Plant cell wall polysaccharides induce colony expansion of soil-derived *Flavobacterium* spp.

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Running title: Pectin induces Flavobacteria colony expansion

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Summary

Flavobacterium is a genus, belonging to the *Bacteriodetes* phylum, characterized by a unique gliding motility. They are often abundant in root microbiomes of various plants, but the factors contributing to this high abundance are currently unknown. In this study, we evaluated the effect of various plant-associated poly- and mono-saccharides on colony expansion of two *Flavobacterium* strains. Both strains were able to grow on pectin and other polysaccharides such as microcrystalline cellulose. However, only pectin, a major component of plant cell walls, substantially enhanced colony expansion on solid surfaces in a dose- and substrate-dependent manner (but did not occur on pectin monomers). On pectin, flavobacteria exhibited a bi-phasic behavior, with an initial phase of rapid expansion, followed by growth within the colonized area. Proteomic and gene expression analyses revealed significant induction of carbohydrate metabolism related proteins when flavobacteria were grown on pectin, including selected SusC/D, TonB-dependent glycan transport operons. Our results suggest an unknown linkage between specific glycan associated operons and flavobacterial colony expansion. This may be associated with their capacity to rapidly glide along the root and metabolize plant cell wall carbohydrates, characteristics that are crucial to rhizosphere competence.

Keywords: Flavobacterium, Roots, Rhizosphere, Pectin, TonB

Originality-Significance Statement: This study reveals unique data linking plant glycan metabolism and bacterial motility, providing insight into bacterial-root associations and rhizosphere competence. Specifically, it explores mechanisms

associated with pectin-stimulated colony expansion in root-associated *Flavobacterium* strains. We determined that expansion of colonies on pectin was biphasic in nature, characterized by rapid proliferation followed by biomass accumulation. We demonstrate by proteomic and gene expression analyses that expansion of *Flavobacterium* on pectin strongly induces TonB related transporters, which seemingly play a role in motility in addition to the uptake and metabolism of plant-associated glycans.

Introduction

The complex interactions between plant-associated microorganisms and their hosts (collectively referred to as the "plant holobiont") are crucial for plant health and growth (1–3). Plants modulate the narrow region of soil that is influenced by root secretions, the rhizosphere, by exuding various small molecular weight compounds (rhizodeposites) such as sugars, amino acids and organic acids, by rhizodepositing root cap border cells, and by releasing various mono- and poly-saccharides in their mucilage (3–8). Organic compounds released from the roots transform the rhizosphere into a nutrient rich environment relative to the surrounding bulk soil, which facilitates colonization by soil microorganisms. Rapid growth, coupled to competition for root resources, results in less diverse rhizosphere and rhizoplane microbiomes relative to surrounding bulk soil, a phenomenon known as the "rhizosphere effect" (4, 9, 10). Rhizosphere and root colonizing bacteria have the capacity to outcompete other soil bacteria and thereby proliferate and thrive in the root ecosystem through a combination of specific traits collectively coined "rhizosphere competence", which include: motility, resistance to stress, ability to utilize plant-derived organic compounds, chemotaxis, and

the production of secondary metabolites (5). Some earlier studies demonstrated that rhizosphere bacteria are attracted to the roots by plant-exuded organic acids such as malic, citric or fumaric acid and various amino acids (11–15). Many of these sensed chemo-attractants can also be consumed by the bacteria (16). Although rhizosphere recruitment and colonization mechanisms of certain plant-growth-promoting rhizobacteria (PGPR) have been identified and characterized, those responsible for recruitment of the vast majority of rhizosphere and rhizoplane bacteria is currently an enigma (17–19).

Flavobacterium is a Gram-negative genus of bacteria from the phylum *Bacteroidetes* known to degrade complex organic compounds in freshwater, marine and soil environments (20, 21). It is highly abundant in the rhizoplane of a wide array of plants, in contrast to considerably lower abundance in bulk soil (21–28), suggesting that members of this genus have specifically adapted to root ecosystems. Several root- and soil-derived flavobacteria were found to antagonize various plant pathogens in different crops (29–33), and recently it was discovered that entophytic *Flavobacterium* strains play a fundamental role in antagonizing phytopathogenic fungi, through the production chitinolytic enzymes and specific secondary metabolites (34). Furthermore, a recent study that applied bacterial network analysis, indicated that they are potential drivers of pathogen suppression in root ecosystems (35). In addition, selected members of this genus have defined as plant growth promoting rhizobacteria (PGPR) of various crops (23, 29, 30, 36–38).

Soil flavobacteria have specialized ability to decompose complex plant derived polysaccharides, such as pectin and glucomannan and the ability to secret various carbolytic enzymes via the *Bacteriodetes*-specific type IX secretion system (T9SS) (21, 39–43). Like other *Bacteroidetes*, they contain a myriad of genes that encode

Polysaccharide Utilization Loci (PULs) that are activated specifically to facilitate glycan capture and uptake (44). These PULs include outer membrane protein transducers involved in polysaccharide utilization, which are part of the TonB family, generally referred to as Starch Utilization System (SUS) proteins. Interestingly, comparative genomics revealed that genomes of soil and root-associated flavobacterial strains are significantly enriched with genes associated with plant polysaccharide degradation relative to aquatic strains from this genus, indicating that physiology of this genus is strongly influenced by its ecology (21).

Most terrestrial *Flavobacterium* strains possess a unique gliding mechanism that rapidly propels them over solid and semi-solid surfaces. The proteins that comprise this gliding system are molecularly intertwined with at least fifteen proteins that make up the T9SS, seven of whom are responsible for the secretion of SprB and RemA adhesins which are expressed on the cell surface and involved in gliding (45, 46). We previously demonstrated that this T9SS-gliding complex is crucial for root colonization by flavobacteria, and this colonization was positively linked to the induction of plant resistance to foliar pathogens (31).

Collectively, the above data strongly suggest that terrestrial flavobacterial strains have evolved means that enable them to interact with plant roots, and that these interactions are beneficial to plant health. Nonetheless, the specific mechanisms behind this phenomenon are currently unclear. In this study, we assessed the impact of various plant-derived sugars on the motility and growth dynamics of flavobacteria by coupling conventional petri dish assays and live-imaging fluorescent microscopy with proteomic and gene expression analyses. We demonstrate that pectin, a plant cell wall-associated polysaccharide, facilitates bi-phasic proliferation over solid surfaces through induction of specific TonB-associated glycan uptake operons. *In-planta* extrapolation of these

results, suggests that this link between pectin, motility and carbohydrate metabolism may be fundamental to rhizosphere competence in flavobacteria.

