic regions and coding sequences are frequently correlated in bacterial genomes [30], a direct comparison between the GC content of the *bcs* genes with the GC content of all other chromosomally encoded genes is unlikely to produce a substantially different result. However, we have not yet investigated the regions surrounding each of the *bcs* operons, which may show significant differences from the rest of the chromosomes.

Finally, no significant differences were observed between the GC content of core synthase and acetylation-associated genes within operons ($p \geq 0.5085$), and no significant correlations found between GC content and gene order ($p > 0.05$). This suggests that the acetylation-associated genes had been transferred and maintained with the core synthase genes, rather than as subsequent and independently acquired functions.

3.3. B. avium *197N biofilm formation in static microcosms*

Given the close protein homologies and conserved operon structures seen between the *B. avium* 197N *bcs* operon and the *P.fluorescens* SBW25, *P.putida KT2400* and

P.syringae DC3000 *bcs* operons, we were interested in determining whether *B. avium* 197N expressed cellulose despite the WssC duplication, and whether it also played a role in A-L interface biofilm formation, as it does for some pseudomonads. As *B. avium* 197N has been reported to form limited biofilms at the meniscus of static liquids [26, 27], we decided to use the static microcosm approach we had previously developed to investigate A-L interface biofilm formation by *P.fluorescens* SBW25 and other pseudomonads [13, 23, 24]. In these, competition for O_2 which is otherwise growth-limiting in the liquid column drives biofilm formation at the liquid surface [31], even in the absence of host-derived factors or environmental signals that might normally regulate such activity, including the expression of biofilm-associated EPS.

In order to determine whether *B. avium* 197N could form biofilms in static microcosms, a range of media and incubation temperatures (BHI, KB and LB; 20 – 42°C) were tested, as although *B. avium* 197N is routinely grown in BHI at 37°C, phenotypic differences have been noted under other conditions [9, 10, 32, 33]. We found clear visual evidence of growth (i.e. culture turbidity) and A-L interface biofilm formation, including culture turbidity, films extending across the A-L interface and sunken debris within three days of incubation under all conditions tested (Fig. 4). The biofilms formed in KB microcosms were particularly obvious and robust, and were characterised as physically cohesive (PC)-class / air-liquid-solid surface (A-L-S) interface-type biofilms [23, 24]. In contrast, BHI biofilms were almost transparent with white flecks of material, and LB biofilms were very thin and fragile.

We recovered biofilm material from microcosms and spread it onto plates to investigate colony morphologies. In *P.fluorescens* SBW25 populations, radiation (i.e. mutation) results in significant numbers of biofilm-forming “wrinkly spreaders” (WSs) identifiable by an altered colony morphology [34]. However, only wild-type colony

morphologies were observed from 3 day-old *B. avium* 197N biofilm samples, and no other signs of radiation were seen in KB microcosms incubated for up to 15 days (e.g. in siderophore production, colouration, motility, etc.). This suggests that the biofilm formation we observed in experimental static microcosms is the result of a normal physiological response by *B. avium* 197N, rather than the activity of biofilm-producing mutants. Further testing using modified media containing avian tissue-derived signal compounds, cell cultures, tracheal rings or lung tissue will be required to determine whether biofilm formation is a behaviour more specifically associated with host infection or the colonisation of water, plants, and soils during transmission between hosts.

3.4. Quantitative characterisation of the B. avium *197N biofilm*

In order to place *B. avium* 197N A-L interface biofilm formation in static microcosms into context, we compared *B. avium* 197N with wild-type *P.fluorescens* SBW25 and the *P.fluorescens* SBW25 WS mutant using a range of media and a quantitative combined biofilm assay measuring microcosm growth, biofilm strength and attachment levels [24]. Wild-type *P.fluorescens* SBW25 was included, as it produces a poorly attached, fragile biofilm referred to as the VM biofilm [35], whilst the WS produces a robust, well-attached biofilm in KB microcosms [20, 21, 28, 34].

We modelled biofilm strength using a GLM approach, and both strain and medium were found to be significant ($p \leq 0.0001$, see Suppl. Table S3, Model 1); representative data for KB microcosms are shown in Fig. 5. Although the VM and WS biofilms were significantly different from one another, the *B. avium* 197N biofilms were of intermediate strength (Fig. 5B) and could not be differentiated from either the VM or WS biofilms ($\alpha = 0.05$). The strongest *B. avium* 197N biofilms were produced in KB microcosms, and across all three strains, KB biofilms could be differentiated from BHI and LB biofilms ($\alpha = 0.05$)

which suggests that biofilm formation was influenced by media composition. Overall growth and biofilm attachment levels were not found to have significant effects on biofilm strength ($p > 0.05$) across the three strains tested in these microcosms. However, in previous work, the VM biofilm has been shown to have significantly lower attachment levels than the WS biofilm [35]. Finally, we also modelled biofilm attachment levels, as biomass at the meniscus is often used as a measure of biofilm growth in microtitre plate-based assays [36]. In this, the strain effect was found to be weakly insignificant ($p = 0.0501$, Suppl. Table S3, Model 2), with the *B. avium* 197N biofilm differentiated from the VM biofilm, but neither from the WS biofilm ($\alpha = 0.05$).

