Chapter 1

Cellulose Expression in *Pseudomonas fluorescens* SBW25 and Other Environmental Pseudomonads

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http://dx.doi.org/10.5772/53736

1. Introduction

Bacterial cellulose was first isolated from the air-liquid (A-L) interface biofilm produced by *Bacterium xylinum* in 1886 [1], an acetic acid bacterium strain which would probably now be recognised as *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) or a related species. Over the following century, more acetic acid bacteria and additional *Proteobacter* were found to produce cellulose (reviewed in [2-3]). Cellulose-producing bacteria include a mixture of gut commensals, plant and animal pathogens (these are listed in Table 1), and all share soil as a common secondary habitat. It is likely that cellulose provides protection against physical disturbance, predation or other environmental stresses common to these diverse environments. The biochemistry of bacterial cellulose expression has been studied extensively for *Gluconacetobacter,* and this understanding has been used as a model for enteric bacteria and pseudomonads [4-5] (for a range of bacterial cellulose reviews, see [2-3, 6-9]). Experimental reports of bacteria expressing cellulose are increasing, as well as the annotation of putative cellulose synthase-like operons in bacterial whole-genome sequences, suggesting that an increasingly wider range of bacteria may be capable of producing cellulose.

Our interest in bacterial cellulose began with the experimental evolution of the soil and plant-associated pseudomonad, *Pseudomonas fluorescens* SBW25 [10-12]. This resulted in a novel biofilm–forming adaptive mutant known as the Wrinkly Spreader (WS) and shown in Figure 1. Subsequent investigation of the WS phenotype identified partially-acetylated cellulose as the main matrix component of the biofilm. The pseudomonads are a highly diverse genus (see recent reviews by [13-14]), and biofilm-formation and cellulose-expression are now known to be common amongst the water, soil, plant-associated and

plant-pathogenic environmental pseudomonads [15]. However, the ecological role of cellulose and the fitness advantage it confers to these bacteria is poorly understood.

Class	Order	Family	Genus	Key habitat
Clostridia	*Clostridales*	*Clostridiaceae*	*Sarcina*	Mammalian intestine commensals
α-Proteobacter	*Rhizobiales*	*Rhizobiaceae*	*Agrobacterium*	Plant pathogens
			Rhizobium	Plant symbionts
	Rhodospirillales	*Acetobacteraceae**	*Gluconacetobacter*	Rotting fallen fruits
β-Proteobacter	*Burkholderiales*	*Alcaligenaceae*	*Alcaligenese*	Opportunistic human pathogens
γ-Proteobacter	*Enterobacteriales*	*Enterobacteriaceae†*	*Enterobacter*	}Mammalian
			Escherichia	} intestinal
			Salmonella	} commensals and pathogens
	Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	Water, soil and plant-associated, including plant, fungal and animal pathogens

Adapted from [2-3]. *, Also known as the acetic acid bacteria; †, Referred to here as the enteric bacteria.

Table 1. Cellulose-expressing bacterial genera

Here we provide a review of our work focussing on biofilm-formation and cellulose expression by SBW25 and other environmental pseudomonads. We do not provide an extensive list of primary literature or current reviews, but hope that the citations we have made will allow others to access the growing wealth of publications relevant to the subjects raised in this review.

2. Bacterial assemblages and biofilms

The formation of biofilms by bacteria is a key strategy in the colonisation of many environments, though biofilms are only one of a range of bacterial assemblages involved in this process. Bacterial assemblages range from isolated surface-attached bacteria, monolayers of associated bacteria forming micro-colonies, larger and more complex structures including differentiated biofilms, as well as poorly-attached or free-floating flocs and slime. At times the differences between assemblage types may be minor and will depend on local environmental conditions. These differences are frequently ignored by many who prefer the simple dichotomy of individual, free-swimming planktonic bacteria verses the structurally complex and genetically-determined biofilms. Here we use the term 'biofilm' to include

partially and fully-saturated aggregations growing on solid surfaces, as well as those that are poorly-attached or 'free-floating', after the early and broad definition of Costerton *et al.* [16].

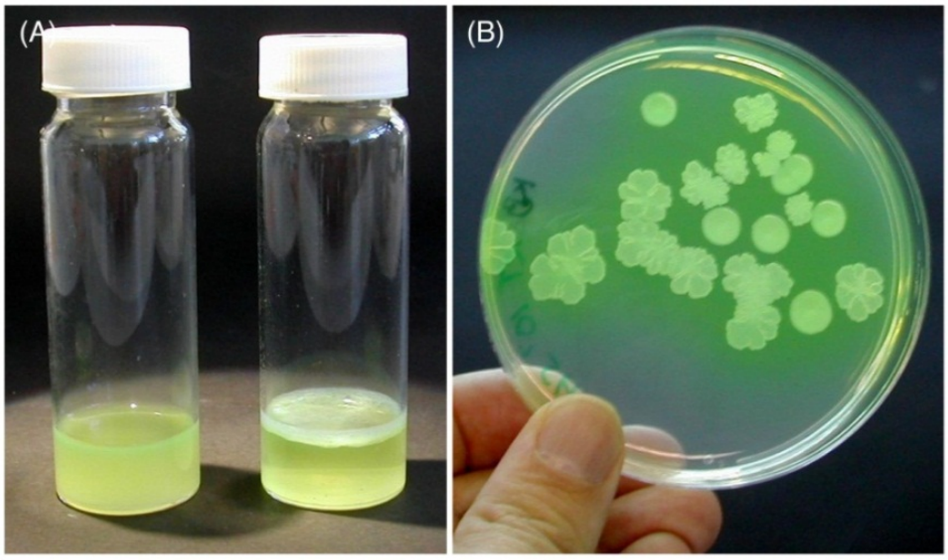

Figure 1. The Wrinkly Spreader mutant of *Pseudomonas fluorescens* SBW25. The Wrinkly Spreader (WS) mutant was isolated from evolving populations of wild-type SBW25 in static King's B microcosms. **(A)** Wild-type SBW25 (*left*) grows throughout the liquid column; in comparison, the WS (*right*) occupies the air-liquid (A-L) interface by producing a robust biofilm 1-2mm thick. **(B)** Wild-type SBW25 (smooth and rounded) and WS (wrinkled) colonies are readily differentiated on agar plates. Images from A. Spiers.

The importance of biofilms (aggregations) in nature is reflected by their prevalence in aquatic, soil, fungal, plant and animal ecosystems, and their role in many chronic human diseases and antibiotic resistance. Many natural biofilms are multi-species structures with complex interactions, and in earlier literature they were often referred to as zoogleal mats. Bacteria found within biofilms are profoundly different from those growing in suspension, differing in both gene expression and physiology and more resistant to desiccation, physical disturbance and predation. A range of biofilm reviews are provided by [16-31].

3. Archetypal 'flow-cell' biofilms

Biofilm research has largely focussed on submerged, solid-liquid (S-L) interface biofilms to provide archetypal models of biofilm structure, function and allow genetic investigation (e.g. *Pseudomonas aeruginosa* PA01 flow-cell biofilms). In these, a surface-attached exopolysaccharide (EPS) polymer matrix-based structure develops away from the solid surface, into the flow of a nutrient and O_2-rich growth medium, and where fluid flow and mass transfer affects biofilm development, structure and rheology (for reviews, see [19, 28-29]).

Biofilm formation begins when planktonic bacterial cells initiate attachment to a solid surface. Attached bacteria start to move across the surface, grow and form micro-colonies, which then develop slowly into the mature biofilm structure in which bacterial cells are embedded in an exopolysaccharide polymer matrix. When conditions become unfavourable within the biofilm, single bacteria or large lumps of biofilm material detach

and move away to colonise new surfaces in more favourable environments (reviewed in [22]). Biofilms of mixed bacterial communities and of individual species that develop on solid surfaces exposed to a continuous flow of nutrients form a thick layer generally described as consisting of differentiated mushroom and pillar-like structures separated by water-filled spaces.