Results

Growth of *Flavobacterium* strains on various carbon sources

The growth of flavobacteria was evaluated on minimal and rich media amended with different carbon sources. *F. johnsoniae* was motile and grew on chitin, pectin and microcrystalline cellulose but not on arabinose (**FigS1**). Subsequently, we evaluated growth of flavobacteria on rich media (PY2 agar) coated with selected sugars (**Fig1A**) to evaluate how specific carbon sources flavobacterial proliferation. Colony expansion of *F. johnsoniae* on PY2 agar media coated with pectin was close to five times higher than on the same media coated with other analyzed mono- and polysaccharides or with double distilled water (DDW, control) ($p<0.05$, Tukey-Kramer HSD test) (**Fig1B**). Wild type (WT) and gliding/typeIX secretion system mutants (ΔgldJ) of *F. johnsoniae* and the pepper root isolate *Flavobacterium* sp. F52 were inoculated in the center of PY2 agar media amended with or without pectin (**Fig1C**). When grown on pectin, WT colonies of both *Flavobacterium* strains significantly expanded after 48 hours of incubation, while growth was reduced in the control without pectin (DDW). In contrast, gliding mutant (ΔgldJ) colonies of both flavobacterial strains did not expand (**Fig1C,D**), indicating that the gliding apparatus is a prerequisite for pectin-induced colony expansion.

Dose-dependent pectin facilitated colony expansion

To determine whether expansion on pectin is dose dependent, *F. johnsoniae* and *Flavobacterium* sp.F52 strains were inoculated at the center of PY2 agar media plates streaked with pectin at final concentrations of 0.5, 1, 2 and 4%. For all the examined pectin concentrations colonies radiated along the pectin streaks, but expansion was more significant on 2% and 4% pectin ($p<0.05$,Tukey-Kramer HSD test) (**FigS2**).

Since galacturonic acid and rhamnose are the two major components of pectins, we examined the colony expansion of *F. johnsoniae* on PY2 agar coated with 2% titrated galacturonic acid, 2% rhamnose, a combination of galacturonic acid and rhamnose, 2% pectin and DDW. *F. johnsoniae* expansion on galacturonic acid, rhamnose (alone or combined) did not facilitate significant colony expansion ($p<0.05$,Tukey-Kramer HSD test) in contrast to colonies that were grown on pectin (**Fig2A, B**).

Temporal dynamics of pectin induced *F. johnsoniae* colony expansion

The expansion of green fluorescent protein (GFP)-labeled *F. johnsoniae* on PY2 agar coated with glucose, microcrystalline cellulose, glucomannan or pectin, was visualized at a higher resolution using time-lapse microscopy. Colony morphology after 32 hours, on each tested substance is presented in **Fig3A**.

Growth dynamics were clearly affected by the sugar type. Expansion on pectin was characterized by a relatively long lag phase. However following this stage, colonies rapidly expanded and after 18 hours, the fluorescent signal of colonies grown on pectin surpassed both the control (without sugar amendment) and the other amended compounds (according to Tukey-Kramer HSD test). Growth on pectin facilitated multiple ring-like structures that resemble previously described “raft” structures (47) (**Fig3B, Supplementary movie 1**). Conversely, expansion of colonies grown on glucose and the control did not display this bi-phasic expansion (**Supplementary**

movie 1). Bacterial colonies grown on glucose expanded the least resulting in compact, small colonies with less ringed structures, suggesting that glucose has an inhibitory effect on *F. johnsoniae* motility and possibly growth as was previously demonstrated (**Fig3C**) (48, 49). The most significant expansion observed between 6 to 20 hours was on cellulose microcrystalline, but the colonies grown on glucomannan and pectin proliferated at later times, and the colonies grown on pectin reached the greatest intensity at 44 hours (**Fig3C**), thereby surpassing the colonies grown on other sugar sources ($p<0.05$,Tukey-Kramer HSD test). The high sensitivity of the fluorescence detection enabled us to visualize lateral colony expansion on cellulose microcrystalline, which was less visible on agar plates (**Fig1A).**

Colonies grown on pectin were bi-phasic, with initial phase of rapid expansion, followed by an enhanced growth phase where cells appeared to proliferate within the expanded area (**Supplementary movie 1**). An initial peak in fluorescence occurred at 20 hours, and a second peak at 36 hours (**Fig3D**). After 20 hours of growth, total fluorescence was highest in cells grown on pectin, glucomannan and microcrystalline cellulose, and lowest on glucose ($p<0.05$,Tukey-Kramer HSD test).

Next, we estimated the velocity of colony expansion on the selected mono- and polysaccharides by measuring the time it took the colonies to cross three radials (3mm, 6mm and 9mm), and subsequently calculating the mean velocity from circle to circle (**Fig3E**). Initially, in the first 1.5mm radius the estimated colony expansion velocity was similar on all substances, however, between 1.5-3mm, colony expansion on pectin and glucomannan increased. Colony expansion velocity slowed slightly in the outer circle with pectin, glucomannan still exhibiting the fastest expansion rates of all the tested mono- and polysaccharides (**Fig3F**). Thus, *F. johnsoniae* expanded faster on pectin than on any of other tested carbon sources (**Fig3G**). Collectively, we conclude

that growth of *F. johnsoniae* on pectin is bi-phasic. Following an initial lag phase, there was a rapid expansion phase, followed by a slower growth phase where cells appear to spread less and replicate more gaining biomass.

Specific TonB/Sus transducers are expressed in response to growth on pectin

In order to gain deeper insight into the molecular mechanisms associated with flavobacteria colony expansion on pectin, we conducted a proteomic assay in which we examined differential intracellular protein expression after 44 hr growth between *F. johnsoniae* growing on PY2 agar coated with pectin relative to the same rich media coated with DDW. Expression of eighty-three and forty-three proteins was higher and lower, respectively on pectin relative to the DDW control (**TableS1**). Genomic annotation with various tools revealed a high proportion of unassigned proteins, while a large fraction of the proteins (17%, 22% and 37% of KEGG, SEED and EggNog annotations, respectively) were associated with carbohydrate metabolism (**FigS3A-C**). Of the 25 most markedly pectin-induced proteins identified, 13 were involved in polysaccharide uptake, processing and metabolism, including four Sus C/D related proteins (**Fig4A**). Other pectin-induced proteins included a novel transcriptional regulator (12-fold higher on pectin) and a protein associated with auxin regulation (26-fold higher on pectin) (**Table S1**). Interestingly, none of the differentially synthesized proteins was gliding related (**Table S2**).

Of the 44 previously-described SusC and 42 SusD homologues identified in the *F. johnsoniae* genome (43), 27 SusC and 15 SusD proteins were detected in the proteomic analysis, in addition to 610 proteins encoding flanking genes surrounding these Sus proteins encoding genes that constitute PUL clusters seemingly associated with glycan metabolism. Of these, three SusC and six SusD proteins along with 31 associated PUL

genes encoding proteins (forming 4 gene clusters **Table S3**) were significantly induced in response to growth on pectin in the proteomic analysis (**Table S4**).