3.5. Cellulose expression by B. avium *197N*

We used the cellulose-binding dye calcofluor and fluorescent microscopy to demonstrate that cellulose fibres were present in *B. avium* 197N colonies and biofilm samples (Fig. 6A). However, relatively little cellulose was observed compared to that reported for VM and WS biofilms [21, 35], and none was observed in samples from a cellulose-deficient (CD) mutant we produced by disrupting the *B. avium* 197N *bcs* operon with a *P.fluorescens* SBW25 *wssB*::IS-**Ω**-Km/hah cassette. Additional evidence to support the presence of cellulose at low levels in biofilms was obtained by ELISA using a cellulose binding-domain–containing (SPA-CBD) fusion protein [29]. This showed a 2.5x increase in cellulose levels in semi-purified biofilm matrix samples samples compared to the CD mutant, though this assay was unreplicated and may need further testing. However, cellulose levels were insufficient for detection by FTIR (see Suppl. Fig. S2). Despite this, significant differences in biofilm strength and attachment levels were found between the *B. avium* 197N and CD mutant biofilms ($\alpha = 0.05$) (Fig. 5). These changes may not necessarily be due to the loss of cellulose in the biofilm per se, but might result from the

loss of the cellulose synthase complex which effects other regulatory systems involved in the expression of biofilm matrix components and attachment factors.

We suggest that the low levels of cellulose seen in *B. avium* 197N biofilms produced under the conditions tested here may be the result of either the unusual *wssC* duplication, which might reduce *wss* operon transcription or cellulose synthase complex assembly, or the result of leaky transcriptional or synthase regulation in the absence of the normal signals required to express cellulose. It is possible that host-specific factors are required to induce cellulose expression to higher levels during invasion of avian tissues, or that other environmental signals are required for expression during transmission between hosts for survival in water, soils or plants. Future analysis will require transcriptional analyses of the *wss* operon, plus quantitative measurements of cellulose production, to better understand how and when cellulose production is controlled by this bacterium.

It remains unclear what EPS is being utilised by *B. avium* 197N as the primary biofilm matrix in these microcosms, as *B. avium* 197N lacks a *bps*-like operon required to express the Bps polymer produced by other *Bordetellae* [7, 8, 11]. SEM imaging of wild-type *B. avium* 197N and CD mutant biofilm samples appear very similar (Fig. 6B) and are like those produced by *B. bronchiseptica* RB50 and *B. pertussis* Bp536, for which Bps is required for biofilm formation [26, 27]. *B. avium* 197N biofilms may utilise a similar polysaccharide, eDNA or a proteinaceous attachment factor or adhesin as the primary biofilm matrix component, but further biochemical and genetic analyses will be required to identify the nature of this EPS.

3.6. Involvement of ci-di-GMP in B. avium 197N *in colony morphology and biofilms*

Biofilm formation and cellulose expression in *P.fluorescens* SBW25 is primarily controlled by c-*di*-GMP levels regulated by WspR and other diguanylate cyclases (DGCs)

[34]. DGCs are found in other pseudomonads and *Bordetellae*, and we have noted a close homologue in *B. avium* 197N (WP_012416712; 336 aa, with 63% ID and an E value of 1e-149). In order to determine whether c-*di*-GMP levels may also play a role in *B. avium* 197N biofilm formation or cellulose expression, we tested the effect of overexpression of a constitutively active and dominant WspR19 mutant that induces a phenotype indistinguishable from the WS in *P.fluorescens* SBW25 [28, 37]. As WspR19 is functional in other pseudomonads and in more distantly related bacteria such as *Caulobacter crescentus* CB15 [23, 38, 39], it might also have an impact on the phenotype of *B. avium* 197N regardless of the activity of WP_012416712.

We first tested whether WspR19 affected *B. avium* 197N swimming motility [10], as the expression of a similar DGC in *B. bronchiseptica* 9.73 was found to repress swimming motility through the elevation of c-*di*-GMP levels [40]. WspR19 was found to partially repress swimming to 11 – 18%% of the levels shown by *B. avium* 197N carrying the control plasmid ($p \leq 0.0001$). We then investigated whether WspR and WspR mutants affected colony morphology, including the uptake of Congo red stain used to assess cellulose and attachment factor expression [20, 21, 28, 35]. WspR19 was found to have a consistent impact on *B. avium* 197N colony morphology, resulting in larger and slightly rougher colonies with higher levels of Congo red staining compared to wild-type *B. avium* 197N and the CD mutant (Fig. 6C). In comparison, *B. avium* 197N colonies expressing the wild-type WspR protein (WspR12) [28, 41] were only slightly stained by Congo red, and colonies expressing the dominant-negative WspR9 mutant [41] were indistinguishable from *B. avium* 197N, the CD mutant and the plasmid control colonies. Although more cellulose could be observed by fluorescent microscopy in colony material produced by *B. avium* 197N expressing WspR19 compared to either *B. avium* 197N with the control plasmid or the CD mutant (data not shown), it is unclear whether the changes in colony

366 morphology are the result of minor increases in cellulose levels or changes in other EPS or
367 attachment factors which also bind Congo red.