A defining feature of many biofilms is the exopolysaccharide polymer 'slime' that encapsulate the bacteria and provide the main structural component or matrix of the biofilm [20, 22, 24-25]. Although generally assumed to be primarily composed of polysaccharides, e.g. alginate, PEL (a glucose-rich polymer) and PSL (a repeating pentasaccharide containing d-mannose, d-glucose and l-rhamnose) produced by *P. aeruginosa* PA01, PIA (a 28 kDa soluble linear β(1-6)-N-acetylglucosamine) and related PNAG polymer produced by *Staphylococcus aureus* MN8m and *S. epidermidis* 13-1, and PIA-like polymers produced by *Escherichia coli* K-12 MG1655, biofilm matrices can also contain proteins and nucleic acids having significant structural roles (reviewed in [30]). Exopolysaccharides are typically viewed as a shared resource that provides a benefit to the biofilm community by maintaining structure, facilitating signalling, and protecting residents from predation, competition, and environmental stress [20, 22, 32-35].

A second characteristic common to many S-L interface biofilms has been the involvement of quorum sensing in micro-colony development, exopolysaccharide expression, and dispersal. For example, the quorum signalling molecule, acyl-homoserine lactone (AHL), functions as a signal for the development of *P. aeruginosa* PA01 and *Pseudomonas fluorescens* B52 biofilms [36-37]. However, mathematical models based on O_2 and nutrient transport (diffusion) limitation result in similar biofilm architecture (reviewed in [38]), suggesting biofilm development is equally sensitive to environmental conditions as it may be to genetically-determined regulation. Although quorum sensing is important in the development of some biofilms, the bacterial community will exploit all available mechanisms to adapt to local environmental conditions. In order to further understand the development and role of biofilms, the local environment should be considered in terms of ecological landscape theory in which the spatial configuration of the biofilm biomass is shaped by multiple physical and biological factors [39]. It is therefore likely that biofilm formation is the net result of many independent interactions, rather than the result of a unique pathway initiating attachment and terminating with dispersal of mature biofilm communities.

4. Air-liquid (A-L) interface biofilms

In contrast to the archetypal S-L interface biofilms, bacterial biofilms also form at the air-liquid (A-L) interface of static liquids and are sometimes referred to as 'pellicles' [30]. Perhaps the earliest experimental observations of these were made for *Bacterium aceti* and *B. xylinum* in 1886 [1, 40]. Both bacteria were isolated from beer undergoing acetic fermentation in which alcohol is converted into acetic acid. *B. aceti*, an acetic acid bacterium whose modern name is unclear, was found to produce a greasy-looking biofilm which varied in thickness from an 'almost invisible film' to a paper-thick structure

depending on the growth medium [40]. In contrast, the *B. xylinum* isolate, which would probably now be recognised as a *Gluconacetobacter* spp. produced a 'vinegar plant' described as a jelly-like transparent mass at the bottom of the liquid, but under favourable conditions it could also produce a robust gelatinous A-L interface biofilm up to 25 mm thick [1].

Vinegar plants are generally a consortia of acetic acid bacteria and yeasts which produce a zoogleal mat or mixed-species biofilm, and were traditionally used to produce vinegar from beer, cider or wine. Acetic fermentation is initiated by a starter culture known as the 'mother' and obtained from a previous vinegar in a process known as back-slopping [41]. A similar starter often referred to as a 'tea fungus' is used today to produce Kombucha, a carbonated cider-like drink from a sugary solution containing black tea (see the description given in [42]). Acetic acid bacteria, including *Gluconacetobacter* spp., can be isolated from these and similar consortia where they are responsible for the cellulose matrix-based biofilm (see an early review of the acetic acid bacteria by [43]). These artificially-maintained *Gluconacetobacter* spp. are probably better adapted to growth in static liquid conditions than environmental isolates recovered from rotting fallen fruit [44] and under the right conditions, some can produce a gelatinous 'plug' up to 20 mm deep in 10-12 days [45]. In these, cellulose expression and probably growth, is restricted to a thin 50-100 μm deep zone at the top, where it is limited by O_2 diffusing from above and nutrients diffusing through the mature biofilm from below [45]. The growing biofilm is maintained in position by the accumulation of small CO_2 bubbles and by pressing against the walls of the container as it develops.

We expect that smaller-scale A-L interface biofilms might also occur in a wide range of natural environments, such as the partially-saturated fluid-filled pore networks of soils, in temporary puddles collecting on plants and other surfaces after rainfall, water-logged leaf tissues, or in small protected bodies of water such as ponds where the surface is not disturbed by wind or currents. In these environments, biofilm development would be restricted by a combination of nutrient availability, O_2 diffusion, physical disturbance, as well as microbial competition and predation by protists and nematodes.

A-L interface biofilms are readily produced in experimental static liquid-media microcosms [5, 11, 15], and an example of the *P. fluorescens* SBW25 Wrinkly Spreader A-L interface biofilm is shown in Figure 1. In a survey of environmental pseudomonads using nutrient-rich liquid King's B microcosms, we categorised A-L interface biofilms on the basis of phenotype and physical robustness into the physically cohesive (PC), floccular mass (FM), waxy aggregate (WA) and viscous mass (VM)-class biofilms [15, 46]. The characteristics of these biofilm-types are summarised in Table 2 (see also Figure 2). A-L interface biofilm formation appears to be an evolutionary deep-rooted ability amongst bacteria, presumably with significant ecological advantages. In experimental microcosms, increases in competitive fitness of biofilm-formers have been observed compared to non-biofilm–forming strains, whilst the cost to being a biofilm-forming mutant in an environment not suited to these structures is also measurable [5, 47-49].

	Waxy aggregate (WA)	Floccular mass (FM)	Physically cohesive (PC)	Viscous mass (VM)
Occurrence	Rare	Common	Common	Common
Structure	Single-piece rigid and brittle structure	Multiple flocs	Single-piece flexible and elastic structure	Large viscous mass
Strength	Strong	Medium	Strong	Weak
Resilience	Good, disruption produces smaller fragments	Good, disruption produces flocs that are hard to destroy	Very good, hard to break into smaller fragments	Very poor, disruption solubilises the structure
Attachment	High	Medium	High	Poor
Matrix	No evidence for EPS, possible cell-to-cell interactions	Observed	Observed	Observed

Biofilm attributes compiled from [15, 46, 73]. Strength, ability to withstand weight applied to the top of the biofilm; Resilience, response to applied physical disturbance such as gentle or vigorous mixing; Attachment, connection to the microcosm vial walls in the meniscus region; Matrix, evidence of EPS from behaviour of samples during microscopy; Cellulose, evidence from Calcofluor-staining and fluorescent microscopy.

Table 2. Different classes of air-liquid (A-L) interface biofilms produced by environmental pseudomonads

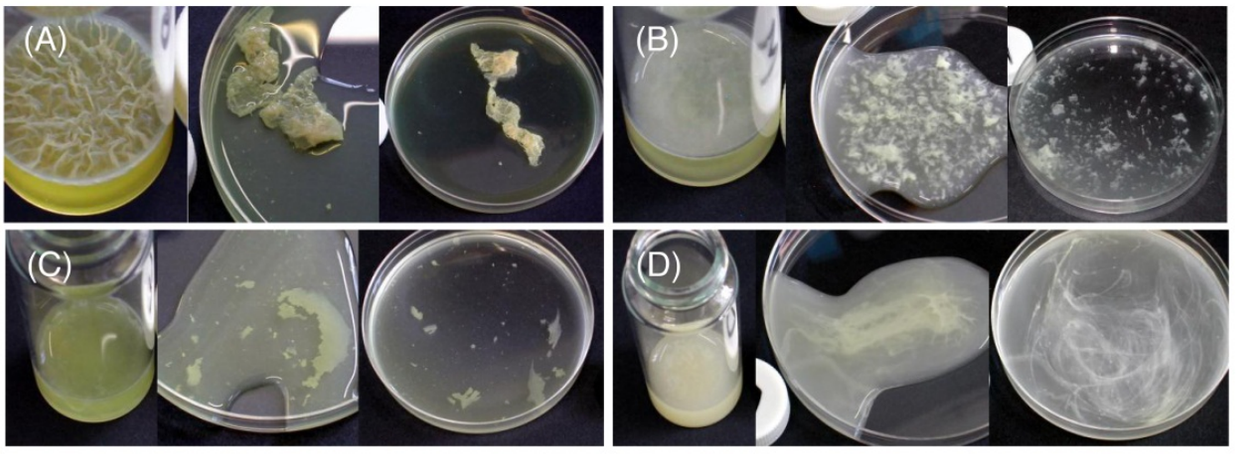

Figure 2. Air-liquid (A-L) interface biofilms. A-L interface biofilms produced by environmental pseudomonads can be categorised into four biofilm types according to visual phenotype, robustness and resistance to physical disturbance. These are the **(A)** Physically cohesive (PC), **(B)** Floccular mass (FM), **(C)** Waxy aggregate (WA), and **(D)** Viscous mass (VM) types. Shown are biofilms in static King's B microcosms *(left)*, after pouring into petri dishes (*middle*), and after vigorous mixing (*right*). Figure adapted from [15].