In order to validate the proteomic results, *F. johnsoniae* cells were again grown on PY2 agar coated with pectin or DDW as described in the proteomic analysis, and the expression of 8 genes (**Table S5**) were evaluated by quantitative real time PCR. Pectin did not facilitate expression of remA (WP_012022896.1) encoding for an adhesion protein involved in gliding motility (not evaluated in the proteomic analysis since it's an extra-membranal protein), indicating that synthesis of this protein is not enhanced on pectin at the tested time point. Expression of genes encoding for the novel transcriptional regulator (Trans. Regul WP_012022876.1), pectate lyase (Pec lyase WP_012026072.1), putative auxin-regulated annotated protein (Auxin WP_012023111.1) and tonB 2144 (WP_012026069.1), were also not differentially expressed on pectin-coated media despite the fact that they were significantly induced on pectin in the proteomic analysis. Among the examined TonB/SusC related genes, tonB 260 (WP_012026229.1) and sus73 (SHH12854.1) were significantly upregulated (60-100 fold, $p<0.05$ Tukey-Kramer HSD test) while TonB 445 (WP_012022294.1) was substantially upregulated (10-20 fold) but not statistically significant (**Fig4B**). tonB260 and sus73 are part of an 18-gene cluster, of which all of the encoded proteins were upregulated in the presence of pectin in the proteomic analysis (**cluster 1-Table S3**). Using two different prediction tools, the TonB260 and Sus73 encoding genes were mapped to the same operon together with a gene encoding for the hydrolytic enzyme polygalacturonase that cleaves the α (1-4) bonds between adjacent galacturonic acids within the homogalacturonic acid backbone of pectin (**Table S6**).

Discussion

Roots modulate their microbiome by secreting exudates, mucilage and other components that can attract or deter bacteria (8, 47, 50), and specific root exudates were found to serve as chemoattractants for microbes that colonize roots (51). However, this seems to be the tip of the iceberg, and little is known about the plant-associated factors that recruit bacteria from the ecologically complex rhizosphere to the surface and endosphere of roots.

Bacterial colonization of plant roots is a multi-stage process that involves recognition, movement towards attractants and attachment (15). Once attached, persistence and propagation on plant roots is dependent on metabolic fitness (52, 53)**,** resistance to plant protective mechanisms and inter-microbial interactions. The abundance of bacteria in rhizosphere soil is about 10^2 higher than in the rhizoplane and root endosphere (54, 55). This exemplifies the extreme selective pressure of this highly competitive environment and the fitness requirements of the root-colonizing microbiome**.**

Members of the genus *Flavobacterium* are highly abundant and substantially enriched (relative to the rhizosphere soil) on, and within roots of several plant species (25, 32, 33, 56, 57), and certain strains have been found to augment plant health and suppress pathogens in a variety of plants (29, 32, 33, 35). In this study, we explored the effect of plant-derived mono- and polysaccharides on colony expansion of flavobacteria, in attempt to elucidate mechanisms associated with plant root-flavobacterial interactions. It was previously shown that *F. johnsoniae* can effectively grow on polysaccharides such as pectin and glucomannan as single carbon sources (43). Collectively, results from this study demonstrate that flavobacteria not only metabolize complex plant cell wall-derived glycans such as pectin, glucomanan and microcrystalline cellulose, but

that these polysaccharides (particularly pectin) also facilitate rapid spreading of flavobacteria over solid surfaces. We postulate that this plant cell wall glycan recognition-uptake mechanism is critical for proliferation of flavobacteria on plant roots.

Interestingly, our results demonstrated that even when carbon was not limited, plant cell wall glycans and especially pectin, facilitated colony expansion of terrestrial flavobacterial strains over solid surfaces. While the specific correlation between expansion on pectin and the unique flavobacterial gliding motility mechanism was not determined in this study, it is evident that the latter is required, because ΔgldJ mutants did not expand on pectin. Propagation on pectin occurred in a concentration dependent manner, and the response was specifically dependent on the mother compound, as the response did not occur with bacteria grown on the pectin monomers D-galacturonic acid and L-rhamnose. In contrast, growth on glucose was found to inhibit propagation of flavobacteria as was previously demonstrated by others (48, 49). Specifically, glucose was shown to inhibit colony spreading via MfsA, (49), which encodes a major facilitator superfamily (MFS) transporter and creation of raft microstructures, but does not inhibit gliding motility in general (47, 48).

Patterns and extent of *F. johnsoniae* colony expansion was dictated by the carbon source supplemented to the growth media. The expansion of flavobacterial colonies on rich media coated with plant-derived polysaccharides (pectin and glucomanan) was rapid, patchy, non-uniform and sparse relative to colonies grown on rich media coated with glucose or DDW. The rapid expansion of flavobacteria on pectin was strongly linked to induction of TonB-related proteins, which undoubtedly assist in binding (recognition), uptake and metabolism of this glucan. Therefore, we hypothesize that for root-associated flavobacteria, pectin is not merely a carbon source but also an

environmental cue. To the best of our knowledge, this is the first time that a specific root-associated factor was found to be specifically linked to flavobacterial spreading.

There are only a few examples of plant cell wall-associated polysaccharides that facilitate root bacteria colonization and proliferation. Flexibacter FS-1 is not able to glide on agarose (unless supplemented with cold water-soluble fraction of agar) but can glide on agarose amended with 1% pectin, while galacturonic acid does not induce a similar effect (58). Purified Arabidopsis polysaccharides (arabinogalactan, pectin, or xylan) triggered biofilm and pellicle formation in *B. subtilis* when added to rich media and induced root colonization (6). Similarly, addition of pectin and sucrose to the media of the PGPR *Bacillus amyloliquefaciens* strain SQY 162, increased bacterial abundance, induced biofilm formation and improved the ability of the amended bacterium to suppress tobacco bacterial wilt disease (59). In the symbiotic nitrogen fixing bacterium *Rhizobium leguminosarum*, glucomannan-mediated attachment was important for legume infection and nodulation (12).

We did not observe any evidence of chemotactic behavior towards pectin, and therefore postulate that propagation on pectin requires direct contact between the flavobacteria and the glycan. This is logical given the large size and low solubility of this plant polysaccaride. Based on proteomics and subsequent qPCR (quantitative real-time PCR) gene expression validation, we believe that this phenomenon is dictated by the induction of TonB-associated *F. johnsoniae* PULs, suggesting a link between niche recognition, colony expansion and metabolic fitness. Similar induction of TonB and PUL was observed in marine flavobacteria as response to phytoplankton blooms characterized in decomposition of alga-derived organic matter (60).

