Regulation of the *Pantoea agglomerans* type III secretion system by plant-exuded sugars

by
Daniel Bacher

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
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degree of

Honors Baccalaureate of Science in Biochemistry and Molecular Biology
(Honors Scholar)

Presented May 26, 2023
Commencement June 2023

AN ABSTRACT OF THE THESIS OF

Daniel Bacher for the degree of Honors Baccalaureate of Science in Biochemistry and Molecular Biology presented on May 26, 2023. Title: Regulation of the *Pantoea agglomerans* type III secretion system by plant-exuded sugars.

Abstract approved:_____

Jeff Anderson

Pantoea agglomerans pathovar *betae* (*Pab*) is a bacterium that causes galls on the roots of beet plants, resulting in reduced marketability of beet roots and crop loss. *Pab* uses a type III secretion system (T3SS) to infect and cause galls on beets. The T3SS is a molecular syringe-like structure that injects proteins, termed "effectors" into plant cells. Once inside the cells, effectors target and suppress the plant's immune signaling pathways, allowing the pathogen to avoid the host immune response. How *Pab* regulates the deployment of its T3SS during the infection process is poorly understood. For other T3SS-containing plant pathogens, plant-derived metabolites act as signals that induce transcription of genes encoding for T3SS regulatory proteins. In this study, we identified metabolites exuded from beet seedlings using gas chromatography-mass spectrometry (GC-MS). Among these metabolites, we identified several plant-exuded sugars. We then tested if the T3SS regulatory genes *hrpXY*, *hrpS*, and *hrpL* are induced by these sugars, as well as by several sugars not found in beet exudate. We found that *hrpXY* and *hrpL* were both strongly induced in response to each of the sugars we tested, regardless of whether the sugar was found in beet exudate or not. In contrast, *hrpS* expression was not strongly induced in response to any sugars tested. We also found that the growth of *Pab* was induced by the same sugars, and that the amount of growth and *hrp* transcription were positively correlated. The close correlation between sugar induced T3SS induction and growth suggests a potential link between rates of sugar catabolism or sugar uptake in determining the relative response of *Pab* to the various sugars tested.

Keywords: Type III Secretion System, *Pantoea agglomerans*, virulence, chemical signaling, gene expression, *hypersensitive response and pathogenicity* cluster, transcriptional reporter assay

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Daniel Bacher, Author

Regulation of the *Pantoea agglomerans* type III secretion system by plant-exuded sugars

ABSTRACT

Pantoea agglomerans pathovar *betae* (*Pab*) is a bacterium that causes galls on the roots of beet plants, resulting in reduced marketability of beet roots and crop loss. *Pab* uses a type III secretion system (T3SS) to infect and cause galls on beets. The T3SS is a molecular syringe-like structure that injects proteins termed "effectors" into plant cells. Once inside the cells, effectors target and suppress the plant's immune signaling pathways, allowing the pathogen to avoid the host immune response. How *Pab* regulates the deployment of its T3SS during the infection process is poorly understood. For other T3SS-containing plant pathogens, plant-derived metabolites act as signals that induce transcription of genes encoding for T3SS regulatory proteins. In this study, we identified metabolites exuded from beet seedlings using gas chromatography-mass spectrometry (GC-MS). Among these metabolites, we identified several plant-exuded sugars. We then tested if the T3SS regulatory genes *hrpXY*, *hrpS*, and *hrpL* are induced by these sugars, as well as by several sugars not found in beet exudate. We found that *hrpXY* and *hrpL* were both strongly induced in response to each of the sugars we tested, regardless of whether the sugar was found in beet exudate or not. In contrast, *hrpS* expression was not strongly induced in response to any sugars tested. We also found that the growth of *Pab* was induced by the same sugars, and that the amount of growth and *hrp* transcription were positively correlated. The close correlation between sugar induced T3SS induction and growth suggests a potential link between rates of sugar catabolism or sugar uptake in determining the relative response of *Pab* to the various sugars tested.

INTRODUCTION

Pantoea agglomerans pathovar *betae* (*Pab*) is a bacterium that is pathogenic towards sugar beet, an important crop used globally for animal feed and sugar. *Pab* forms galls on the roots of beet plants, and these galls can affect crop viability and cause significant losses for farmers. *P. agglomerans* likely became a plant pathogen by acquiring a pathogenicity plasmid (pPATH) that carries multiple genes required for virulence (Manulis et al., 1991). However, it is not well understood why the host range of *Pab* is restricted to beets. Studying the interaction between *Pab* and the host beet plants may lead to new disease prevention strategies that protect against infection and improve crop yields.

Pab uses a type-III secretion system (T3SS) to cause galls on host plants. The T3SS is a syringe-like molecular structure that injects proteins into the host cells to suppress immunity and may also contribute to gall formation (Nissan et al., 2017). The T3SS of *Pab* is not expressed in the absence of its host, so there must be a way for *Pab* to recognize the host plant. How host recognition is achieved by *Pab* is currently unknown. In *Pab*, the T3SS is encoded by genes within the *hypersensitive response and pathogenicity* (*hrp*) cluster on the pPATH plasmid (Manulis et al., 1991). The gene cluster includes four genes, *hrpX*, *hrpY*, *hrpS*, and *hrpL*, that encode for the components of a transcriptional regulatory cascade that regulates transcriptional activation of the *hrp* box promoter (Mor et al., 2001, Nizan-Koren et al., 2002) **(Figure 1)**. HrpXY is a known two-component protein system that directs expression of *hrpL* which encodes

for the protein HrpL, an alternative sigma factor that activates expression of the T3SS. HrpX is a sensor kinase which, upon sensing a yet unknown signal, possibly a plant-derived signaling molecule, phosphorylates HrpY. HrpS is an intermediate between HrpY and HrpL and is an enhancer-binding protein that likely functions as a transcriptional regulator of *hrpL* (Wei et al., 2000). The *hrp* box is a cis regulatory element that HrpL binds to, found within the promoter region of all known T3SS effector genes (Shen & Keen, 1993, Xiao & Hutcheson, 1994).

The gall-forming ability of *Pab* is dependent on expression of genes within the *hrp* cluster, but most *hrp* genes are only expressed when *Pab* are in contact with the host plant, suggesting *Pab* has a mechanism for sensing and responding to the environment encountered in the host. This mechanism, however, remains unknown.

Host signals that induce virulence have been identified for other plant pathogenic bacteria. In *Pseudomonas syringae* pv tomato strain DC3000, a pathogen of tomato and the model plant Arabidopsis, specific organic acids, amino acids, and sugars found in fluid exuded from plants (exudate) strongly induce *hrp* gene expression (Anderson et al., 2014; Turner et al., 2020). In the fire blight pathogen *Erwinia amylovora*, phenolic compounds derived from plants have been shown to induce T3SS-associated genes, including the *hrp* pathway (Khokhani et al., 2013). *Erwinia amylovora* is a closely related bacteria within Erwiniaceae family with *P. agglomerans* that infects plants in the Rosacea family, such as apples and pears.