5. Experimental evolution and the Wrinkly Spreader

Many aspects of the ecological and mechanistic bases of evolution have been investigated by the experimental evolution of bacteria (reviewed in [50-52]). The adaptive radiation of the soil and plant-associated pseudomonad, *P. fluorescens* SBW25 [10, 12], has been investigated in some detail using experimental King's B microcosms (see Figure 1) following the first report by Rainey and Travisano [11]. These can be incubated with shaking to provide a homogenous environment, or statically without physical disturbance to provide a heterogeneous environment. The initial wild-type SBW25 colonists of static microcosms rapidly establish a gradient in which O_2 drops to < 0.05% of normal levels below a depth of 200μm [53]. This gradient produces heterogeneity in the microcosm and defines three niches for colonisation and adaptation: the A-L interface, the liquid column, and the vial bottom. In contrast, microcosms subject to constant and vigorous mixing do not develop an O_2 gradient or different niches. Wild-type SBW25 rapidly radiates to produce a range of phenotypically distinguishable mutants (morphs or morphotypes) to occupy the different niches [11]. This diversification is reproducible and occurs rapidly, typically within ~100 generations and 1-3 days. The main morphotypes recovered from evolving populations of wild-type SBW25 in static microcosms include the Wrinkly Spreaders (WS) which produce a wrinkled colony morphology and colonise the A-L interface through the formation of a biofilm (Figure 1); the Smooth (SM) morphs, including wild-type SBW25, which produce round, smooth colonies and colonise the liquid column, and the Fuzzy Spreaders (FS) which are characterised by fuzzy-topped colonies and colonise the anoxic bottom of static microcosms [11].

In an effort to understand the mechanistic basis of the adaptive leap of wild-type SBW25 from the liquid column-colonising SM-morph to the WS A-L interface niche specialist, the underlying molecular biology of the WS phenotype was investigated. This work, described in the following section, ultimately showed that the evolutionary innovation was the use of cellulose to produce a physically robust and resilient biofilm which allowed the colonisation of the A-L interface. Competitive fitness experiments have demonstrated that the WS has a significant fitness advantage over non-biofilm–forming strains in static microcosms [5, 11, 49]. Simplistically, WS cells were able to intercept O_2 diffusing across the A-L interface from the atmosphere before non-biofilm–forming competitors could do so lower down the liquid column, and as a result, WS populations could grow more rapidly than non-WS populations [53]. In contrast however, the WS do not enjoy a fitness advantage in shaken microcosms where the O_2 concentrations are uniform or on agar plates where the WS phenotype is unstable [5, 48-49].

6. Cellulose expression in *P. fluorescens* SBW25

In order to understand the underlying mechanistic basis of the WS phenotype, a mini-transposon screening approach using mini-Tn*5* was adopted to identify critical genes and pathways [5]. Mini-transposon insertions typically destroy the function of the targeted gene, and the disruption of critical genes in the WS would be expected to result in mutants that produced rounded, smooth (SM)-like colonies rather than the typical WS colony. Plates

containing hundreds or thousands of WS colonies could be easily screened for a few SM-like colonies which could then be isolated for further examination.

This approach allowed the identification and sequencing of the SBW25 *wss* operon containing ten genes (*wssA-J*) required for the WS phenotype and is shown in Figure 3 (*wss* is an acronym for WS structural locus, responsible for the production of the main structural component required for the WS phenotype) [5]. Overall, the *wss* operon showed strong similarity to the cellulose biosynthetic clusters originally identified as the *acs* operon in *Gluconacetobacter hansenii* (formerly *Acetobacter xylinus*) ATCC 23769 [54] and subsequently annotated as the *yhj* operon in the whole-genome sequence of *Escherichia coli* K-12 [55]. Most *acs* (*Acetobacter* cellulose-synthesizing) homologues are now referred to as *bcs* (bacterial cellulose synthesizing) genes as we do here (*yhj* has no meaning). The degree of homology between the *wss*, *bcs* and *yhj* genes at the amino acid level strongly suggested that the SBW25 *wss* operon encoded a functional cellulose synthase, and the predicted functions of the Wss proteins are listed in Table 3.

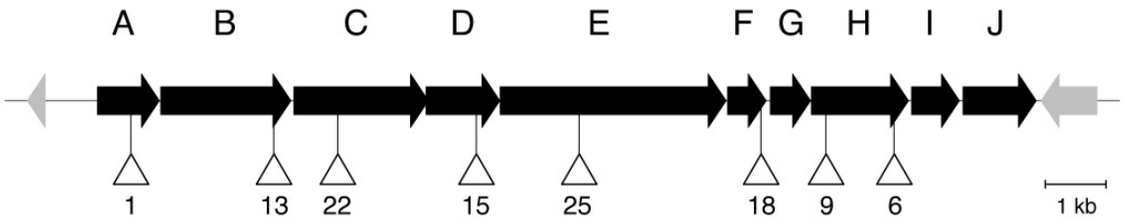

Figure 3. Structure of the cellulose biosynthesis operon. The *Pseudomonas fluorescens* SBW25 cellulose synthase is encoded by the *wss* operon (*wssA-J*, black arrows). The core synthase is composed of WssBCDE subunits and the associated acetylation activity produced by WssFGHI. WssA and WssJ may be involved in the correct cellular localisation of the Wss complex, though WssJ is functionally redundant. The locations of key mini-Tn*5* transposon insertions are indicated (open triangles). WS-1, 13, 22, 15 & 25 mutants are unable to express cellulose. WS-18, 9 & 6 mutants express un-acetylated cellulose. Upstream of the *wss* operon is $tRNA^{Thr}$ and downstream a hypothetical protein of unknown function (grey arrows). Scale bar: 1 kb. Figure adapted from [5].

However, the SBW25 *wss* operon showed two notable differences to the *G. xylinus bcs* and *E. coli yhj* operons. First, the *wss* operon contains two MinD-like homologues, WssA and WssJ, not previously recognised as having a role in cellulose synthesis. WssJ shows 51% identity at the amino acid level with WssA, but only short sections of similarity at the nucleotide level and does not appear to be a simple repeat of the *wssA* gene sequence. As MinD is involved in cell division and determining cell polarity [56], WssA and WssJ were proposed to ensure the correct spatial localization of the cellulose synthase complex at the cell poles [5]. Subsequently, the WssA-homologue, YhjQ (BcsQ), has been shown to be essential for this in *E. coli* K-12 [57]. Secondly, the *wss* operon includes three genes, *wssGHI*, that shares homology with the alginate acetylation proteins of *P. aeruginosa* FRD1, AlgFIJ [58].

In order to demonstrate that the SBW25 *wss* operon encoded a functional cellulose synthase, and to determine the role of the alginate acetylation-like *wssGHI* genes, cellulose expression in the WS and mini-Tn*5* mutants was examined by a variety of techniques, including Congo

red colony staining, fluorescent microscopy, enzymatic digestion and structural analysis of purified matrix material [5, 59-60].