Their high energetic cost and substrate specificity of TonB transducers explains why the genes encoding them were induced by pectin and not constitutively expressed (61, 62). Nonetheless, the specific regulatory mechanism responsible for facilitating this response and the subsequent linkage to the induced gliding motility in flavobacteria remain an enigma. This is especially true in view of the fact that pectin did not induce expression of remA, the lectin binding, flavobacterial cell surface adhesin involved in gliding (63). The gliding machinery might be involved in the earlier phase of the response to pectin, in which we observed intensive bacterial motility. Alternatively, pectin might facilitate gliding in a protein expression-independent manner, or induce other components of the gliding machinery not identified in the proteomic analyses.

Previous experiments showed that *Pseudomonas putida* mutants that lacked TonB, were deficient in their capacity to uptake iron and displayed impaired seed colonization, again linking TonB to metabolic and functional fitness (64). However, knock-out of specific tonB genes might be less effective in *F. johnsoniae* due to the multitude of predicted tonB genes in its genome, which suggests a high level of functional redundancy (43). Similar functional redundancy was shown in proteomic analysis of the soil bacterium *Cytophaga hutchinsonii* where multiple cellulolytic enzymes were induced after growth on microcrystalline cellulose (65).

In *Xanthomonas campestris pv. Campestris*, pectate sensed by specific TonB-dependent receptors triggered secretion of extracellular polygalacturonate. This resulted in pectin degradation and generation of oligogalacturonides (OGA) that are recognized as damage-associated molecular patterns (DAMPs), facilitating the initiation of the plant defense mechanisms (66). In light of our results and the findings in *X. campestris*, we speculate that sensing, uptake and degradation of pectin by TonB and associated proteins, results in OGA release, and plant immune response activation.

This pathway may explain the biocontrol and improved plant disease resistance induced by flavobacteria in various plant types. Further experiments will be needed to support these assumptions.

Beside TonB related proteins, pectin induced additional protein targets. The up regulation of auxin-regulated protein is especially interesting since auxin is a major phytohormone responsible for plant growth and development demonstrating again a possible connection between pectin sensing and flavobacterial-plant interactions. The additional upregulation of the putative transcriptional regulator may suggest that pectin induces a downstream response.

Gene expression (qPCR) analysis did not completely correlate with the proteomics analysis, when comparing flavobacterial colonies growing on media amended with pectin relative to the control without pectin. The discrepancy between mRNA and protein expression might be explained by post-translational modifications or regulation affecting protein stability, degradation and complex formation, as shown in similar cases (67, 68).

Live-imaging fluorescent microscopy revealed that *F. johnsoniae* growth on PY2 media coated with different mono- and polysaccarides was bi-phasic in nature, with an initial phase of rapid expansion, followed by biomass production within the colonized area. This bi-phasic growth pattern, which was most pronounced on pectin, resembles previously described models in motile *E. coli*, which depicted an initial expansion phase, where "pioneer" bacteria with high velocity advance in front of the colony, followed by a second phase, where "settler" bacteria grow and replicate locally (16, 69). Collectively, we hypothesize that plant-derived polysaccharides and especially pectin

may serve as signal that facilitates the expansion of "pioneer" cells, and later as carbon sources that support growth of "settlers".

To summarize, we found that pectin, a prominent plant cell wall polysaccharide, facilitates expansion of flavobacteria on solid surfaces, even in the presence of nutrient-rich media. We postulate that pectin may be a "missing link" in elucidating the mechanisms responsible for rhizosphere competence in flavobacteria, *i.e.* their capacity to efficiently colonize and proliferate in the root niche. Thus, in the root environment, plant cell wall polysaccharides, and specifically pectin, may not only serve as a nutrient source for flavobacteria, but also as a potential cue for colonization and rapid expansion along the root surface. The interaction between pectin (and potentially other root glycans) and flavobacteria is mediated by induction of TonB/SusC operons and other associated PULs that facilitate metabolism of pectin.

Experimental Procedures

Bacterial Strains and growth conditions

Flavobacterial strains targeted in this study included *F. johnsoniae* strain UW101 (ATCC17061), a gliding/secretion impaired (ΔgldJ) *F. johnsoniae* strain UW101, *Flavobacterium* sp. F52, isolated from the roots of a greenhouse pepper (70, 71), and a gliding/secretion impaired (ΔgldJ) *Flavobacterium* sp. F52 strain. For live imaging microscopy (see below) the fluorescent strain *F. johnsoniae* UW101 WT+pAS43 (Flavo GFP) Flavo-ErytRFlavo-CefR, and the gliding/secretion impaired *F. johnsoniae* UW102-48 (ΔgldJ)+pAS43 (Flavo GFP) Flavo-ErytR Flavo-CefR were used. Erythromycin, 100 ug/ml was added to the media of the GFP labeled bacteria.

Flavobacteria strains were grown in CYE medium (Casitone, 10 mg/ml, yeast extract at 5mg/ml, and 8 mM $MgSO_4$ in 10 mM Tris buffer [pH 7.6]) at 30°C, as previously

described (72). To observe colony-spreading, bacteria were grown on PY2 agar medium (2 g peptone, 0.5 g yeast extract,10 mg/ml agar, pH 7.3) at 30°C (73).

Organic amendments to growth media

A suite of mono- and polysaccharides were amended to growth media in various configurations (as described below), to evaluate colony spreading dynamics of the selected flavobacterial strains. The following substances were used in this study: pectin (Sigma P9135), arabinose (Sigma A9462), D(+)glucose (Fischer scientific 10373242), microcrystalline cellulose (partially depolymerized cellulose synthesized from an α-cellulose precursor, Merck 102331), D(-)arbinose (Acros 161451000), glucomannan (Megazyme P-GLCML), chitin (Sigma C7170), L-rhamnose (Sigma 83650) and D(+)-galacturonic acid monohydrate (Sigma 48280-5G-F). All substances were dissolved and suspended to 2% final concentration in DDW. Glucose, arabinose, rhamnose and galacturonic acid (titrated to pH 7) were dissolved and filtered through a 0.22-micron filter. Pectin was dissolved in DDW heated at 80°C, and subsequently filtered through 0.45-micron filters. Chitin, microcrystalline cellulose, and glucomannan were mixed with DDW heated to 50°C and then autoclaved for 20 min.