368 The expression of WspR mutants in *B. avium* 197N also affected biofilm strength,
369 with strain, medium and growth all found to have significant effects ($p \leq 0.0001$, Suppl.
370 Table S3, Model 3). In particular, strains could be differentiated into three groups, with B.
371 avium 197N carrying the control plasmid or expressing WspR9 having the least effect on
372 strength, WspR12 having an intermediate effect, and WspR19 having the greatest effect
373 on strength ($\alpha = 0.05$). The strongest biofilms were produced in KB microcosms by *B.*
374 *avium* 197N expressing WspR19 which were significantly stronger than the plasmid control
375 (2.5 x, $p = 0.0353$), though growth was significantly reduced (0.6 x, $p = 0.0002$). This
376 suggests that WspR19 has a negative pleiotropic effect when expressed in *B. avium* 197N,
377 as it does in *P.fluorescens* SBW25 [37]. Although the strain was weakly insignificant ($p <$
378 0.0727) when attachment was modelled (Suppl. Table S3, Model 4), it seems likely that
379 biofilm formation by *B. avium* 197N in static microcosms under the conditions tested here
380 is regulated to some degree by c-*di*-GMP levels.

381 In conclusion, bacterial cellulose synthase *bcs* operons are common amongst the
382 *Proteobacteria*, though acetylation-associated genes are rare and have only been
383 identified in a select group of pseudomonads and a few other species including *B. avium*
384 197N. In this work, we confirm that *B. avium* 197N is able to express cellulose at low
385 levels, though it was not necessary for the formation of A-L interface biofilms in static
386 microcosms, and further investigation is required to identify the primary matrix components
387 utilised by *B. avium* 197N under the conditions tested here and to identify the
388 environmental signals and regulatory mechanisms which control expression under natural
389 conditions. Although cellulose might be used during infection of the upper respiratory
390 tract of hosts, it may also be important for survival between hosts in water, soil or on plant

surfaces, and this might explain why *bcs* operons are carried by environmental bacteria as well as pathogenic and commensal intestinal bacteria.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Author's contributions

KMcL and AOF contributed equally to this work as first co-authors. KMcL, OM and AJS designed the experiments. AOF, KZ, DF and AJS carried out the bioinformatics analyses. KMcL, CI, AK, IUM, OM and SR carried out the experiments. KMcL, SMH and AJS analysed the data. AJS and OM prepared the manuscript with comments from all authors. AJS, YD and OM were responsible for the management of this project, with AJS leading.

Supplementary material

Methods: Construction of pAS296 and the cellulose-deficient (CD) mutant; Fourier transform infrared spectroscopy (FTIR); plasmid isolation, electroporation and strain manipulation; scanning electron microscopy (SEM); and swimming motility.

Fig. S1. The *B. avium* 197N *bcs* operon shows DNA level similarities with those found in key pseudomonads.

Fig. S2. FTIR does not differentiate between wild-type *B. avium* 197N and cellulose-deficient (CD) mutant biofilm samples.

Table S1. *Proteobacteria* having convincing bacterial cellulose synthase (*bsc*) operons.

Table S2. Representative *Proteobacteria* having WssB homologues.

Table S3. General linear modelling of biofilm strength and attachment levels.

References

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Figure Legends

Fig. 1. **Bacterial cellulose synthase (*bcs*) operon structures.** *Bcs* operons have been identified in the whole-genome sequences of a wide range of bacteria. In *P.fluorescens* SBW25 the genes are annotated as *wssA – J* and are predicted to form the core cellulose synthase complex (dark blue) including an endoglucanase (light blue) and associated acetylation subunits (green), and to be involved in the localization of the complex at the periplasmic membrane (mauve) (top section). Shown also are the *bc*s operons of *P. putida* KT2400, *P.syringae* DC3000 and *B. avium* 197N, which also contains a duplication of a truncated version of *wssC* (blue stripes). The scale bar indicates 1 kb.

Fig. 2. **Phylogenetic analyses of representative WssB homologues.** Shown here is a phylogenetic tree showing the relationships between WssB proteins from the key strains *B. avium* 197N, *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 (bold), as well as a number of other bacteria in which *wss*-like *bcs* operons have been identified. A number of *Komagataeibacter* spp. sequences were used to root the tree (indicated by the black square), and these α-group sequences represented by *Gluconacetobacter xylinus* E25 (light blue) were found to cluster separately from the other β and γ-group sequences. Most

γ-group *Pseudomonas* spp. sequences clustered together (light green). Although the β-group sequences root at the base of the *Pseudomonas* spp. cluster, the *Bordetella* spp. sequences including *B. avium* 197N (purple) clustered separately from the *Burkholderia* spp. (light orange). This NJ tree is based on COBALT multiple alignments of the coding sequences listed in Table 1. Some sequences have not been labelled in the tree for clarity. The distance bar indicates 0.1 units.

Fig. 3. **GC content of the *bcs* operons.** An analysis of the *bcs* operons from *B. avium* 197N, *P.fluorescens* SBW25, *P.putida KT2400* and *P.syringae* DC3000 reveals substantial variation in gene GC content within operons. The GC contents of individual genes (grey circles), the mean with SE bars for each operon (squares) and each genome (triangles) are shown. See the main results section for statistical comparisons.