In this study, we used identified gas chromatography-mass spectrometry (GC-MS) to identify soluble metabolites exuded from beet seedlings. Among the metabolites identified we identified multiple sugars as components of the exudate. We used a GFP transcriptional reporter plasmid to test the expression of *Pab* genes *hrpXY*, *hrpS*, and *hrpL* in response to a panel of simple sugars, including several sugars identified in the beet exudate. By testing changes in the expression of genes in the *hrp* regulatory cascade of the *Pab* T3SS, we identified sugars that are potentially significant in helping the bacteria to recognize host cells and express its T3SS, thus giving us information into how the *Pab* T3SS may be regulated. We also examined whether these same genes are induced by beet seedling exudate.

METHODS

Preparation of Arabidopsis and beet exudates. Beets (*Beta vulgaris*) were grown in a 10-14 light-dark cycle on 0.5X Murashige and Skoog (MS) medium solidified with 2.87% plant agar and supplemented with 20 g/L sucrose. To produce exudate, each seedling was soaked in 1 mL of deionized water for 24 hours. The exudate was filter-sterilized and stored at -20°C until further use. Arabidopsis (*Arabidopsis thaliana*) exudate preparation was the same, except seedlings used were one week old.

Bacterial storage and culture conditions. Bacterial stocks were stored in 25% glycerol at -80°C. *Pantoea agglomerans* pv *betae* (*Pab*) 4188 were cultured on Luria-Bertani (LB) rich medium solidified with 1.5% agar (Luria & Burrous, 1957). *Pseudomonas syringae* DC3000 were cultured on King's B (KB) rich medium solidified with 1.5% agar (King et al., 1954). The LB and KB agar were supplemented with 30 μg/mL kanamycin antibiotic for plasmid selection and 50 μg/mL rifampicin antibiotic for strain selection. Bacteria were grown on agar for 24 hours at 28°C prior to use.

Gibson assembly of *hrpS*$_{promoter}$:*gfp* (1 kb) plasmid. *E. coli* carrying the plasmid pPROBE-NT (Miller et al., 2000) were inoculated into LB broth supplemented with 30 µg/mL kanamycin for plasmid selection, and the inoculated cultures were grown at 28°C for 24 hours. The Qiagen plasmid midi kit (#12123) was used to purify the pPROBE-NT plasmid from the cultured bacteria, and the purified plasmid DNA was digested with the SmaI endonuclease. *Pab* genomic DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit (#27106) from an overnight culture of *Pab*. Oligonucleotides A and B were used to PCR amplify a region approximately 1 kb upstream of *hrpS* from the isolated gDNA **(Table S1)**. The NEBuilder Hi-Fi DNA Assembly Master mix (NEB #E2621S) was used to ligate the PCR product into the linearized pPROBE-NT, with the product containing the 1 kb *hrpS*$_{promoter}$ upstream of *gfp*. The assembled *hrpS*$_{promoter}$:*gfp* pPROBE-NT construct plasmid was mixed with chemically competent DH10β *E. coli* cells and incubated at 42°C for 45 seconds. After heat shock, the cells recovered in LB broth for 1 hour at 28°C, then plated on LB agar with 50 µg/mL rifampicin and 30 µg/mL kanamycin. After 24 hours, transformants were confirmed by colony PCR using oligonucleotides C and D **(Table S1)** and by Sanger sequencing to confirm the presence of the *hrpS*$_{promoter}$ insert.

Triparental mating to clone pPROBE-NT *hrpS*$_{promoter}$:*gfp* into *Pab*. The *Pab* 4188 parental strain, *E. coli* DH10β pPROBE-NT *hrpS*$_{promoter}$:*gfp* strain, and *E. coli* pRK600 helper strain bacteria were grown on LB agar containing, 50 µg/mL rifampicin, 30 µg/mL kanamycin, and 30 µg/mL chloramphenicol, respectively. Each bacterial culture was centrifuged, and the resulting bacteria pellet resuspended in 1 mL of sterile deionized water. To transfer the plasmids from *E. coli* to *Pab* via conjugation, 100 µL of the *Pab* parent strain, 20 µL of the DH10β pPROBE-NT *hrpS*$_{promoter}$:*gfp* strain, and 20 µL of the pRK600 helper strain were mixed, and 20 µL of the mixture was plated on a nitrocellulose membrane placed on the surface of an LB agar plate supplemented with 50 µg/mL rifampicin and 30 µg/mL kanamycin. After 24 hours at 28°C, the bacteria were resuspended in water and 1x, 10x, and 100x (volume/volume) dilution series were plated again on LB agar containing 50 µg/mL rifampicin and 30 µg/mL kanamycin. Transformants were confirmed by colony PCR using oligonucleotides C and D **(Table S1)** and positive colonies were stored at -80°C in 25% glycerol stocks.

Microplate reader Absorbance and GFP fluorescence assays. To assay for *hrp*$_{promoter}$:*gfp* expression, *Pab* were scraped from the surface of the agar and resuspended into 1 mL of sterilized deionized water. The bacteria were washed three times in 1 mL of water and adjusted to an optical density at 600 nm (OD_{600}) of 1.0. In a 96-well plate, a 10 µL aliquot of 1.0 OD_{600} bacteria was inoculated in a T3SS-inducing minimal medium (Huynh et al., 1989) supplemented with either 25 mM of fructose, glucose, galactose, mannitol, or sucrose or with 30 µL beet exudate (final concentration of 30%). Culture density ($Absorbance_{600}$) and GFP fluorescence (485 nm excitation, 535 nm emission) of each well was measured every half-hour using a Tecan Spark 10M plate reader. The plate was kept at 25°C and shaken in between reads. To measure the expression of promoter:*gfp* constructs, fluorescence values were divided by Abs_{600} values to account for growth of the bacteria in the media, and Abs_{600}-adjusted fluorescence values from wells inoculated with pPROBE-NT empty vector strains were subtracted from wells inoculated with *hrp*$_{promoter}$:*gfp* constructs strains to account for background fluorescence.

RESULTS

Beet exudate contains an array of sugars detected by gas chromatography-mass spectrometry. We prepared beet exudate by soaking beet seedlings in water for 24 hours. We then used gas chromatography-mass spectrometry (GC-MS) to identify metabolites present in the beet exudate. The exudate contained many known plant-derived metabolites, including sugars, organic acids, and amino acids **(Table 1, S2)**.

Upstream promoter regions of *hrp* genes can be fused to *gfp* using a Gibson assembly-based cloning method. To assay the expression of *hrp* genes in the presence of beet exudate and specific plant-derived metabolites, we generated transcriptional reporter plasmids that contain the upstream promoter region of *hrpXY*, *hrpS*, or *hrpL* fused with the green fluorescent protein gene (*gfp*). The *gfp* gene encodes for a GFP protein that fluoresces under certain wavelengths of light. Therefore, the amount of GFP fluorescence from bacteria carrying the *hrp*:*gfp* transcriptional reporter can be used to report the levels of *hrp* transcription under different conditions **(Figure 2A)**. For each *hrp* transcriptional reporter produced, we PCR-amplified a roughly 400 bp promoter region upstream of each gene. To study *hrpS* expression in more detail, we also PCR-amplified a larger 1 kb region upstream of the *hrpS* gene. An example of our PCR products is shown in **Figure 2D**. The oligonucleotides we used to PCR-amplify each promoter region are listed in **Table S1**.