Flavobacterial growth on various sugars

The selected sugars were amended to the PY2 agar plates in two manners: (i) to test the effect of specific carbon sources on the directional proliferation of flavobacteria, 10 µl of the selected compounds were thinly applied along a line projecting outward from the center of the petri dish using a pipetor (**Fig2S**); (ii) to test the effect of specific carbon sources on the general proliferation of flavobacteria, 500µl were uniformly smeared over the entire petri dish (**Fig1A, C, Fig2A**). Non-soluble substances such as microcrystalline cellulose and chitin, were vigorously vortexed and dispensed using a

cut tip. Where two sugars were used (*i.e.* rhamnose+ galacturonic acid) we added 250µl of each. In all cases, plates were left to dry overnight after adding organic amendments. Flavobacteria were incubated on CYE media overnight, colonies were harvested and subsequently diluted in 200ul saline to 0.6-1 OD ($5x10^9$-$1.12\ x10^{10}$ cells), vortexed well, and 2ul were spotted in the center of PY2 agar covered or streaked with the selected sugars as indicated above. Plates were left to dry for 15min and then incubated for 48 hours at 30°C. The colony area or the length of expansion (in cm) were measured using Fiji (74) and statistics was calculated using JMP®, Version Pro 14 (SAS Institute Inc., Cary, NC, 1989-2019). Differences between length/area were considered as significantly different when $p<0.05$ in Tukey HSD test unless indicated differently.

RNA extraction

To assess the expression of selected genes in the presence of pectin, total RNA was extracted from *F. johnsoniae* cells grown for 48 hours at 30°C on PY2 plates covered with 500µl of DDW or 2% pectin as described above. For each plate, bacteria were suspended in 1 ml cold (4°C) PBS buffer, washed once in cold PBS, centrifuged for 2min at 18,000g, resuspended in TE buffer supplemented with 0.4mg/ml lysozyme and then incubated for 10 min at room temperature. RNA was subsequently extracted from cells using the TRIzol reagent (TRIzol® InvitrogenTM, #15596026), following the manufacturer's instructions. Residual DNA was removed from the RNA samples by digesting with RQ1 DNAse (Promega M6101A) at 37 °C for 40 min. For the real time experiments, cDNA was synthesized using 50ng of DNAse treated RNA, with 1ul of random primers (Promega C118A). Synthesis of single strand cDNA was achieved using ImProm-IITM Reverse-Transcriptase (Promega, Madison, WI, United States).

The integrity and concentration of the extracted RNA and cDNA, was examined with a QubitTM 3.0 Fluorometer (Thermo Fisher Scientific, United States) using reagents and protocols supplied by the manufacturer, and by electrophoresis of samples on a 0.8% agarose gel.

Live Imaging Fluorescent Microscopy Experiments.

To visualize the effect of different mono- and polysaccharides on *F. johnsoniae* colony expansion, we filled the wells of a 24 well plate with 500µl of PY2 agar. Subsequently, 10ul of the mono- and polysaccharides (2%) or the DDW control were gently applied to the wells in triplicates, plates were rotated for 1hour and dried overnight at room temperature. A 30G needle (KDL 16-0310) was used to seed the bacteria in the center of each well. Microscopic imaging was performed using a NIKON eclipse Ti microscope (Nikon, Japan) equipped with a ProScan motorized XY stage, an HF110A system (enabling rapid switching of emission filters)(Prior Scientific, MA, USA) and a temperature-controlled stage incubator (LAUDA ECO RE 415, Korea). Bright field illumination was provided by a cool LED pE-100A (Cool LED, UK). Excitation light for epifluorescence microscopy was provided by a Spectra X light engine (Lumencor, USA). Imaging was performed using a long working distance 40x objective (NA 0.6) (Nikon, Japan). Images were captured at 2 hr intervals for 44 hr using an ANDOR zyla 5.5 MP sCMOS camera (Oxford Instruments, UK).

Images were processed using the NIS elements AR 4.6 (64 bit) software package (Nikon, Japan) and Fiji (74). Fluorescence level was normalized to the initial measured value (to avoid differences in the initial number of seeded bacteria) and to the maximal fluorescence on PY2 media amended with DDW (to reduce variability of GFP fluorescence levels between movies). The population growth dynamic of GFP- *F.*

johnsoniae on each substance computed using Fiji's time series analyzer plugin, and average fluorescence density profiles of the expanding population over time was quantified using JMP®, Version Pro 14 . (SAS Institute Inc., Cary, NC, 1989-2019).

Proteomics Sample preparation

Samples were subjected to in-solution tryptic digestion using a modified Filter Aided Sample Preparation protocol (FASP). Sodium dodecyl sulfate buffer (SDS) included: 4% (w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT. Urea buffer (UB): 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.0 and 50mM Ammonium Bicarbonate. Bacterial cell pellets were lysed in SDS buffer. Lysate was centrifuged at 16,000 g for 10min. 50 ug total protein were mixed with 2 mL UB, loaded onto 30 kDa molecular weight cutoff filters (Sartorius VS15RH22) and centrifuged. 1.5 ml of UB were added to the filter unit and centrifuged at 14,000 g for 40 min. Proteins were alkylated using iodoacetamide (10 mM final concentration) and washed twice with Ammonium Bicarbonate. Trypsin was then added (50:1 protein amount:trypsin) and samples incubated at 37°C overnight, followed by a second trypsin digestion for 4 hours at 37°C. Digested peptides were then collected in a clean tube by centrifugation, acidified with trifloroacetic acid, desalted using HBL Oasis (Waters 094225), speed vaccumed to dryness and stored in -80°C until analysis. All chemicals used were from Sigma Aldrich, unless stated otherwise. Sample preparation and initial statistical analysis was performed at the Nancy and Stephen Grand Israel National Center for Personalized Medicine, The Weizmann Institute of Science, Rehovot, Israel.

Liquid chromatography and proteomics analysis

Each sample was loaded and analyzed using split-less nano-Ultra Performance Liquid Chromatography (10 kpsi nanoAcquity; Waters, Milford, MA, USA). The mobile phase was:

A) H_2O + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online using a Symmetry C18 reversed-phase trapping column (180 µm internal diameter, 20 mm length, 5 µm particle size; Waters). The peptides were then separated using a T3 HSS nano-column (75 µm internal diameter, 250 mm length, 1.8 µm particle size; Waters) at 0.35 µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 20%B in 155 min, 20% to 90%B in 5 min, maintained at 90% for 5 min and then back to initial conditions. ULC/MS grade solvents were used for all chromatographic steps.Each sample was analyzed on the instrument separately in a random order in discovery mode. Raw data was searched against the *F. johnsoniae* protein databases, to which a list of common lab contaminants was added. Database search was done using the Mascot algorithm, and quantitative analysis was performed using the Expressionist software from GeneData. As a rule of thumb we consider significant differences to be >1 peptide per protein, fold change >2 or <0.5, and <0.05 p-value. Proteins were functionally annotated using RAST (Rapid Annotations using Subsystems Technology) (75).