Protein (synonyms)	Function (Accession No.)
WssA (BcsQ, YhjQ)	Cellulose synthase-associated positioning subunit (CAY46577.1): MinD–like ATPase involved in the appropriate spatial localisation of the cellulose synthase complex.
WssB (BcsA, YhjO)	Cellulose synthase subunit (CAY46578.1): catalytically-active subunit responsible for the polymerisation of UDP-Glucose into cellulose. Predicted integral transmembrane protein, contains conserved D residue, QXXRW, HAKAGN and QTP motifs, a PilZ domain. Binds c-*di*-GMP.
WssC (BcsB, YhjN)	Cellulose synthase subunit (CAY46579.1): unknown function (originally thought to bind c-*di*-GMP). Often fused with WssB.
WssD (Orf1, YhjM)	Cellulose synthase subunit (CAY46580.1): Endo-1,4-D-glucanase (D-family cellulase).
WssE (BcsC/S, YhjI)	Cellulose synthase subunit (CAY46581.1): unknown function. Includes a putative signal peptide.
WssF (BcsX)	Cellulose synthase-associated acetylation subunit (CAY46582.1): suggested function is to present acyl groups to WssGHI.
WssG	Cellulose synthase-associated acetylation subunit (CAY46583.1): AlgF-like protein involved in the acetylation of cellulose. Includes a putative signal peptide.
WssH	Cellulose synthase-associated acetylation subunit (CAY46584.1): AlgI-like protein involved in the acetylation of cellulose. Predicted integral transmembrane protein.
WssI	Cellulose synthase-associated acetylation subunit (CAY46585.1): AlgJ-like protein involved in the acetylation of cellulose. Localised to the periplasm.
WssJ	Cellulose synthase-associated positioning subunit (CAY46586.1): MinD-like ATPase like WssA but apparently functionally redundant.

G. xylinus Bcs and *E. coli* Yhj synonyms are provided in parentheses. Function suggested from Wss experiments and Wss homologue investigations. AlgFIJ homologues are from *P. aeruginosa* FRD1 [58].

Table 3. Predicted functions of the *Pseudomonas fluorescens* SBW25 Wss proteins

The diazo dye, Congo red (CR), had been used previously to stain bacterial colonies expressing cellulose [61], and we used this technique to show that WS, WS-18, WS-6 and WS-9, but not WS-1, WS-13, WS-22, WS-15 and WS-25 mutants, appeared to express cellulose on King's B plates [5, 59]. WS and WS-18 biofilm material were subsequently stained with the more specific fluorescent dye, Calcofluor, and examined by fluorescent microscopy (Figure 4). This showed that the biofilm was dominated by an extensive network of extracellular cellulose, with fibres ranging from 0.02 μm to over 100 μm thick. In

places, the fibres appeared to form large clumps of material and in other places forming thin films, with bacterial cells associated with the fibres and found within the voids [59]. In comparison, in colonies the cellulose fibres appear to collect above the mass of the colony (Figure 5). Scanning electron microscopy images of WS biofilms suggest a lattice-work of pores (Figure 6) which might be the result of constant growth at the top surface of the biofilm which slowly displaces older strata deeper into the liquid column [53]. Rough calculations of the density of WS biofilms suggest that they were > 97% liquid, which is in agreement with the finding that microbial amorphous celluloses are very hydrophilic, with gels having a water holding capacity of 148 – 309 g water / g dry cellulose [62-63]. Recent rheological tests have shown that the WS biofilm structure is a classic viscoelastic solid (gel-like) material (AK & AJS, unpublished observations). The structural integrity of WS biofilms could be destroyed by incubation with cellulase, adding support to initial conclusions that the major matrix component of the WS biofilm, expressed by the *wss* operon, was cellulose or a cellulose-like polymer [5, 59].

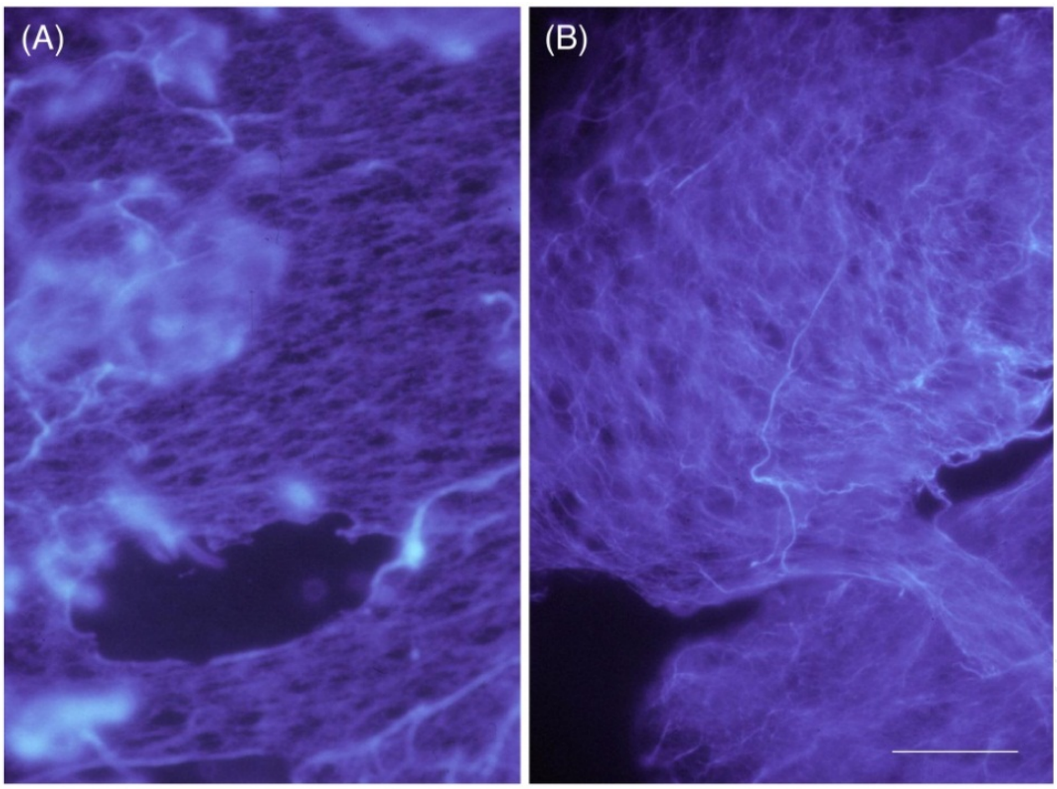

Figure 4. Fluorescent microscopy of WS biofilms. The cellulose fibre matrix of the Wrinkly Spreader (WS) biofilm can be visualised by staining with Calcofluor and fluorescent microscopy. Shown are two images showing the highly hydrated and fibrous nature of the WS biofilm. Scale bar: 100 μm. Images from A. Spiers.

WS and WS-18 biofilms were subsequently purified in order to determine the chemical identity of the matrix component. Carbohydrate analysis indicated that both samples contained ~75% glucose (Glu) and ~25% rhamnose (Rha) [59]. The latter could be explained as coming from contaminating Rha-containing A-band LPS which is highly conserved amongst the pseudomonads [64]. Linkage analyses of derivatized WS samples by GC-MS

identified a major peak corresponding to 4-Glu, and minor peaks corresponding to 2,4-Glu, 3,4-Glu and 4,6-Glu, which is consistent with a β(1-4)-linked glucose polymer, i.e. cellulose. In contrast, the WS-18 material did not contain 2,4-Glu, 3,4-Glu or 4,6-Glu derivatives, suggesting that the *wss* alginate acetylation-like genes were responsible for the acetylation of glucose residues at the 2, 3, and 6 Carbon positions. This was further supported by [^{1}H]-NMR analysis which confirmed the presence of acetylated hexose residues in the WS extract, with 14% of the glucose residues estimated to be modified with one acetyl group [59]. Although cellulose is readily acetylated by chemical treatment, we are not aware of any other reports of biologically-produced acetylated cellulose.