The plasmid used for our promoter:*gfp* constructs, pPROBE-NT, contains a SmaI restriction endonuclease cut site next to the start of the *gfp* region **(Figure 2B)**. We cut pPROBE-NT with SmaI and saw sufficient resolution on a gel between the cut and uncut versions of the plasmid **(Figure 2C)**. Gibson assembly is a relatively high-throughput method to assemble genes by joining DNA fragments into the pPROBE-NT plasmid backbone that contains DNA overhangs created by an exonuclease. This method has a relatively low error rate, and is efficient, requiring only one step at most points within the procedure (Gibson et al., 2009). We then transformed the assembled pPROBE-NT $hrp_{promoter}$:*gfp* constructs into DH10β *E. coli* cells **(Figure S1)**. We then used the process of bacterial conjugation to transform all of our $hrp_{promoter}$:*gfp* pPROBE-NT constructs from *E. coli* into *Pab* 4188 cells.

***Pab hrpL* transcription is strongly induced by a variety of sugars.** Our GC-MS analysis of beet exudate identified an array of sugars and sugar alcohols, including both simple saccharides and disaccharides. We selected two of these metabolites: fructose and sucrose, for further screening **(Table 1)**. We also selected sugars glucose and galactose that were not detected in the beet exudate, as well as mannitol. The purpose for selecting additional sugars was to assess if sugar-induced *hrp* expression only occurs in response to sugars found in beet exudate.

To determine if individual sugars can induce *hrpL* expression in *Pab*, we cultured our $hrpL_{promoter}$:*gfp* reporter strain in various minimal media each supplemented with only one type of sugar. The cultures were maintained in wells of a 96-well assay plate, and a plate reader was used to measure both GFP fluorescence at multiple time points post-inoculation. To assess bacterial growth, the plate reader also measured the Absorbance at λ=600 nm of each culture at each time point. Each of the sugars tested significantly induced *hrpL* expression within six hours

after inoculation **(Figure 3AB)**. The timing of maximal expression for each sugar varied across independent replicates of the experiment **(Figure 3A)** **(Figure S2)**. Despite this variability, fructose and mannitol consistently led to the highest levels of *hrpL* expression. In comparison, galactose and sucrose induced *hrpL* at a mid-range level, whereas glucose consistently led to a relatively weak but still statistically significant level of *hrpL* induction **(Figure 3B)**.

***Pab hrpXY* transcription is strongly induced by a variety of sugars.** We tested if the sugars would induce *hrpXY* expression in our $hrpXY_{promoter}$:*gfp Pab* strain as well. Like *hrpL*, all the sugars tested increased GFP fluorescence within 6 hours after treatment. Like *hrpL* expression, time points for maximum expression varied across independent replicates, and the levels of maximal expression also varied somewhat across independent replicates **(Figure 4A)** **(Figure S3)**. Across all replicates, fructose, galactose, mannitol, or sucrose into minimal medium led to a significant increase in *hrpXY* expression relative to a control with just minimal medium, while in two of three replicates, glucose also led to a significant, but smaller increase in expression, much like the results seen when treating *Pab* $hrpL_{promoter}$:*gfp* with glucose **(Figure 4)** **(Figure S3)**.

***Pab hrpS* transcription is not strongly induced by sugars.** We also tested if the same sugars induce *hrpS* expression in our *Pab* 0.4 kb $hrpS_{promoter}$:*gfp* reporter strain. In contrast to *hrpL* and *hrpXY*, fructose did not strongly induce the expression of *hrpS* based on levels of GFP fluorescence from this reporter strain (**Figure 5**). A small statistically significant increase in fluorescence was measure in response to minimal medium containing 50 mM fructose, but low overall expression of the promoter was seen compared to the *hrpXY* and *hrpL* promoters **(Figure 5)**. It is possible that our 0.4 kb *hrpS* promoter construct may be missing one or more of the important control elements. Therefore, we increased the region of upstream *hrpS* promoter to 1 kb in size; however, we did not observe any significant induction of *hrpS* expression using thus second reporter construct. In fact, a small decrease in GFP fluorescence was observed in response to fructose, glucose, galactose, mannitol, or sucrose **(Figure 6)**. It is possible that even our 1 kb promoter is still missing important control elements that could be within the open reading frame or further upstream of the 1 kb region selected.

***Pab* can use a variety of sugars as growth substrates.** We also assessed sugar-induced growth of *Pab* by measuring the Abs_{600} of *Pab* cultured in the same sugar-containing minimal media used for *hrp* expression. Each of the sugars tested significantly increased the growth of *Pab* (pPROBE-NT empty vector) relative to bacteria cultured in a minimal medium without sugar. Fructose and mannitol nearly doubled the peak Abs_{600} reached by the cultures, while galactose or sucrose caused a 1.75-fold increase in the peak Abs_{600}. Glucose caused only a 1.3-fold increase in the peak Abs_{600} **(Figure 7)**.

The magnitude of sugar-induced expression of *hrpXY* and *hrpL* and the magnitude of sugar-induced growth of *Pab* are positively correlated. When the Abs_{600} values for sugars inducing $hrpL_{promoter}$:*gfp* expression are plotted against the GFP fluorescence values obtained in the same well, a strong linear fit is obtained ($R^2 = 0.9117$). The same is true for sugars inducing $hrpXY_{promoter}$:*gfp* expression ($R^2 = 0.9725$) **(Figure 8)**. This strong correlation suggests that sugars that *Pab* prefers to use for energy metabolism also induce *hrpL* and *hrpXY* expression.

Autofluorescence of beet exudates limits the use of GFP as a transcriptional reporter. We also cultured our *Pab* *hrp*$_{promoter}$:*gfp* strains in minimal medium supplemented with beet exudate. Adding beet exudate into minimal media to 30% (volume/volume) increased the initial fluorescence at time t=0 of the experimental run, even in cultures of *Pab* carrying a pPROBE-NT empty vector **(Figure 9)**. Additionally, adding exudate to minimal medium supplemented with fructose did not significantly increase the expression of *hrpL*$_{promoter}$:*gfp* **(Figure 10A)** or the expression of *hrpXY*$_{promoter}$:*gfp* **(Figure 10B)**.