Fig.4. ***B. avium* 197N forms biofilms at the air-liquid (A-L) interface of static microcosms**. Shown are representative images of *B. avium* 197N biofilms *in* situ in BHI, KB and LB microcosms (A – C, top row) and of biofilm material after pouring into petri dishes (bottom row). Biofilms become progressively more robust with longer incubation periods, but tend to detach from the meniscus region and sink to the bottom of the vials. Microcosms were incubated at 20°C for three days before imaging.

Fig. 5. **The *B. avium* 197N biofilm is intermediate in strength but with high levels of attachment.** Biofilm formation in static microcosms can be quantified by

using a combined biofilm assay in which microcosm growth (A), biofilm strength (B) and attachment levels (C) are determined. Shown here are data for *P.fluorescens* SBW25 which produces the VM biofilm (white bars), the WS which produces the WS biofilm (black), *B. avium* 197N (indicated by 'Wt' for wild-type, dark grey), and the *B. avium* 197N cellulose-deficient (CD) mutant (light grey) incubated in KB microcosms at 20°C for three days before assay. Means and SE are shown. Significant differences between means were observed for growth ($p < 0.0001$), strength ($p < 0.0001$) and attachment ($p = 0.0005$); means within assays not connected by the same letter are significantly different ($\alpha = 0.05$).

Fig. 6. **Cellulose is expressed by *B. avium* 197N but is not essential for biofilms.** Cellulose fibres forming the matrix of the *P.fluorescens* SBW25 WS mutant are readily visualised by fluorescent microscopy after staining with calcofluor (A). In comparison, only low levels of cellulose expression can be detected in *B. avium* 197N biofilm samples and none in the cellulose-deficient (CD) mutant even at higher magnification. Qualitative comparison of scanning electron microscope images of *B. avium* 197N and CD mutant biofilm samples show no obvious differences in structure (B), suggesting that the presence of cellulose has little apparent impact on biofilm structure. Congo red, which also binds cellulose and other compounds, including proteinaceous attachment factors, stains colonies of *B. avium* 197N expressing the constitutively active diguanylate cyclase WspR19 mutant *in trans* (C). However, little staining is apparent in colonies of wild-type *B. avium* 197N, *B. avium* 197N transformed with pVSP61 (the control plasmid), pVSP61-WspR9, pVSP61-WspR12 or the CD mutant.

Table 1. Bacteria and plasmids.

Strains and plasmids	*Comment*	*Source / Reference*
Bordetella avium 197N	Wild-type strain (ATCC BAA-1003), Km^S, Tc^S.*	ATCC [9, 10]
Ba. 197N cellulose deficient (CD) mutant	*Ba.* 197N::*wssB*::ISΩ-Km/*hah* constructed using pAS296, Km^R.	This work
Escherichia coli K12 DH5α	Standard host for maintaining plasmids.	Gibco-BRL, UK
Pseudomonas fluorescens SBW25	Wild-type strain expressing low-levels of cellulose and producing a weak, viscous mass (VM)-class biofilm.	[42]
Pf. SBW25 Wrinkly Spreader (WS) mutant	*Pf.* SBW25 *wssF* (*S301R*) over-expressing cellulose and producing a robust, physically cohesive (PC)-class biofilm.	[20]
pAS296	Suicide plasmid designed to disrupt *Pf.* SBW25 *wss*-like *bcs* operons derived from pNR9.3, Km^R.	This work
pNR9.3	A self-replicating *wssB*::IS-**Ω**-Km/hah mini-transposon construct, Km^R.	S. Giddens
pVSP61-Tc	A modified version of the pVSP61 broad host-range cloning vector, Km^R and Tc^R.	[28, 41]
pVSP61-WspR9-Tc	pVSP61-Tc expressing the dominant-negative diguanylate cyclase mutant, WspR9 (*G296R*), Km^R and Tc^R.	[41]
pVSP61-WspR12-Tc	pVSP61-Tc expressing the wild-type diguanylate cyclase, WspR12, Km^R and Tc^R.	[28, 41]
pVSP61-WspR19-Tc	pVSP61-Tc expressing the constitutively-active diguanylate cyclase mutant, WspR19 (*R129C*), Km^R and Tc^R.	[28, 41]

All plasmids were maintained in *Ec.* K12. ATCC, American Type Culture Collection. Km and Tc were used at 50 and 12.5 $\mu g.ml^{-1}$, respectively, to select for plasmids in *Ec.* K12, and at 100 and 12.5 $\mu g.ml^{-1}$ for *Ba.* 197N strains, respectively. *, Antibiotic susceptibility using MASTRING M13, M14, and M51 disks was determined for *Ba.* 197N (S, Sensitive; R, Resistant): Ampicillin (R), Cephalexin (R), Cephalthin (R), Chloramphenicol (S), Ciprofloxacin (R), Coliston sulphate (S), Cotrimoxazole (R), Erythromycin (R), Fusidic acid (R), Gentamycin (S), Nalidixic acid (R), Nitrofurantoin (R), Novobiocin (R), Oxacillin (R), Penicillin (R), Streptomycin (S), Tetracycline (S), Trimethoprim (R) (these are in agreement with [33].

Table 2. Homology between *Pf.* SBW25 Wss-like bacterial cellulose synthase (Bcs) complex subunits.