Twenty-five pectin-induced proteins were selected based on high fold change between PY2 agar coated with pectin vs. PY2 agar coated with DDW and statistical significance (proteins without annotation were removed from this analysis). Gene abundance across the dataset was normalized to 100% for each gene, and a heatmap was created using studio.plot.ly (https://plotly.com/).

Quantitative PCR Assessment of Gene Expression Levels

The expression of 10 genes encoding for proteins found to be significantly induced on pectin in the proteomic analyses were analyzed using qPCR. Primers for qRT-PCR experiments (**Table S5**) were constructed based on the *F. johnsoniae* genome sequence and were pre-designed using the PrimerQuest® tool (Integrated DNA Technologies,

USA). Triplicate cDNA samples for each of the treatments (with or without pectin) were diluted and 2ng was used in a 20 µl final reaction volume together with 10ul Fast SYBR^{TM} green PCR master mix (Thermo Fisher scientific), 100 nM each of forward and reverse primers, DDW and 1ul of template cDNA. Amplification was carried out on a StepOnePlus real-time PCR thermocycler (Applied Biosystems, Foster City, CA, United States) using the following program: heat activation of DNA polymerase at 95°C for 3 min and 40 cycles at 95°C for 5 sec for denaturation and primer annealing and extension at 60°C for 30 sec. A melting curve was produced to confirm a single gene-specific peak and to detect primer-dimer formation by heating the samples from 60 to 95°C in 0.3°C increments. Gene amplification was confirmed by detection of a single peak in the melting curve analysis. For each gene, PCR gene amplification was carried out using three independent biological replicates. Expression of each of the targeted genes was normalized to that of three alternative housekeeping genes (16S rRNA, DNA gyrase subunit B (*gyr*B, EC 5.99.1.3) , and the electron transfer flavoprotein, alpha subunit (ETF) threonine synthase (EC 4.2.3.1). These genes were selected because there was no detected difference in their expression when grown on PY2 media amended with pectin *vs.* DDW in the proteomic analysis. The relative abundance of each target gene relative to a reference gene was determined according to the method described previously (Livak and Schmittgen2001). Concentrations and ΔΔCT values were calculated and analyzed with the StepOne software v2.3 (Applied Biosystems, Foster City, CA, United States). Concomitant "no-RT" reactions, lacking reverse transcriptase, were performed for each sample and run to confirm absence of DNA contamination, as well as no template controls (NTCs) to confirm lack of contamination. Reaction efficiency was monitored by means of an in internal standard curve using a 10-fold dilution of DNA ranging from 0.01-10ng of DNA per

reaction, in triplicates. Efficiency was between 92.1 and 97.2% for all primers, and R2-values were greater than 0.99. Data analysis was conducted using the StepOne software v2.3 (Applied Biosystems, Foster City, CA, United States).

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Figures

Fig1- Impact of sugars on proliferation of flavobacteria. Image (A) and quantification (B) of colony area of *F. johnsoniae* colony expansion on PY2 agar amended with different mono- and polysaccharides: arabinose, glucose, microcrystalline cellulose (M.Cellulose), pectin and no sugar control, after 48hr incubation at 30°C (N=4). Image (C) and quantification (D) of colony expansion on PY2 agar of wild type (WT) and gliding mutants (ΔgldJ) of *F. johnsoniae* and *Flavobacterium* sp. F52, amended with DDW or 2% pectin after 48hr incubation at 30°C (N=3). Colony area was measured using Fiji. Letters indicate statistical significance calculated using ®JMP Pro14, was considered significant if $p<0.05$ by Tukey