DISCUSSION

In this study, we used a GFP transcriptional reporter assay to test whether various sugars can induce the expression of T3SS-regulating genes *hrpL*, *hrpXY*, and *hrpS* in *Pantoea agglomerans*. By fusing the *gfp* gene to the promoter regions of *hrpL*, *hrpXY*, and *hrpS* in a broad host plasmid pPROBE-NT, and by introducing the plasmids into *Pab*, we monitored the expression level of these genes based on the levels of GFP fluorescence in *Pab* cultures. The main advantage of GFP fluorescence detection over other methods to detect transcription is that it provides a way to monitor gene expression from the same culture over time without the need to extract and analyze mRNA from the treated cells (Soboleski et al., 2005). When designing each promoter:*gfp* reporter, a primary consideration was how much of the upstream promoter of each gene to include in the constructs. In studies of *Pantoea stewartii*, gene expression was observed with reporter constructs that included 200 bp upstream of *hrpL* and *hrpXY*, and roughly 1 kb upstream of *hrpS* (Merighi et al., 2006). We observed sugar induced expression of all three genes with constructs that included roughly 400 bp upstream of each open reading frame **(Figures 3-5)**. However, we measured much weaker promoter activity from the 400 bp *hrpS*$_{promoter}$:*gfp* construct. It is possible that there may be a transcription control element that is not captured in the 400 bp upstream region. To test this possibility, we made a second *hrpS*$_{promoter}$:*gfp* construct that included a 1 kb region upstream of *hrpS*. Interestingly, we saw even lower promoter activity from this promoter **(Figure 6)**, though it should be noted that this promoter was tested with a lower concentration of sugar compared to the tests conducted with the 400 bp *hrpS*$_{promoter}$:*gfp* construct. While this may account for the difference in promoter activity, these results would seem to suggest that there is not an additional binding site within the 1 kb region upstream of the *hrpS* open reading frame.

Because the T3SS regulatory system is initiated by HrpX, which is a predicted sensor kinase protein possessing a sensor domain, it is possible that the system is initiated by HrpX recognizing a signal from the plant host. We predicted that T3SS gene expression changes in *Pab* would be induced by plant-exuded metabolites, like what is observed with T3SS genes in *Pseudomonas syringae* (Anderson et al., 2014, Turner et al., 2020), and that these changes would be captured by changes in GFP fluorescence over time as the bacteria are inoculated in medium containing the metabolites.

To identify plant-exuded metabolites that *Pab* may encounter during the infection process, we soaked beet seedlings in water and used gas-chromatography-mass spectrometry (GC-MS) to identify the metabolites exuded by the seedlings into this solution. We found a range

of simple sugars present in the beet exudate **(Table 1)**, as well as several other metabolites **(Table S2)**.

T3SS genes in many plant pathogenic bacteria, including *P. syringae* are induced by simple sugars (Turner et al., 2020), but it has not yet been reported if simple sugars induce T3SS genes in *Pab*. To investigate this, we used our $hrp_{promoter}$:*gfp* constructs to test if *Pab* T3SS regulatory genes are induced by the beet-exuded sugars sucrose and fructose. We also tested the sugars glucose, galactose, and sugar alcohol mannitol, since these have also been reported to induce T3SS genes in *P. syringae* (Turner et al., 2020). We found that *Pab hrpL* and *hrpXY* were significantly induced by all tested sugars, and that varying levels of induction were seen in response to different sugars. We observed the same pattern of induction for both *hrpXY* and *hrpL*, with fructose and mannitol being the strongest inducers and glucose being the weakest. Sucrose and galactose induced *hrpXY* and *hrpL* to similar intermediate levels as well. There was no significant difference between the effect seen with fructose compared to the effect seen with mannitol, or the effect seen with sucrose compared to the effect seen with galactose **(Figures 3-4)**.

We also found that *Pab* growth was induced by the same sugars that induced *hrpL* and *hrpXY* expression **(Figure 7)**. Notably, the increased culture density and increased GFP fluorescence from bacteria carrying either the $hrpL_{promoter}$:*gfp* or $hrpXY_{promoter}$:*gfp* were strongly correlated with each other **(Figure 8)**. The variation in the level of effect from different sugars may be due to differences in rates of sugar uptake into the cell, as highly hydrophilic compounds like sugars cannot freely diffuse across cell membranes and must use active transport. Fructose and mannitol appear to be preferred by *Pab* for both growth and T3SS gene expression, possibly because they enter the cell more easily than other sugars. This would also explain the observed correlation between T3SS gene expression and growth induction, as the levels of sugar inside a cell would increase its potency in use for both signaling and energy production. All of the sugars tested induced both *hrp* expression and bacterial growth. Taken together with the result that *Pab* shows no preference for beet exuded or non-beet exuded sugars, this may suggest that the signal which activates transcription of *hrpXY* and *hrpL* may be a common product of catabolism of these sugars, rather than the sugars themselves.

While we have determined that *hrpXY* and *hrpL* in *Pab* are induced in the presence of sugars, we still do not know at what part in the *hrp* transcriptional regulatory pathway the signals enter. Because *hrpL* is activated downstream of *hrpXY*, we can predict that *hrpXY* induced transcription will also induce transcription of *hrpL*. We can test this by testing the transcription of our $hrpL_{promoter}$:*gfp* in a genetic background in *Pab* where *hrpXY* is removed. In *Erwinia amylovora*, silencing *hrpX* or *hrpY* both reduce *hrpL* transcription drastically (Wei et al., 2000). This suggests that sugar-induced transcription of *hrpXY* directly leads to expression of *hrpL*. Similarly, we may expect that *hrpS* transcription would be directly induced by *hrpXY* as well, however we did not see strong *hrpS* transcription induced by sugars **(Figures 5-6)**, which suggests that *hrpS* transcription is not related directly to *hrpXY* induction. In contrast to *hrpL*, in *E. amylovora*, neither silencing *hrpX* nor *hrpY* has a strong effect on *hrpS* expression (Wei et al., 2000). If we were to do the same experiments in *Pab*, obtaining a similar result would suggest

that *hrpS* transcription is not induced by *hrpXY* transcription. Because we don't see strong *hrpS* expression with sugars, another option would be to overexpress *hrpXY* and examine the effect on *hrpS* transcription. If overexpressing *hrpXY* does not lead to increased expression of *hrpS*, it would further confirm that *hrpXY* transcription does impact *hrpS* transcription. Overall, our results suggest that *hrpXY* transcription is a point where signals may enter the *Pab* T3SS regulatory pathway. However, discovering additional sites where signals may enter will require further experimentation.

Since beet-exuded sugars induce T3SS expression in *Pab*, we also tested if exudate from beet seedlings can induce the expression of *hrpXY*, *hrpS*, and *hrpL*. Unfortunately, beet exudate fluoresces at the same wavelength as GFP **(Figure 9)** which makes capturing changes in *gfp* expression difficult with beet exudate as a treatment condition. Adding exudate to fructose led to a statistically significant decrease in both *hrpXY* and *hrpL* expression **(Figure 10)**, though we predict that this result is explained by the auto-fluorescence seen in the exudate. To avoid this problem, one option would be to make transcriptional reporter assays using non-fluorescence-based methodologies, such as a β-galactosidase reporter (Miller, 1972), or using RNA-seq or RT-qPCR to directly measure *hrp* transcription levels. While these methods would make it more difficult to track expression over a time course within the same cultures, if after 8 hours we still saw significantly reduced *hrpXY* and *hrpL* expression when treated with the beet exudate, it would suggest that there is a specific transcriptional repression effect from some element of the exudate.