			Localisation	*Core synthase subunits*				*Acetylation-associated subunits*				*Localisation*
		Feature	WssA	WssB	WssC	WssD	WssE	WssF	WssG	WssH	WssI	WssJ
Reference :	*Pseudomonas fluorescens* SBW25	PFLU :	0300	0301	0302	0303	0304	0305	0306	0307	0308	0309
		Length :	344	739	755	436	1279	221	221	468	374	324
		GC content :	62.7	63.0	62.0	62.8	63.2	59.8	63.1	62.4	64.5	61.1
Compared with :	*Bordetella avium* 197N	BAV :	2632	2631	2630/2629*	2628	2627	2626	2625	2624	2623	*NP*
		Length :	255	733	764/753	398	1323	221	222	469	389	
		% Identity :	36	60	44/51	47	42	56	50	66	51	
		E value :	3e-42	0	0/0	9e-116	0	3e-80	3e-52	0	5e-112	
		GC content :	61.7	60.4	63.9/61.7	64.3	64.8	58.9	65.2	60.6	63.6	
	Pseudomonas putida KT2440	PP :	2634	2635	2636	2637	2638	*NP*	*NP*	*NP*	*NP*	*NP*
		Length :	235	865	760	370	1172					
		% Identity :	28	56	34	38	25					
		E value :	0.007	0	5e-122	2e-75	3e-44					
		GC content :	65.7	64.9	64.3	66.1	65.7					
	Pseudomonas syringae DC3000	PSPTO :	1026	1027	1028	1029	1030	1031	1032	1033	1034	*NP*
		Length :	381	739	751	403	1230	221	224	471	383	
		% Identity :	37	69	57	54	52	71	53	85	62	
		E value :	5e-69	0	0	6e-147	0	9e-110	2e-66	0	2e-155	
		GC content :	61.0	60.3	61.7	65.0	63.9	60.5	62.5	62.0	63.8	

The following features of the proteins listed are: genome coding sequence (PFLU, BAV, PPU and PSPTO), protein length (amino acids), BLAST % amino-acid identity and E values compared to the *Pf.* SBW25 protein, and GC content of the gene. *, *Ba.* 197N has a duplication of *wssC* which is annotated as *wssC2* (BAV2630) and *wssC1* (BAV2629). NP, No homologue present in that gene cluster / operon.

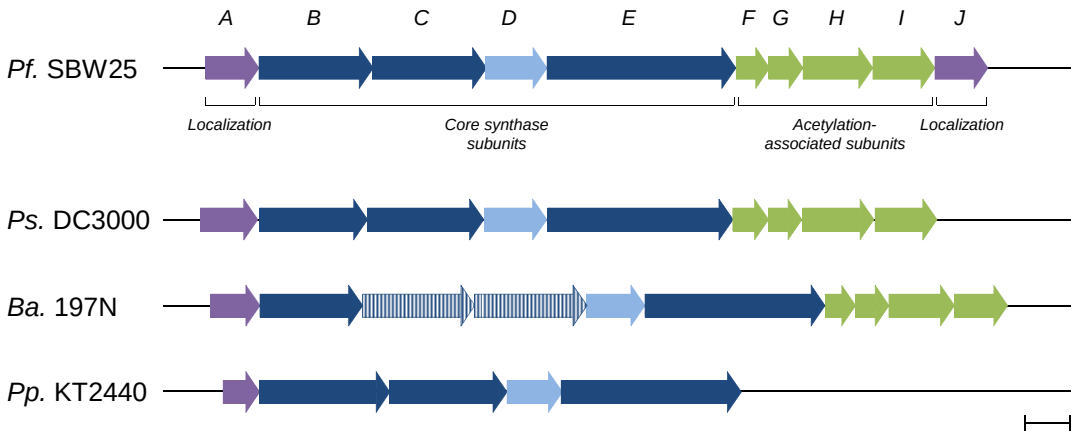

A
B
C
D
E
F
G
H
I
J
Pf. SBW25
Localization
Core synthase subunits
Acetylation-associated subunits
Localization
Ps. DC3000
Ba. 197N
Pp. KT2440