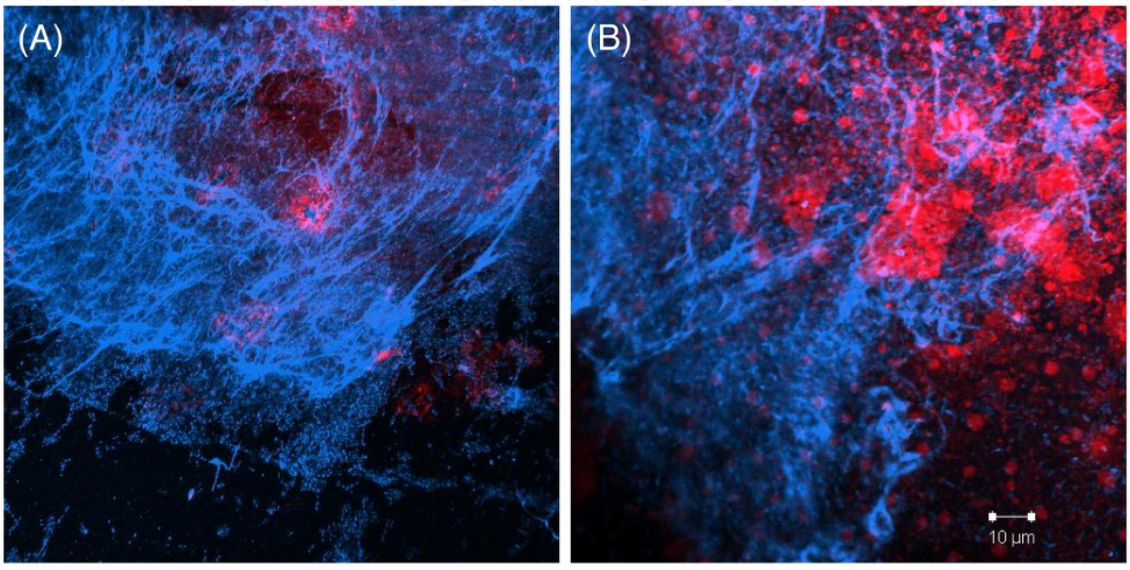

Figure 5. Inducing cellulose expression with c-*di*-GMP. An increase in c-*di*-GMP levels can induce cellulose expression in some pseudomonads. Shown are confocal laser scanning microscopy (CLSM) images of colony material from **(A)** *Pseudomonas fluorescens* SBW25 and **(B)** *P. syringae* DC3000 expressing the constitutively-active DGC response regulator WspR19 *in trans* which increases c-*di*-GMP levels, visualised with Calcofluor for cellulose (blue) and ethidium bromide for bacteria (red). Scale bar: 10 µm. Images from O. Moshynets.

The partial acetylation of the cellulose fibres expressed by the WS clearly had an impact on colony morphology and biofilm strength. Colonies produced by WS-18 were readily differentiated from WS and wild-type SM-like colonies, whilst the WS-18 biofilm was ~ 4*x* weaker than the partially-acetylated structure produced by the WS, suggesting that acetylation increased the connectivity of cellulose fibres within colonies and the biofilm matrix [59-60]. Incubation with EDTA reduced WS-18 biofilm strength, whilst incubation with some diazo dyes increased WS-18 biofilm strength compared to WS biofilms, suggesting that fibre interactions could be altered by sequestering Mg^{2+} and coating cellulose fibres with dyes [60]. A lipopolysaccharide-deficient mutant which produces a very weak biofilm compared to both WS-18 and WS, was also affected by EDTA and diazo dyes, indicating that the partially-acetylated cellulose fibres also interacted with the lipopolysaccharide on the surface of cells or associated with cell debris to further strengthen the biofilm structure [60].

Individual cellulose polymers can also interact directly to produce a number of different forms or allomorphs. *G. xylinus* produces two crystalline allomorphs, known as cellulose I and II, which requires the cellulose synthase-associated BcsD subunit that couples cellulose polymerisation and crystallization [54, 65]. However, SBW25 lacks a BcsD homologue and therefore can only produce non-crystalline amorphous cellulose.

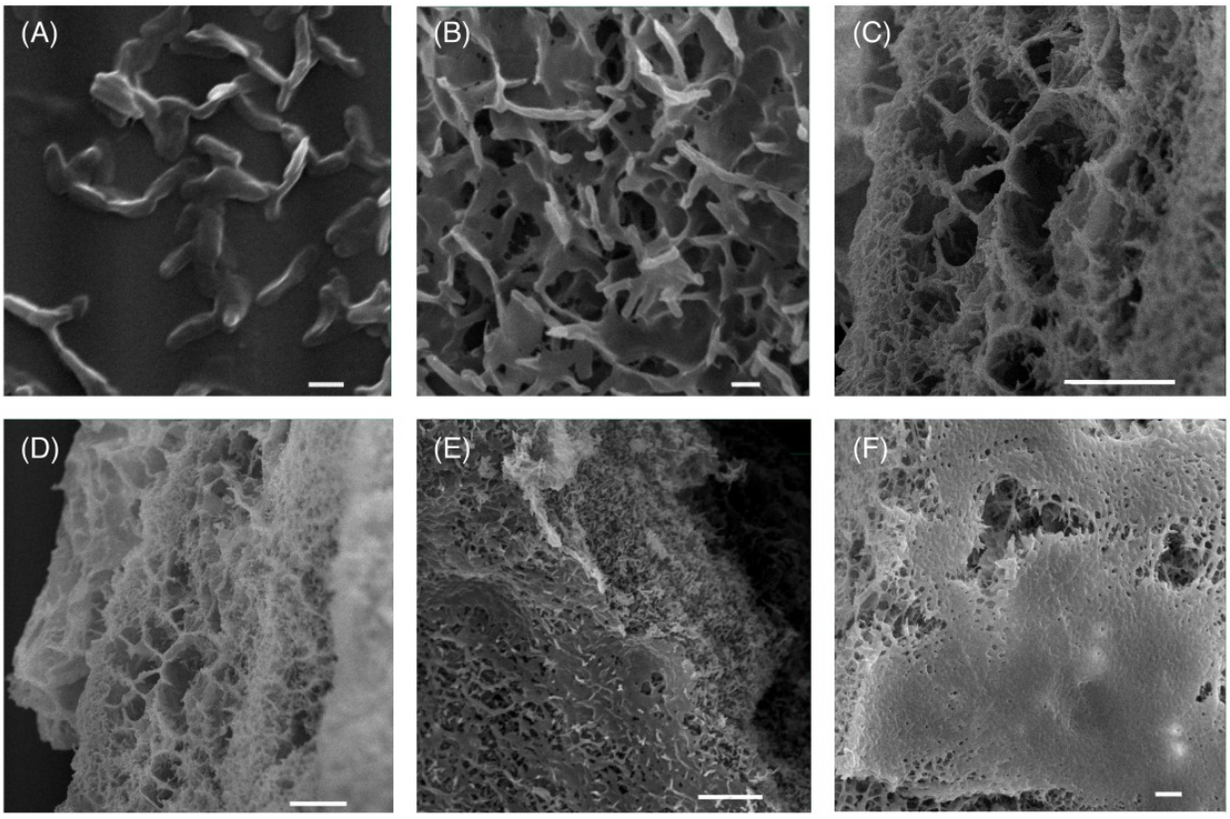

Figure 6. WS biofilm ultrastructure. Scanning electron microscopy (SEM) images of Wrinkly Spreader (WS) biofilms suggest a porous but robust structure. Shown here are a series of SEM images of decreasing magnification, from **(A)** single cells to **(F)** large pieces of biofilm. Images were obtained after freeze-drying and shadowing with gold. Scale bars: A & B, 1μm; C – F, 10 μm. Images from O. Moshynets.

Following the analysis of the WS mini-Tn*5* mutants, a further round of mini-transposon mutagenesis was undertaken using IS*phoA*/hah [66]. This mini-transposon allowed both the impact of polar and non-polar insertions to be assessed; the former destroy the function of the disrupted gene as well as any down-stream expression, whilst the latter destroys gene function but leaves down-stream expression unaffected (this is possible after Cre-mediated deletion of the central portion of the IS*phoA*/hah cassette which leaves a 63-codon in-frame insertion). WS IS*phoA*/hah mutants were recovered for each of the *wss* genes, except *wssJ*. Each of the polar IS*phoA*/hah insertions and corresponding non-polar Cre-deletions in *wssA-E* resulted in the loss of cellulose expression, whilst polar and non-polar mutants in *wssF-I* resulted in a WS-18–like phenotype [47, 59]. Finally, a WS *wssJ* deletion mutant was constructed and shown to have no impact on cellulose expression, suggesting that the final gene of the *wss* operon might be functionally redundant [47].