CONCLUSION

While it is known that transcription of T3SS elements is induced in several plant pathogens in response to plant-derived metabolites, in the beet-infecting *Pab*, transcriptional activation of the T3SS has not been characterized. Our transcriptional reporter screens revealed that when treated with sugars, *Pab* highly increases transcription of genes encoding its T3SS. Our GFP reporter assays revealed that for the genes *hrpXY* and *hrpL*, which are T3SS regulators, sugars strongly induce transcription of these genes. The transcriptional induction seen for these genes is not specific to sugars which are present in beet exudate, possibly suggesting that the signal that induces transcription is a common metabolite of sugar catabolism. The levels of sugar-induced transcription of *hrpXY* and *hrpL* were positively correlated with sugar-induced growth of *Pab*. This correlation may be explained by different rates of sugar uptake into *Pab* cells. Through future genetic screening, we can further characterize how *Pab* uses plant-derived signals to induce transcription of its T3SS.

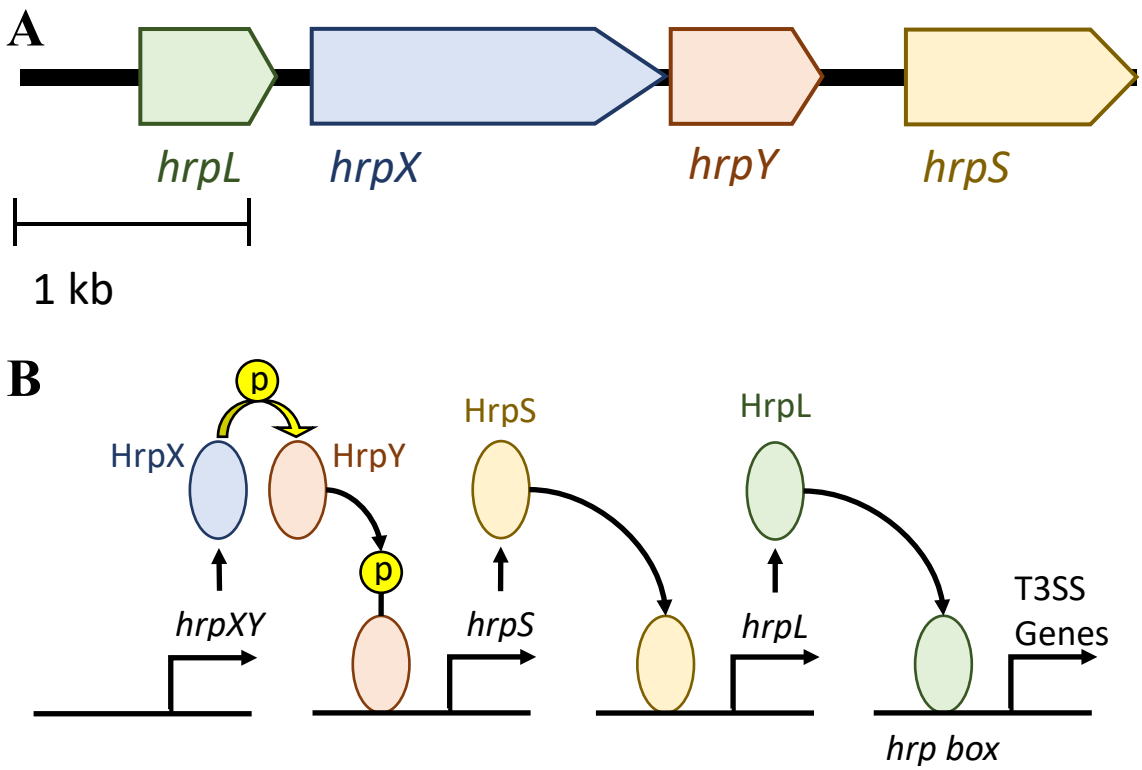


Figure 1 – The *Pantoea agglomerans hrp* genes are part of a transcriptional regulatory pathway that controls the expression of genes encoding the type III secretion system in. (A) Gene model of *hrp* genes on the pPATH$_{Pab}$ plasmid. (B) Signaling pathway for *hrp* signaling cascade, where HrpX phosphorylates HrpY in a two-component system, leading to the downstream induction of *hrpS*, then *hrpL*, which binds to the *hrp* box promoter which regulates the expression of the genes in the type III secretion system (T3SS) in *P. agglomerans pv. betae* (*Pab*).