Figure 1

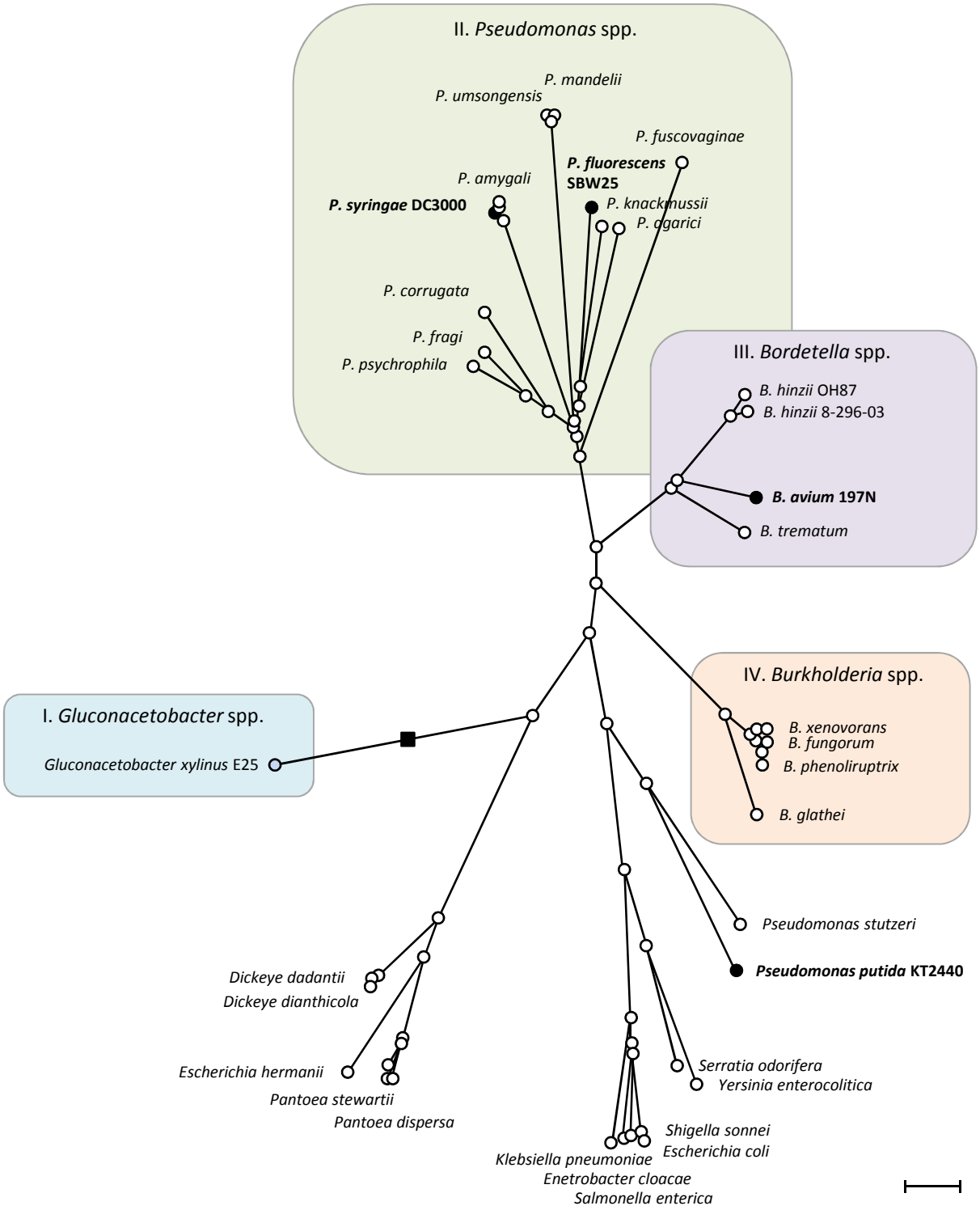

II. Pseudomonas spp.
P. mandelii
P. umsongensis
P. fuscovaginae
P. fluorescens SBW25
P. amygali
P. syringae DC3000
P. knackmussii
P. agarici
P. corrugata
P. fragi
P. psychrophila
III. Bordetella spp.
B. hinzii OH87
B. hinzii 8-296-03
B. avium 197N
B. trematum
IV. Burkholderia spp.
B. xenovorans
B. fungorum
B. phenoliruptrix
B. glathei
I. Gluconacetobacter spp.
Gluconacetobacter xylinus E25
Pseudomonas stutzeri
Pseudomonas putida KT2440
Dickeye dadantii
Dickeye dianthicola
Escherichia hermanii
Pantoea stewartii
Pantoea dispersa
Serratia odorifera
Yersinia enterocolitica
Shigella sonnei
Escherichia coli
Klebsiella pneumoniae
Enetrobacter cloacae
Salmonella enterica

Figure 2

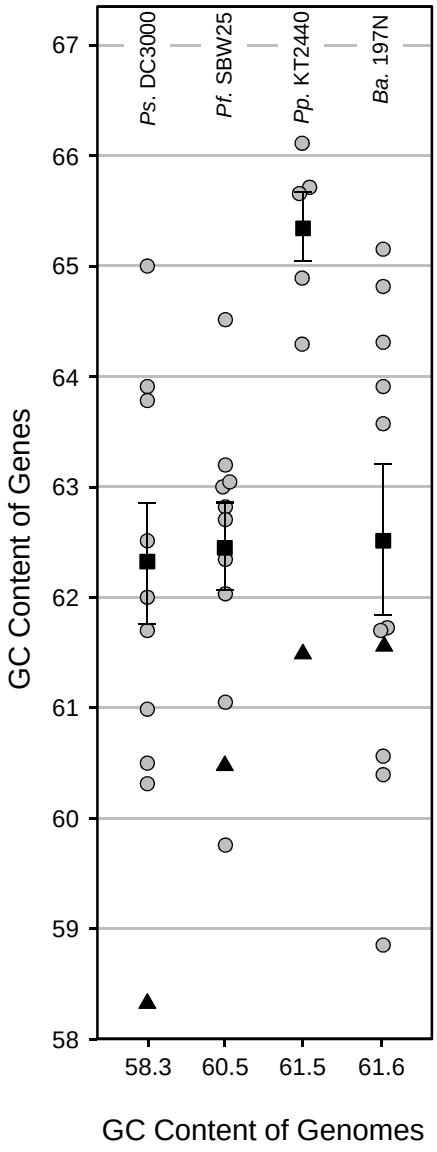

Ps. DC3000
Pf. SBW25
Pp. KT2440
Ba. 197N
67
66
65
64
63
62
61
60
59
58
GC Content of Genes
58.3
60.5
61.5
61.6
GC Content of Genomes