Examination of other mini-Tn*5* mutants also lead to the identification of the *wsp* regulatory operon of seven genes, *wspA-E & R* (*wsp* is an acronym for WS phenotype locus, responsible for the regulation of the WS phenotype) [67]. The function of these have been modelled on the *Escherichia coli* Che chemosensory system (reviewed in [68]) to provide a mechanistic explanation of the induction of the WS phenotype [67] (a schematic of this is shown in Figure 7). In this the methyl-accepting chemotaxis protein (MCP) WspA forms a membrane-bound complex with two scaffold proteins, WspB and WspD, plus the histidine kinase WspE. In the absence of an appropriate environmental signal, the complex is silent and does not activate the associate response regulator, WspR, by phosphorylation. The system is controlled by a negative feedback loop mediated by the WspC methyltransferase and WspF methylesterase. WspC constitutively antagonises WspF, and in wild-type SBW25 the activities of the two are balanced, preventing the activation of WspR and allowing the Wsp complex to oscillate between active and inactive states. WspR is a di-guanylate cyclase (DGC) response regulator, and the phosphorylated active form, WspR-P, synthesizes c-*di*-GMP (bis-(3'-5')-cyclic dimeric guanosine monophosphate) from GTP [69]. In this model, we hypothesised that mutations in WspR which stimulated DGC activity without requiring phosphorylation, or mutations inhibiting WspF function, would result in an increase in c-*di*-GMP production. This would then lead to the activation of the WS phenotype through the direct stimulation of the cellulose synthase complex, rather than up-regulated *wss* transcription [5] (the second component required for the WS phenotype, the pilli-like attachment factor, has not yet been identified and it is not known how it might be regulated by c-*di*-GMP). Several mutants of WspR had been engineered, and the effect of the constitutively-active mutant WspR19 [70] on cellulose expression by wild-type SBW25 is shown in Figure 5.

This model for the activation of the WS phenotype has been confirmed through the identification and testing of WspF mutations found in a number of independently-isolated Wrinkly Spreaders [67]. Interestingly, no naturally occurring WspR mutants have been identified yet, despite the fact that engineered WspR mutants like WspR19 were found to show the predicted phenotype [60, 69-71]. Mutations in other operons leading to the activation of the AwsR and MwsR DGCs can also induce the WS phenotype [47, 72]. These different routes activating the WS phenotype can be seen as an example of parallel evolution leading to new A-L interface biofilm-forming genotypes in static microcosms [72].

During the molecular investigation of the WS phenotype, the non-biofilm–forming wild-type SBW25 was modified by the insertion of a constitutive promoter to increase the levels of *wss* operon transcription. This mutant, JB01, was found to produce a very weak biofilm, poorly attached to the microcosm vial walls and to express similar amounts of cellulose as the WS [5, 60]. Subsequently, we found that wild-type SBW25 could be non-specifically induced by exogenous Fe to produce a phenotypically-similar biofilm referred to as the VM biofilm [73] (see below for a description of Viscous mass (VM)-class biofilms). However, VM biofilm-formation was not the result of an increase in *wss* transcription, and as yet, no link has been identified between Fe regulation and cellulose expression. We speculate that the induction of the VM biofilm is due to a minor perturbation of the Wsp system or the cellulose synthase complex itself that allows activation despite sub-critical levels of c-*di*-GMP.

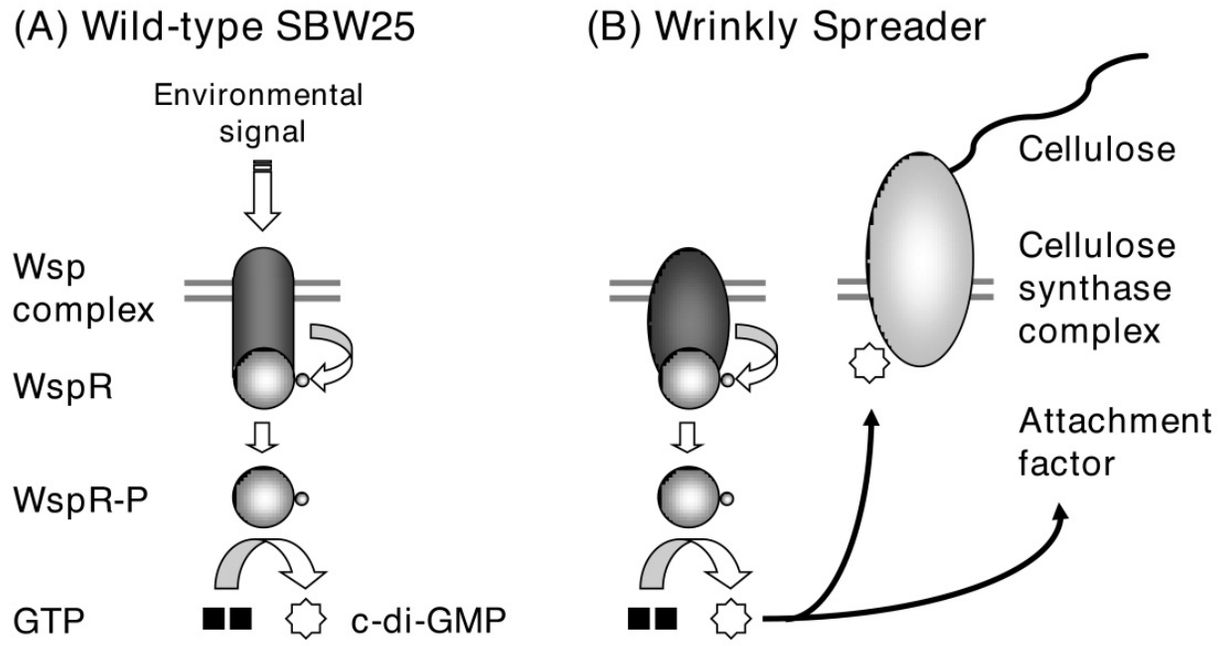

Figure 7. Activation of the WS phenotype. The Wrinkly Spreader (WS) phenotype is controlled by the membrane-associated Wsp complex and associated DGC response regulator WspR. **(A)** In wild-type *Pseudomonas fluorescens* SBW25, when an appropriate environmental signal is received the Wsp complex phosphorylates WspR which then results in the production of c-*di*-GMP. However, in the absence of signal, the Wsp complex is silent and c-*di*-GMP levels remain low. **(B)** In the Wrinkly Spreader a mutation in a Wsp subunit (WspF) alters the sensitivity of the Wsp complex such that it activates WspR in the absence of the environmental signal. The resulting increase in c-*di*-GMP activates the membrane-associated cellulose synthase complex to produce cellulose, and also activates the unidentified attachment factor that is also required for the WS phenotype.

7. Biofilm formation and cellulose expression amongst other pseudomonads

Having discovered that *P. fluorescens* SBW25 could express cellulose in the WS biofilm (and subsequently in the VM biofilm), we were interested to see if related environmental pseudomonads could produce similar cellulose-matrix–based A-L interface biofilms. We therefore undertook a survey of environmental pseudomonads, including water, soil, plant-associated and plant pathogenic isolates (we did not include human or other animal pathogens in this survey) [15]. The ability of each to produce an A-L interface biofilm was assessed in static King's B liquid media microcosms. Importantly, this assay did not differentiate between isolates that constitutively produced biofilms, with those that might utilise quorum sensing-like signalling to initiate biofilm-formation, or those that had mutated into a biofilm-forming genotype. Of the 185 environmental pseudomonads tested, 76% were found to produce observable biofilms within 15 days incubation. The phenotypes of these were variable, with biofilm strengths ranging 1500*x*, but could be categorised into the physically-cohesive (PC), floccular mass (FM), waxy aggregate (WA) and viscous mass (VM)-class biofilms described in Table 2 (see also Figure 2) [15, 46].

Calcofluor-fluorescent microscopy identified cellulose as the matrix component of 20% of the biofilm-forming isolates, indicating that at least seven *Pseudomonas* species were capable of expressing cellulose under the conditions tested. These included *P. corrugata* (tomato pathogens), *P. fluorescens* (plant-associated isolates), *P. marginalis* (alfalfa and parsnip pathogens), *P. putida* (rhizosphere and soil isolates), *P. savastanoi* (olive pathogens), *P. stutzeri* (represented by a single clinical isolate), and *P. syringae* (celery, cucumber, tobacco, and tomato isolates or pathogens) (isolates from another eleven *Pseudomonas* spp. were tested, including *P. aeruginosa* PA01 and PA14, and were not found to produce cellulose). For two of the cellulose-expressing isolates, *P. putida* KT2440 and *P. syringae* DC3000, the whole genome sequences were available and SBW25 *wss*-like cellulose synthase operons had been annotated [74-75], though no experimental reports of either expressing cellulose had been made.