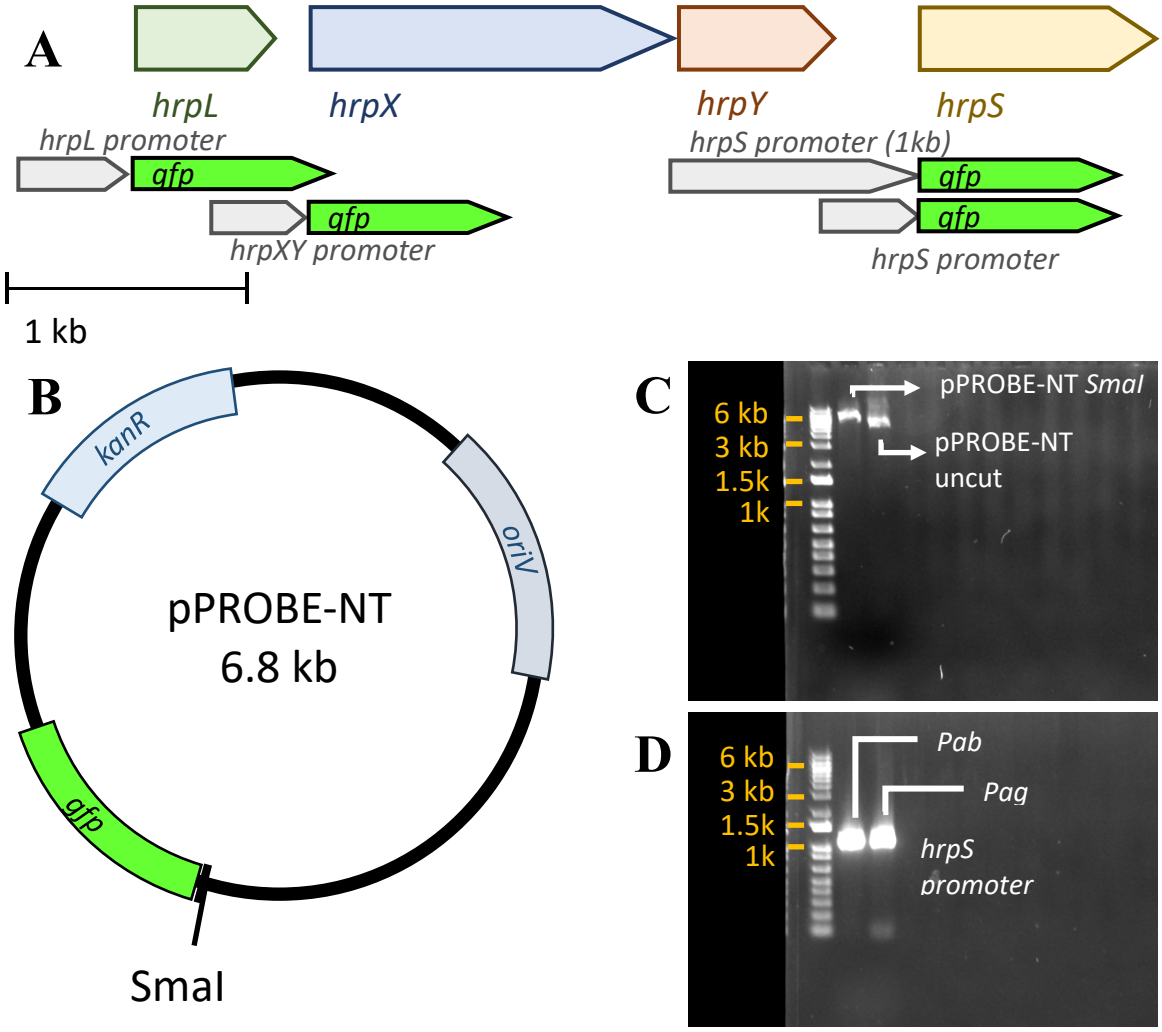


Figure 2 – Gibson assembly methods can be used to generate a plasmid with promoter:*gfp* constructs to be inserted in *Pantoea agglomerans*. (A) Gene model of *hrp* genes on the pPATH$_{Pab}$ plasmid with the promoter regions highlighted, including *gfp* fusion sites. (B) pPROBE-NT Plasmid model with *kanR* resistance cassette, *oriV* site, and SmaI cut site highlighted. SmaI cut site represents where promoter regions from the *hrp* genes are inserted. (C) Electrophoresis result of pPROBE-NT *SmaI* and pPROBE-NT uncut. (D) Electrophoresis result showing successful PCR amplification of the 1 kb *P. agglomerans* pv. *betae hrpS* promoter region to be inserted into the pPROBE-NT vector using restriction cloning, and the 1 kb *P. agglomerans* pv. *gypsophilae hrpS* promoter region.

Sugars	Sugar Alcohols
xylose	D-threitol
D-lyxose	arabitol
6-deoxy-D-glucose	xylitol
fructose**	ribitol
D-mannose	lactitol
cellobiose	palatinitol
D-allose	
sucrose**	
sophorose	
lactose	

Table 1 – Sugars in beet exudate

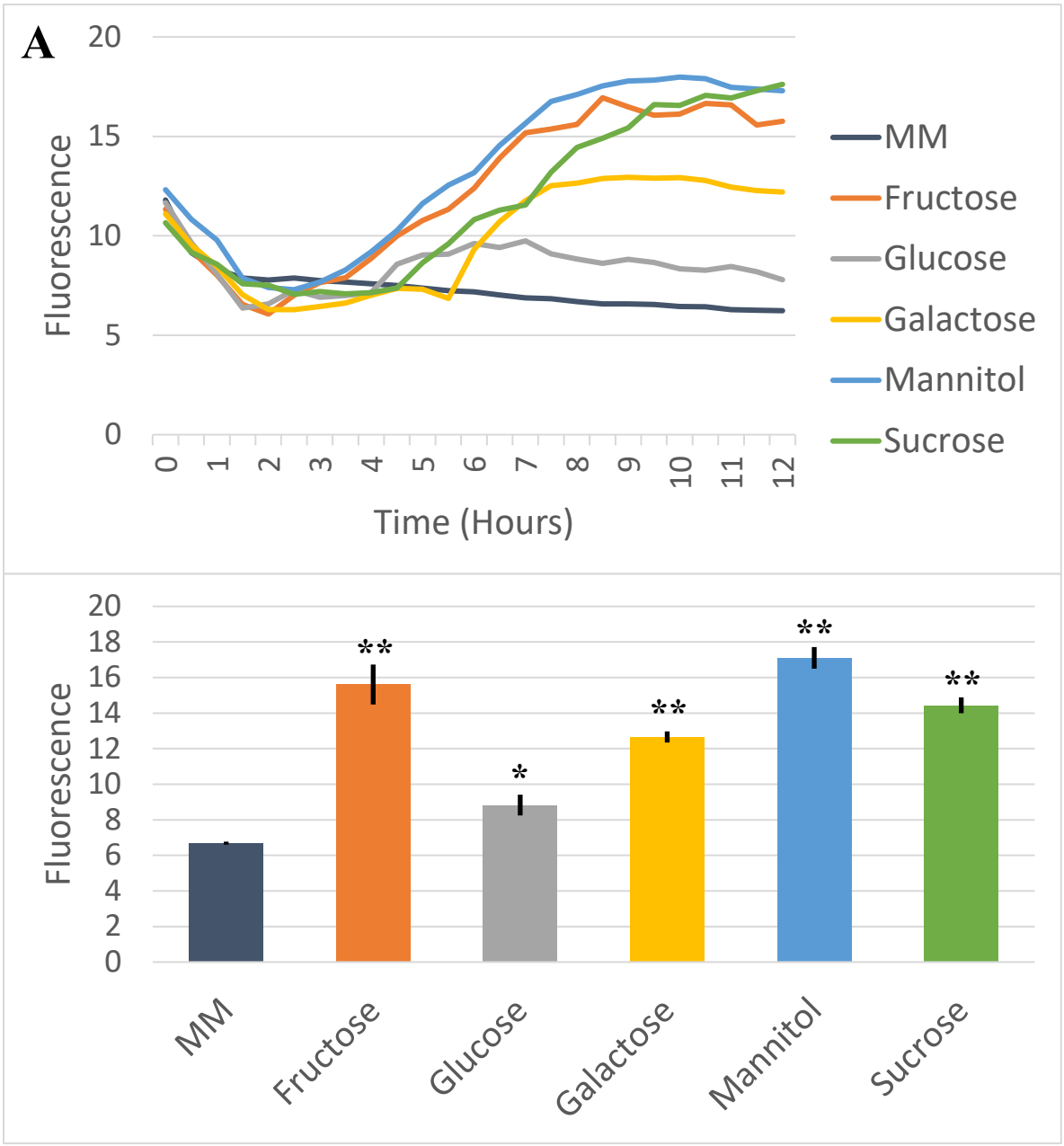


Figure 3 – Expression of *P. agglomerans hrpL*$_{promoter}$*:gfp* is induced by various sugars. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpL*$_{promoter}$*:gfp* construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A) Time course of *Pab hrpL*$_{promoter}$*:gfp* expression. Graphed are average GFP fluorescence values from replicate wells in a single experiment, n=3. (B) Graphed are average GFP fluorescence values ± SE at 8 hours post-inoculation, n=3. Data in panel B are from same experiment shown in panel A. Asterisks indicate a statistically significant difference between sugar and minimal media treatment values based on a t-test comparison (*: $p<0.05$, **: $p<0.01$). Data in this figure are representative of three independent experiments.