Figure 3

(A) BHI microcosms (B) KB microcosms (C) LB microcosms

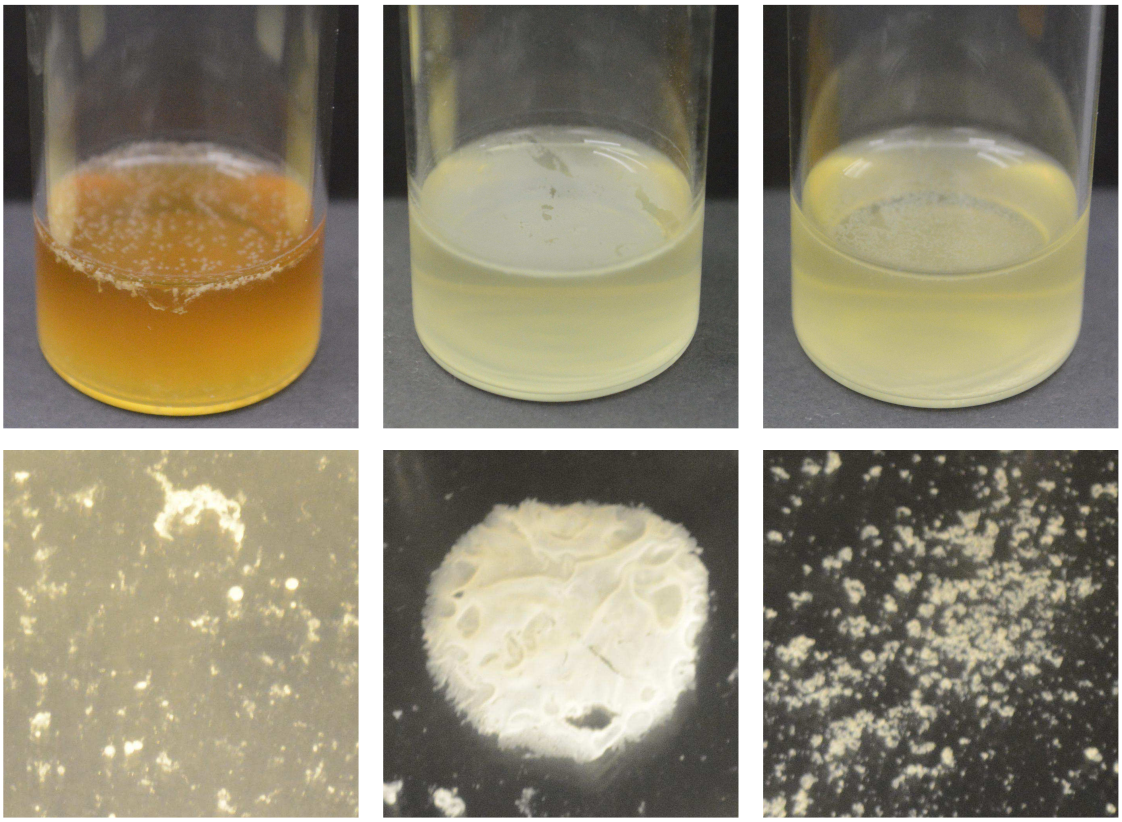


Figure 4

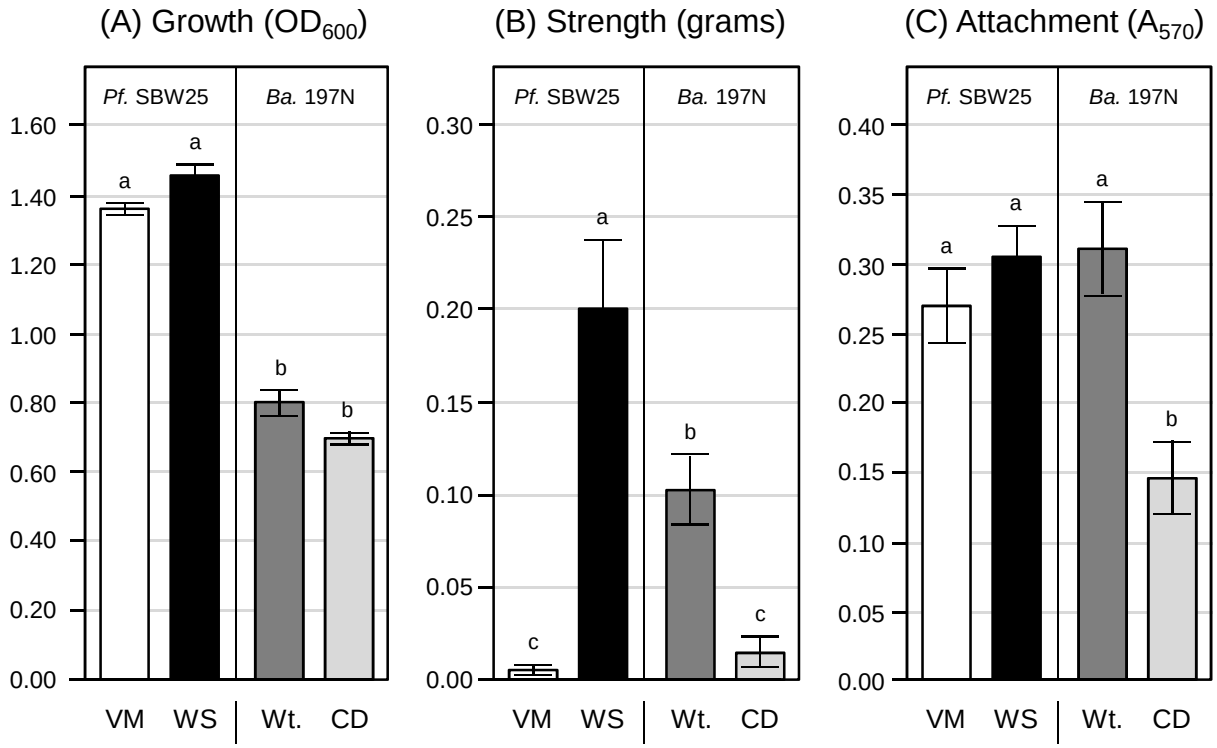
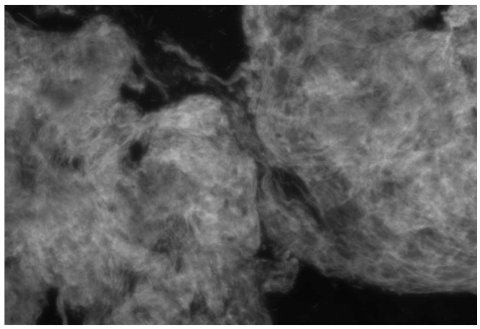
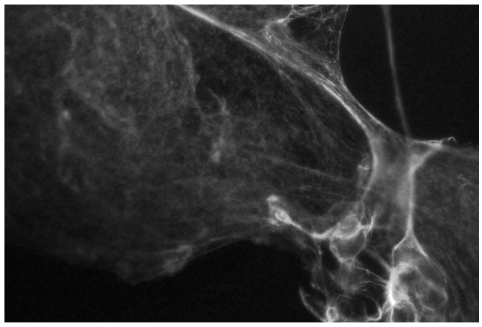

(A) Growth (OD_{600})
(B) Strength (grams)
(C) Attachment (A_{570})
Pf. SBW25
Ba. 197N
VM
WS
Wt.
CD
a
b
c

Figure 5

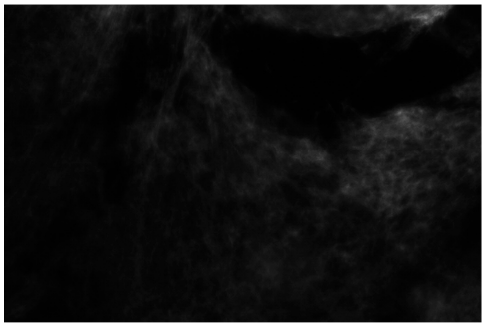