Many environmental pseudomonads can also be induced to form A-L interface biofilms and to express cellulose using WspR19. When expressed *in trans* in wild-type SBW25 it produces the WS phenotype [60, 70], though in other pseudomonads the impact was found to be more variable. In a test of 16 pseudomonads known to form biofilms and express cellulose, WspR19 was found to significantly increase biofilm attachment, strength, and cellulose expression in *P. fluorescens* 54/96, *P. syringae* DC3000, *P. syringae* T1615 and *P. syringae* 6034 [15] (WspR19 induction of cellulose production by SBW25 and DC3000 is shown in Figure 5). WspR19 also induced a WS-like phenotype in *P. putida* KT2440, despite the fact that biofilm-formation or cellulose expression in this pseudomonad had not been observed in the initial survey (cellulose expression was subsequently reported for both wild-type and WspR19-carrying strains under different experimental conditions by [76]). Similarly, nine of ten non-biofilm–forming and non-cellulose expressing *P. syringae* isolates were found to produce biofilms when induced with WspR19, and two of these also expressed detectable levels of cellulose [15]. These findings suggest that biofilm-formation and cellulose expression in pseudomonads closely related to *P. fluorescens* SBW25 are probably controlled by the same c-*di*-GMP–mediated regulatory system or are sensitive to non-specific increases in c-*di*-GMP levels.

Habitat (sample size)	A-L interface biofilms	Evidence of cellulose
Plant pathogens (n = 57)	6%	26%
Plant & soil associated (n = 28)	82%	39%
Scottish soil (n = 73)[a]	95%	76%*
River (n = 57)	82%	5%
Indoor & outdoor ponds (n = 50)[b]	94%	56%
Pitcher plants (*Sarracenia* spp.) (n = 50)[c]	74%	68%
Spoilt cold-stored meat (n = 60)[d]	77%	28%
Mushroom pathogens (n = 26)[e]	77%	69%

* Estimated from a sub-sample of 25 isolates. Data compiled from [15] and unpublished research from **a,** R. Ahmed, AK & AJS; **b,** B. Varun, AK & AJS; **c,** D.S. Kumar, AK & AJS; **d,** M. Robertson & AJS; and **e,** AK & AJS.

Table 4. Prevalence of A-L interface biofilm formation and cellulose expression amongst environmental pseudomonads

We have conducted additional surveys of pseudomonads isolated from other habitats, including pond water, pitcher plant (*Sarracenia* spp.) deadfall trap-water, spoilt cold-stored meat and mushrooms (Table 4). These confirm the wide-spread ability of environmental pseudomonads to form A-L interface biofilms and to express cellulose under the experimental conditions used previously [15]. It is also evident that pseudomonads are capable of producing a wide range of EPS in addition to cellulose, including alginate, levan, marginalan, PEL, PSL, and a number of other polymers, which may be utilised as biofilm matrix components [77-80].

8. Distribution of *wss*-like cellulose synthase operons amongst the proteobacteria

We are undertaking a bioinformatics analysis of all publicly-available fully-sequenced bacterial genomes in order to determine the phylogenetic distribution and structural variation of *P. fluorescens* SBW25 *wss*-like cellulose biosynthetic operons amongst the proteobacteria. Protein (TBLASTN) homology searches were run against the GenBank complete genome database [81] using the SBW25 Wss proteins as the query sequences in October of 2011. From this, we have identified over 50 bacteria with gene clusters that showed significant protein sequence homology (≥ 25% ID) to three or more Wss proteins, including a minimum of two key cellulose synthase subunits, WssB, WssC, or WssE. Putative SBW25 *wss*-like operons were then manually curated for accuracy to provide Wss homologue protein sequences and operon structures. Phylogentic trees were constructed using the multiple sequence alignment program ClustalW 2.0 [82], and neighbour-joining and minimal evolution methods implemented by Geneious 5.5.5 (Biomatters Ltd, NZ).

Although this bioinformatics analysis is on-going and the final results expected to be published elsewhere, we make the following preliminary observations. First, whilst *wssB* tends to be followed by *wssC* in the *wss*-like cellulose synthase operons as found previously [5, 8, 83], there are examples within the *Burkholderia* and in *Cupriavidus metallidurans* CH34 where *wssB* is separated from the rest of the operon. Second, we note that only the *P. fluorescens* SBW25 and *P. syringae* DC3000 *wss* operons include the *wssG-I* alginate acetylation-like genes, but not the closely-related pseudomonad *P. putida* KT2440. This suggests that DC3000 may also be able to express partially-acetylated cellulose, and that *wssF-I* genes may be narrowly restricted to the *P. fluorescens–syringae* complex. Third, there is considerable variation in cellulose synthesis operon structure amongst the *Enterobacteriacea*, with many having two clusters of genes (e.g. *Erwinia billingiae* Eb66, *Klebsiella pneumoniae* MGH78578, *Pantoea* sp. At-9b). Many enteric bacteria also include the additional genes *bcsEFG* which have no *wss* homologues (e.g. *Escherichia coli* K-12 MG1655, *Salmonella enterica* Typhimurium LT2, *Vibrio fisheri* MJ11). These have been reported to be associated with cellulose production in *Salmonella enteritidis* 3934 [4]. However, *Escherichia coli* K-12 DH10B contains only *bcsFG*, raising the possibility that *bcsE-G* are not essential for cellulose production in these bacteria. Although the *Gluconacetobacter* are not closely related to the enteric bacteria, we note that *G. xylinus* NBRC 3288 has a small *wssBCE* operon plus a larger *wssDBCE* operon. It is possible that such duplications might enable higher levels of

cellulose expression under some environmental conditions, or that such gene duplications may persist for some time before deletion.

Finally, the clustering of WssB homologue sequences (Figure 8) generally follows the 16S phylogenetic relationships between bacteria. However, we are surprised to find that *P. fluorescens* SBW25 and *P. syringae* DC300 cluster with many of the *Burkholderia* and *Xanthomonas*, whilst *P. putida* and *P. stutzeri* strains cluster with the enteric bacteria. We have yet to compare the clustering patterns of the WssC, WssD and WssE homologues, where conserved patterns may reflect different functional roles for cellulose and host lifestyles, whilst aberrant placements of single proteins might reflect the random mutation of a phenotype no longer of functional value or under positive selection.