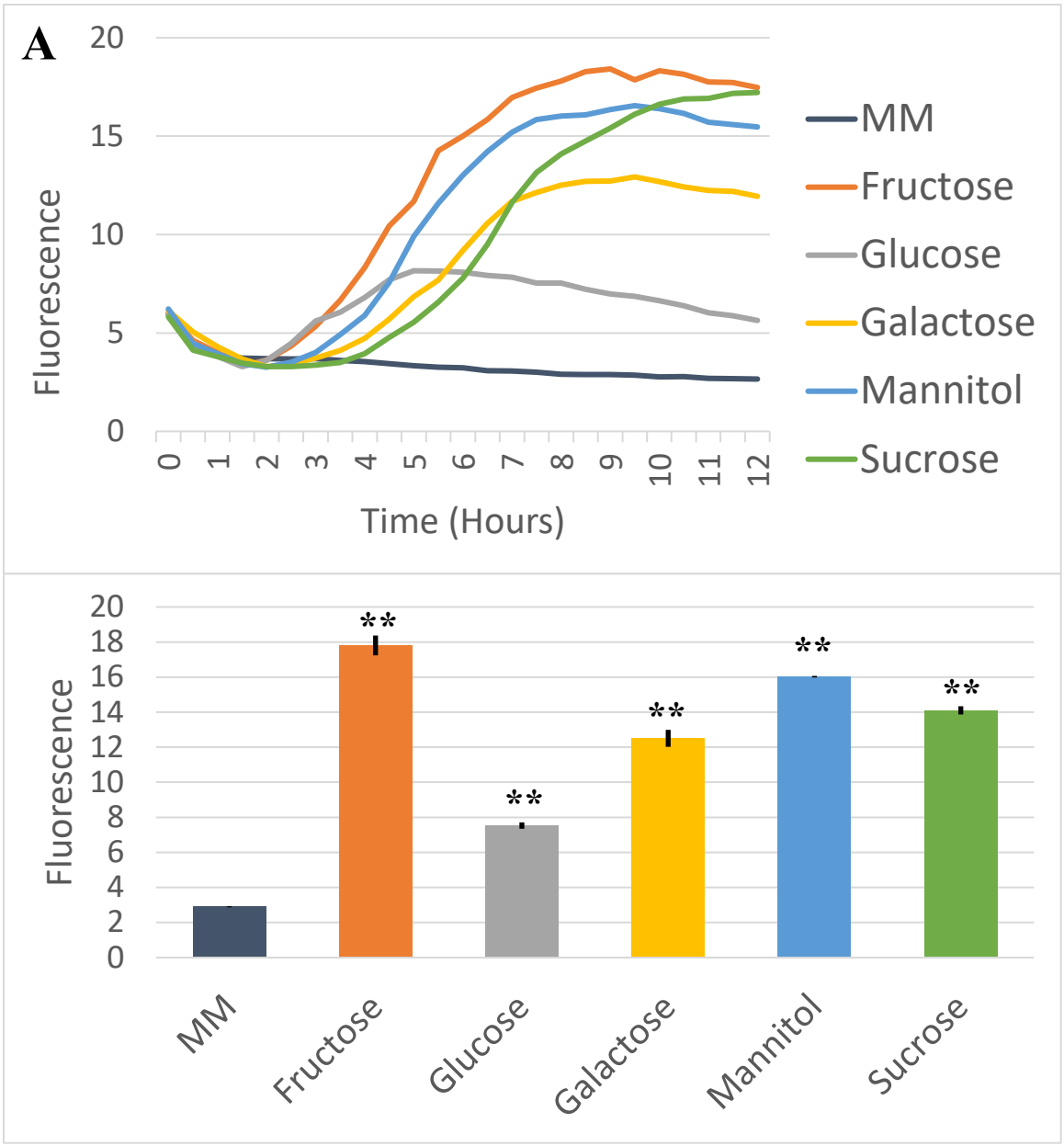


Figure 4 – Expression of *P. agglomerans hrpXY*$_{promoter}$*:gfp* is induced by various sugars. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpXY*$_{promoter}$*:gfp* construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A) Time course of *Pab hrpXY*$_{promoter}$*:gfp* expression. Graphed are average GFP fluorescence values from replicate wells in a single experiment, n=3. (B) Graphed are average GFP fluorescence values ± SE at 8 hours post-inoculation, n=3. Data in panel B are from same experiment shown in panel A. Asterisks indicate a statistically significant difference between sugar and minimal media treatment values based on a t-test comparison (**: $p<0.01$). Data in this figure are representative of three independent experiments.