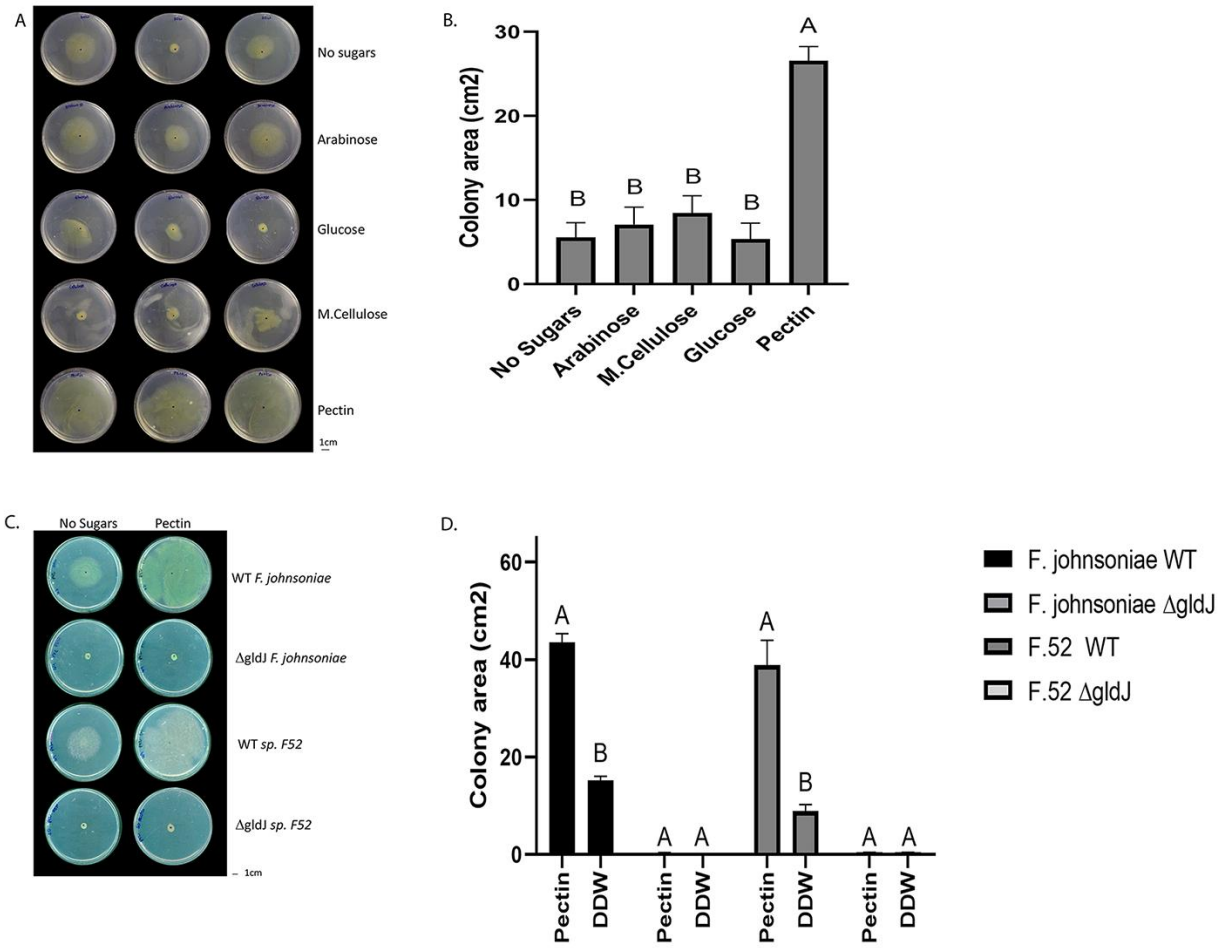


Fig2 - Impact of pectin precursors (galacturonic acid and rhamnose) on proliferation of flavobacteria. (A) *F. johnsoniae* colony expansion on PY2 agar amended with pectin, galacturonic acid, rhamnose, and galacturonic acid and rhamnose or no sugar control incubated at 30°C for 48 h (N=4). (B) Graphic description of colony area based on results from (A). Letters indicate statistical significance, calculated using JMP Pro14®, and differences between treatments were considered significant when $p<0.05$ by Tukey HSD.

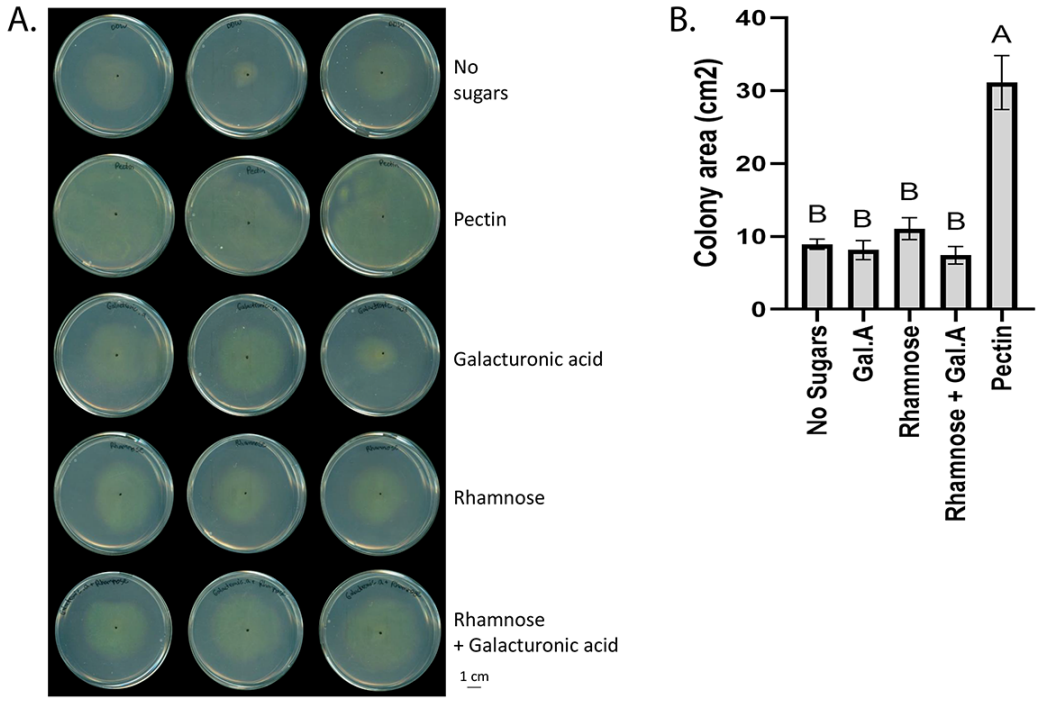
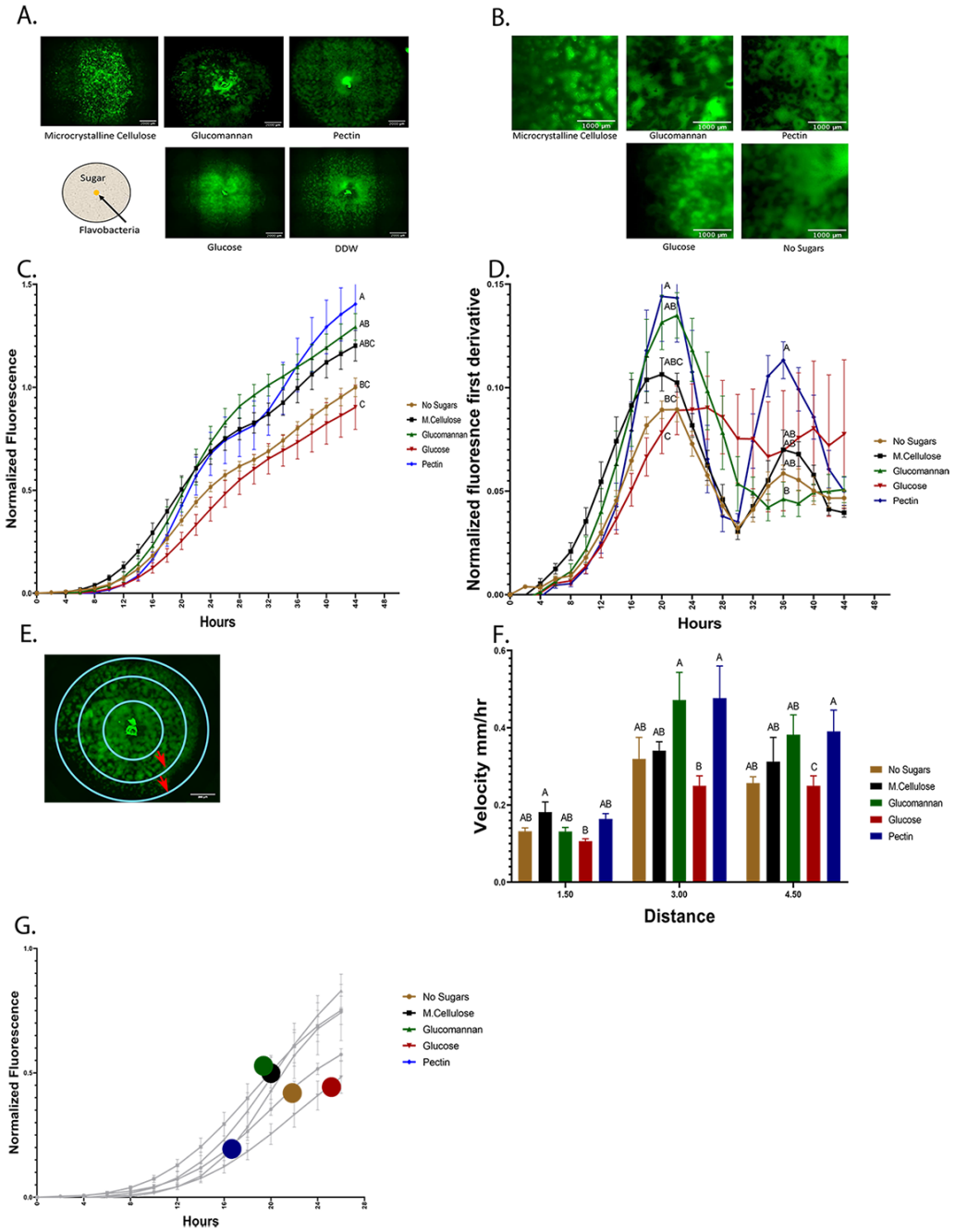