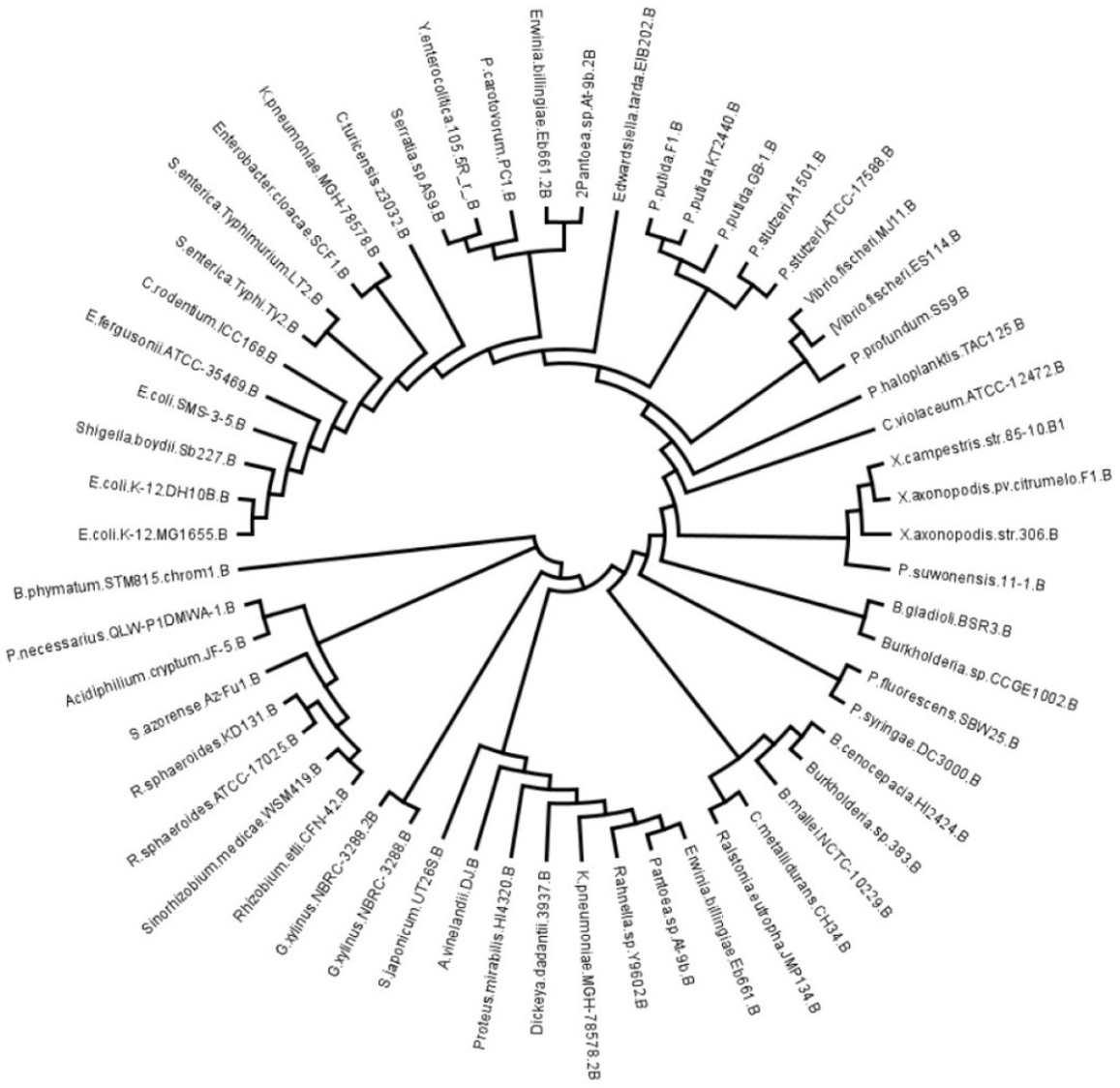

Figure 8. Cladogram of WssB homologues. The structure of the WssB cladogram is similar to that constructed using 16s rRNA sequences, with the enteric bacteria and pseudomonads forming two distinct clusters. Within the pseudomonads, the *P. fluorescens-syringae* complex has diverged earlier than the *P. putida-stutzeri* group. The cladogram was constructed using Geneious 5.5.5 (Biomatters Ltd, NZ) default parameters after multiple sequence alignment of 58 WssB proteins by ClustalW 2.0 [82].

9. Ecological role and fitness advantage of cellulose

Attempts to understand the importance of cellulose expression by *P. fluorescens* SBW25 in soil and the phytosphere have involved investigation of the regulators controlling *wss* operon transcription [5, 84], and measurement of fitness advantage using plant microcosms [85]. Cellulose expression is clearly regulated at two levels in SBW25. First, the activity of the cellulose synthase complex is regulated by c-*di*-GMP levels, but it is not known what environmental signals control WspR, other DGCs or their antagonists, though c-*di*-GMP is known to be involved in a range of surface colonisation and pathogenicity systems in a variety of bacteria (reviewed in [86-87]). Second, cellulose expression is regulated at the level of *wss* operon transcription, with mini-transposon analysis identifying AlgR, AwsR and WspR as positive regulators, and AmrZ and FleQ as negative regulators [5, 84].

AwsXR was a previously unrecognised regulatory system, first identified in SBW25 where the mutational activation of the DGC AwsR results in the WS phenotype [47, 72], though the normal means of regulating AwsR activity remains unknown. FleQ is a c-*di*-GMP–responsive transcriptional regulator, and in *P. aeruginosa* PA01 it controls the hierarchical regulatory cascade for flagella biosynthesis and the repression of the PEL biosynthesis genes [88-89]. AlgR and AmrZ (also referred to as AlgZ) are transcription factors involved in the regulation of a number of systems including alginate biosynthesis and twitching motility [79, 90]. It is possible that AlgR, AmrZ, and AwsR directly repress SBW25 *wss* transcription, whilst AwsX, FleQ, and WspR act indirectly to regulate transcription and therefore cellulose expression [84]. However, no environmental signals have been identified that induce cellulose expression via these repressors.

Sugar beet (*Beta vulgaris*) seedlings have been used to determine the competitive fitness advantage cellulose-expression may provide wild-type SBW25 compared to a cellulose-deficient mutant [85]. In these experiments, seeds were first inoculated with a mixture of wild-type SBW25 and SM-13, a mutant containing a mini-Tn*5* insertion cassette derived from WS-13 [5]. These were then germinated and grown for four weeks in an artificial soil substrate. Bacteria were then recovered from the stems and leaves (the phyllosphere), from roots and adherent vermiculite (the rhizosphere), and from un-planted containers ('bulk soil') to allow the calculation of competitive fitness (W) [91] (we report W for SM-13 *cf* wild-type SBW25 here for clarity). In the phyllosphere and rhizosphere, SBW25 was found to have a significant fitness advantage over SM-13, with $W \approx 1.8$ and $W \approx 1.11$, respectively, but not in bulk soil where $W \approx 1.05$. These findings suggest that the appropriately-controlled expression of cellulose by wild-type SBW25 provides some benefit on plant surfaces. It is possible that the mechanistic nature of this benefit may be an improved tolerance to water-limiting conditions rather than resistance to physical disturbance and predation, as the seedlings were watered using a tray rather than from a sprinkler, and vermiculite was used instead of natural soil.

Comparisons of the survival of wild-type SBW25 and a cellulose-deficient mutant similar to SM-13 under water-limiting conditions have shown that the loss of cell viability is faster for the mutant than for wild-type SBW25 (A Koza & A Spiers, unpublished observations). A

similar observation has been made for *P. putida* mt-2 (the progenitor of KT2440) where water stress was also found to increase cellulose expression [76]. Many bacteria respond to desiccation by producing exopolysaccharides, many of which are hygroscopic and retain water entropically [92], and amorphous cellulose is more hygroscopic and retains more liquid than crystalline cellulose [62]. Support for an anti-predation role for cellulose comes from the finding that the competitive fitness of WS genotypes in static microcosms increases in the presence of the grazing protist *Tetrahymena thermophile* [93].

10. Concluding statement

Bacterial cellulose production and air-liquid (A-L) interface biofilm-formation was first described 1886 for *Bacterium xylinum*, and subsequently observed by ourselves and colleagues in the evolution of the *Pseudomonas fluorescens* SBW25 Wrinkly Spreader (WS) some 116 years later. It is clear that this type of biofilm-formation is common-place amongst the environmental pseudomonads, many of which also utilise cellulose as the main matrix component of the biofilm. The fitness advantage of cellulose matrix-based biofilm-formation by SBW25 in static microcosms is well-proven, but the fitness in natural environments, and the true function of cellulose, is poorly researched and not yet understood. It may be that bacterial cellulose is used to form small biofilms in water bodies, acting to retain bacteria at the A-L interface or to maintain them on solid surfaces against water flow. Appositely, cellulose fibres may resist desiccation stress in water-limited environments, or even provide protection from protist and nematode predation. It is of course possible that cellulose performs a number of functions, which might explain the wide distribution of cellulose synthase operons amongst the *Proteobacter* inhabiting a diverse array of environments.

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Acknowledgement

AJS is a member of the Scottish Alliance for Geoscience Environment and Society (SAGES) and AK was a SAGES-associated PhD student with AJS. KZ undertook his MSc research

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project with AJS. AF is currently undertaking his MSc with AJS and YYD; his preliminary bioinformatics results are presented here and will be published at a later date. Funding from the Royal Society of Edinburgh through the International Exchange Programme supported the collaboration between AJS and OM for this project. The University of Abertay Dundee is a charity registered in Scotland, No: SC016040.

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