(A) Fluorescent microscopy



Pf. SBW25 Wrinkly Spreader (10x)

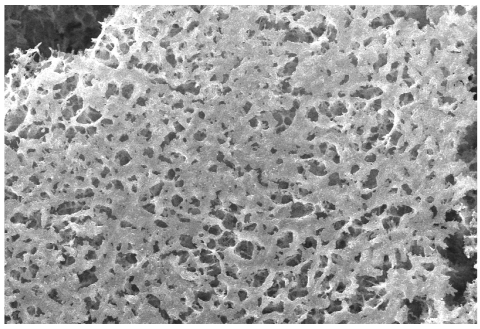


Ba. 197N (40x)

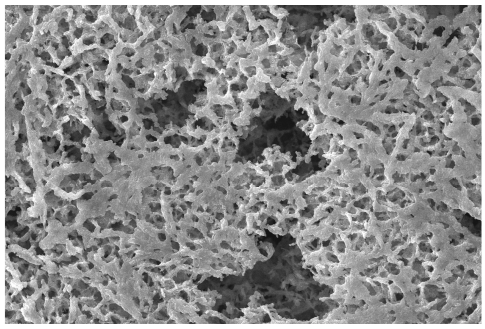


CD mutant (40x)

(B) Scanning electron microscopy

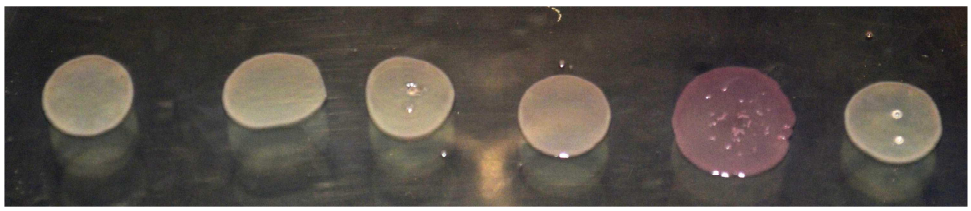


Ba. 197N (500x)



CD mutant (500x)

(C) Congo red plates



Ba. 197N pVSP61 WspR9 WspR12 WspR19 CD

Figure 6