Fig3 - Temporal dynamics of flavobacterial proliferation on polysaccharides using live imaging microscopy. (A) Morphology of GFP-labeled *F. johnsoniae* colonies on selected sugars and polysaccharides. Bacteria were inoculated in the center of PY2 agar coated with the indicated sugars and polysaccharides (schematically described in the insert). Images show colony morphology after 32hr. (B) Enlarged image of GFP-*F. johnsoniae* colony morphology after 20hr of growth on the selected sugars and polysaccharides as indicated in (A). (C) Growth rates of GFP-*F. johnsoniae* colonies on the selected sugars and polysaccharides. Data was normalized as described in the materials and methods section. Differences in the average colony fluorescence intensity after 44 hr was compared and considered significant if $p<0.05$ by Tukey HSD (indicated by letters). Data includes means and data from three biological replicates composed of three technical repeats in each. (D) Temporal dynamics of GFP-*F. johnsoniae* growth rates. Growth was compared at the peaks (20hr and 36hr) and considered significant if $p<0.05$ by Tukey HSD. (E) Schematic diagram showing the three characterized regions of interest (ROI- 1.5, 3 and 4.5 mm radii) used to evaluate of bacterial expansion rates. (F) Estimated expansion rates of GFP-*F. johnsoniae* on the selected sugars and polysaccharides. Velocity was estimated by expansion time in hours taken to cross known ROIs as indicated in E. (G) Estimated expansion time relative to estimated growth of GFP-*F. johnsoniae* on the selected sugars and polysaccharides. Colored circles mark time (h) for bacteria to cross the 4.5mm radius on each substance as calculated in F. Differences between treatments were considered significant when $p<0.05$ by Tukey HSD


A.
Microcrystalline Cellulose
Glucomannan
Pectin
Sugar
Flavobacteria
Glucose
DDW
B.
Microcrystalline Cellulose
Glucomannan
Pectin
Glucose
No Sugars
C.
Normalized Fluorescence
Hours
No Sugars
M.Cellulose
Glucomannan
Glucose
Pectin
D.
Normalized fluoresnce first derivative
Hours
No Sugars
M.Cellulose
Glucomannan
Glucose
Pectin
E.
F.
Velocity mm/hr
Distance
No Sugars
M.Cellulose
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Glucomannan
Glucose
Pectin
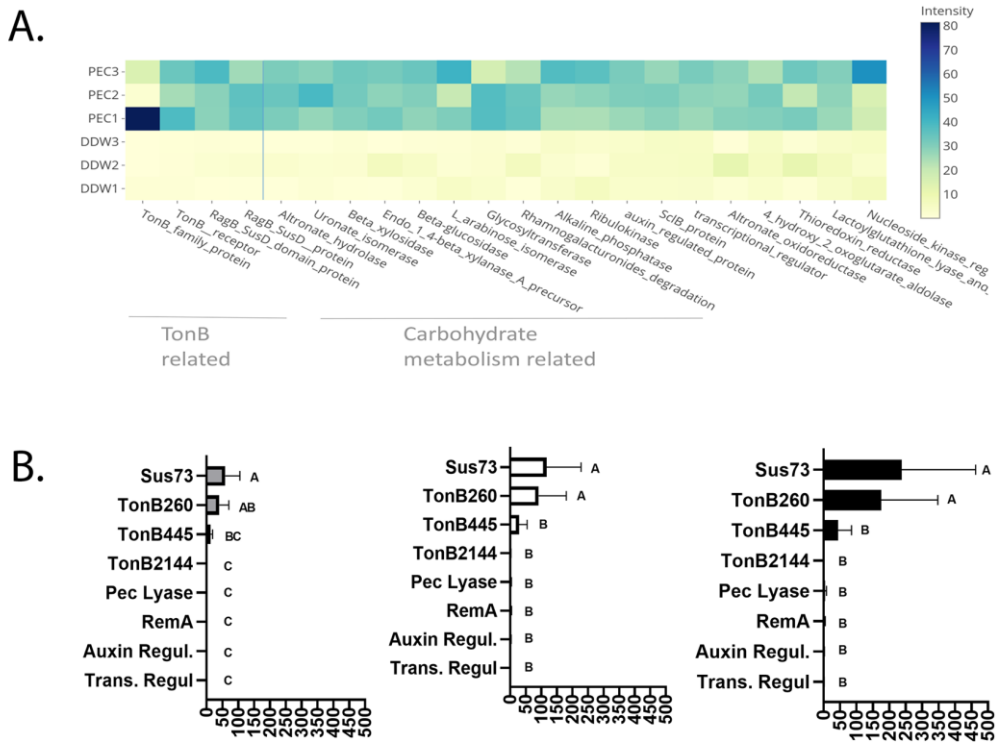
Fig4 - Pectin induced flavobacterial genes and proteins. (A) Differential expression of the 25 most substantial pectin induced proteins based on proteomic analysis of *F. johnsoniae* colonies grown on PY2 medium amended with pectin relative to colonies grown on identical media amended without pectin (DDW). Heat map shows triplicates for each treatment. All described proteins are statistically significant ($p<0.05$). (B) The mRNA expression level of selected proteins (Sus73 (SHH12854.1), TonB260 (WP_012026229.1), TonB445 (WP_012022294.1), TonB 2144 (WP_012026069.1), Auxin regulator (WP_012023111.1), Transcriptional regulator (WP_012022876.1), Pectate lyase (WP_012026072.1) and RemA(WP_012022896.1) shown to be induced in the proteomic analysis described in (A), using quantitative real-time PCR (qPCR). Fold changes in mRNA levels of the target genes were normalized against the 16SrRNA gene (left), the Electron transfer Flavoprotein, alpha subunit (WP_012023552.1) (center), and the DNA gyrase subunit B (WP_012024321.1)(right). Change in target genes fold change RNA expression was calculated using the $2^{-\Delta\Delta CT}$ method and statistical significance ($p<0.05$) by Student T-test. Error bars represent standard errors of six independent experiments based on two independent RNA extraction.