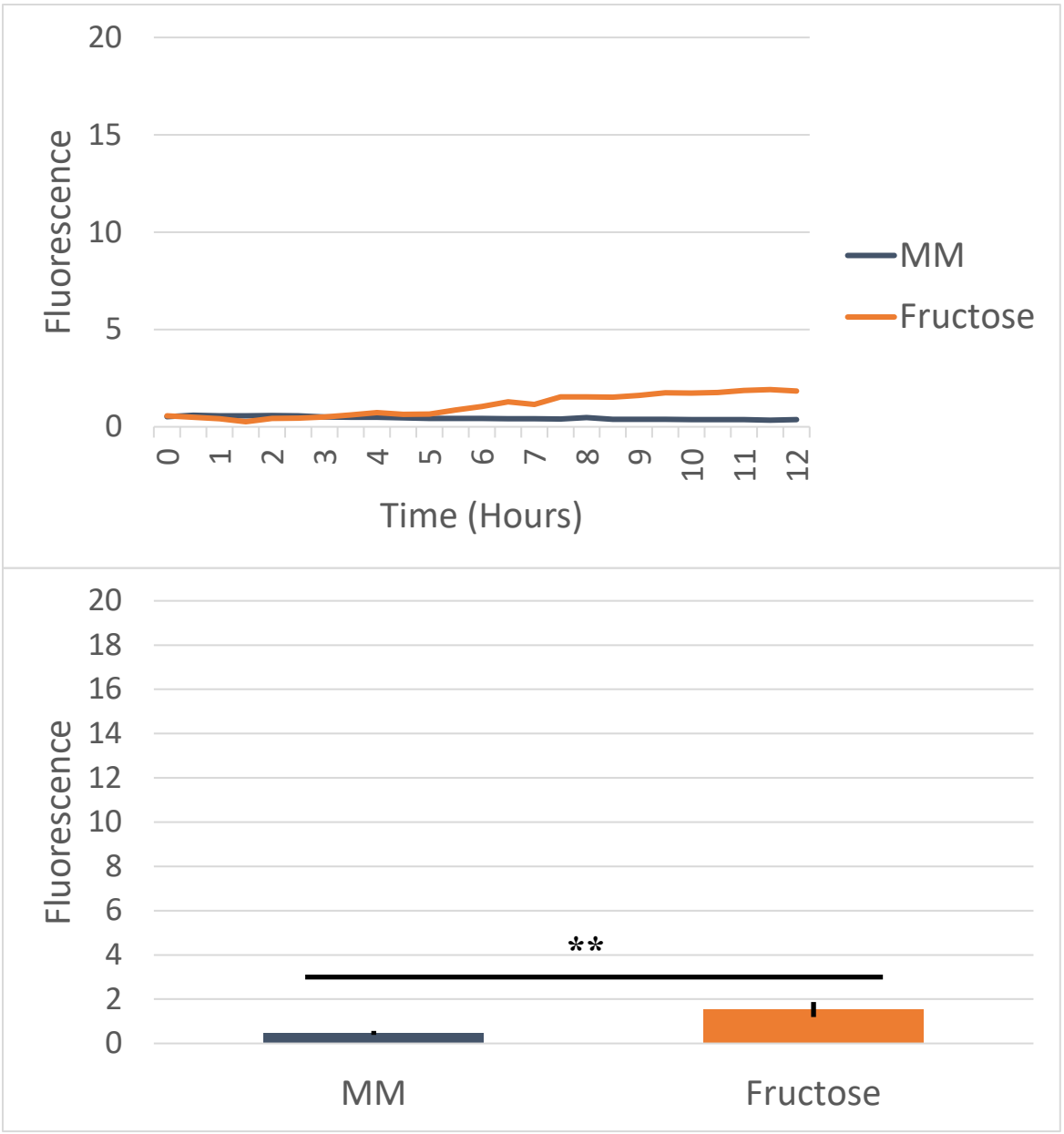


Figure 5 – Expression of *P. agglomerans hrpS*$_{promoter}$*:gfp* (0.4 kb) is weakly induced by fructose. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpS*$_{promoter}$*:gfp* (0.4 kb) construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 50 mM of fructose. (A) Time course of *Pab hrpS*$_{promoter}$*:gfp* expression. Graphed are average GFP fluorescence values from replicate wells pooled across three experiments, n=9. (B) Graphed are average GFP fluorescence values ± SE from data pooled across three experiments at 8 hours post-inoculation, n=9. Asterisks indicate a statistically significant difference between fructose and minimal media treatment values based on a t-test comparison (**: $p<0.01$). Data in panel B are pooled from the same experiments shown in panel A.

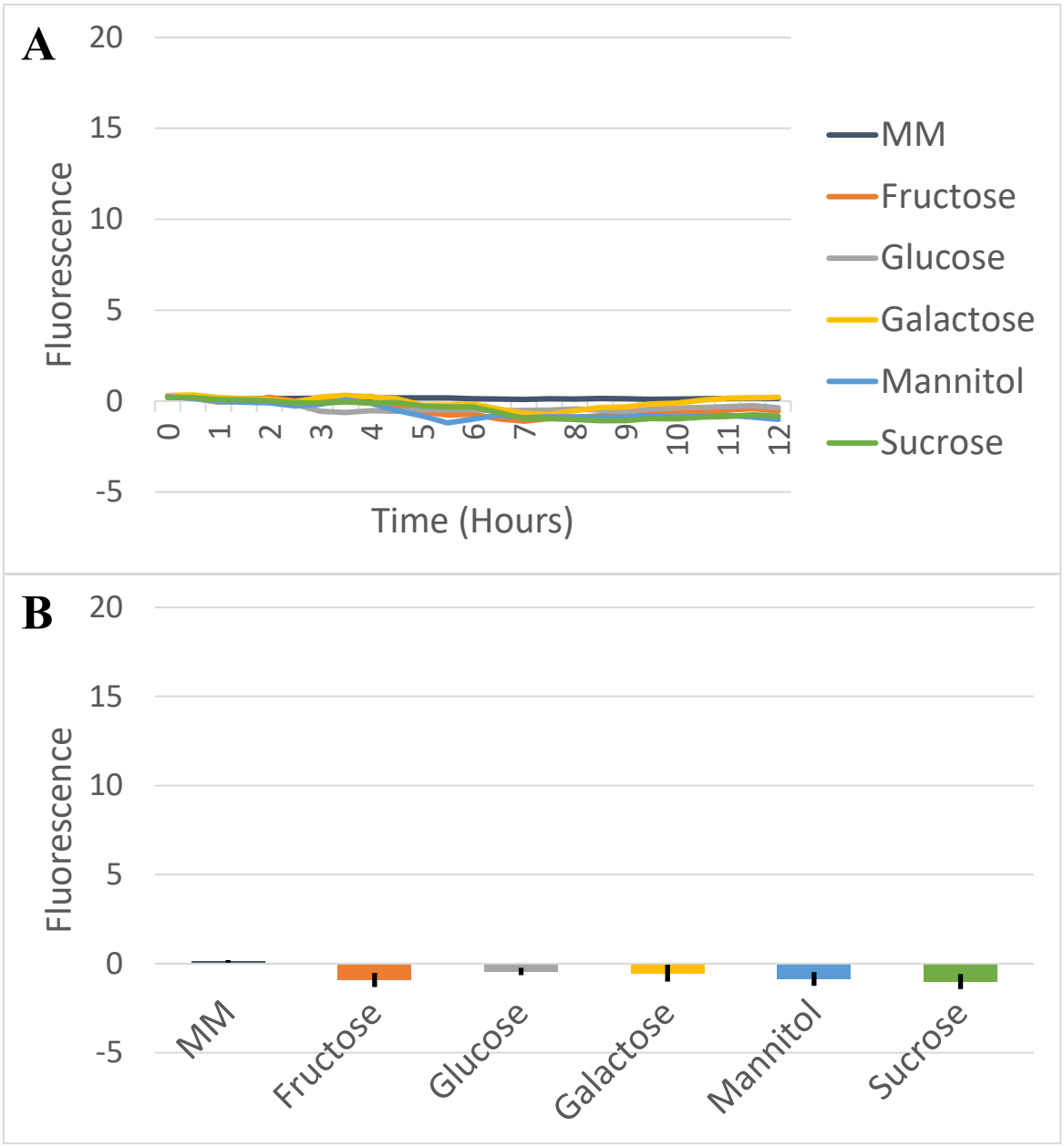


Figure 6 – Expression of *P. agglomerans hrpS*$_{promoter}$*:gfp* (1 kb) is not induced by various distinct sugars. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpS*$_{promoter}$*:gfp* (1 kb) construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A) Time course of *Pab hrpS*$_{promoter}$*:gfp* expression. Graphed are average GFP fluorescence values from replicate wells pooled across two experiments, n=6. (B) Graphed are average GFP fluorescence values ± SE from data pooled across two experiments at 8 hours post-inoculation, n=6. Data in panel B are pooled from the same experiments shown in panel A.

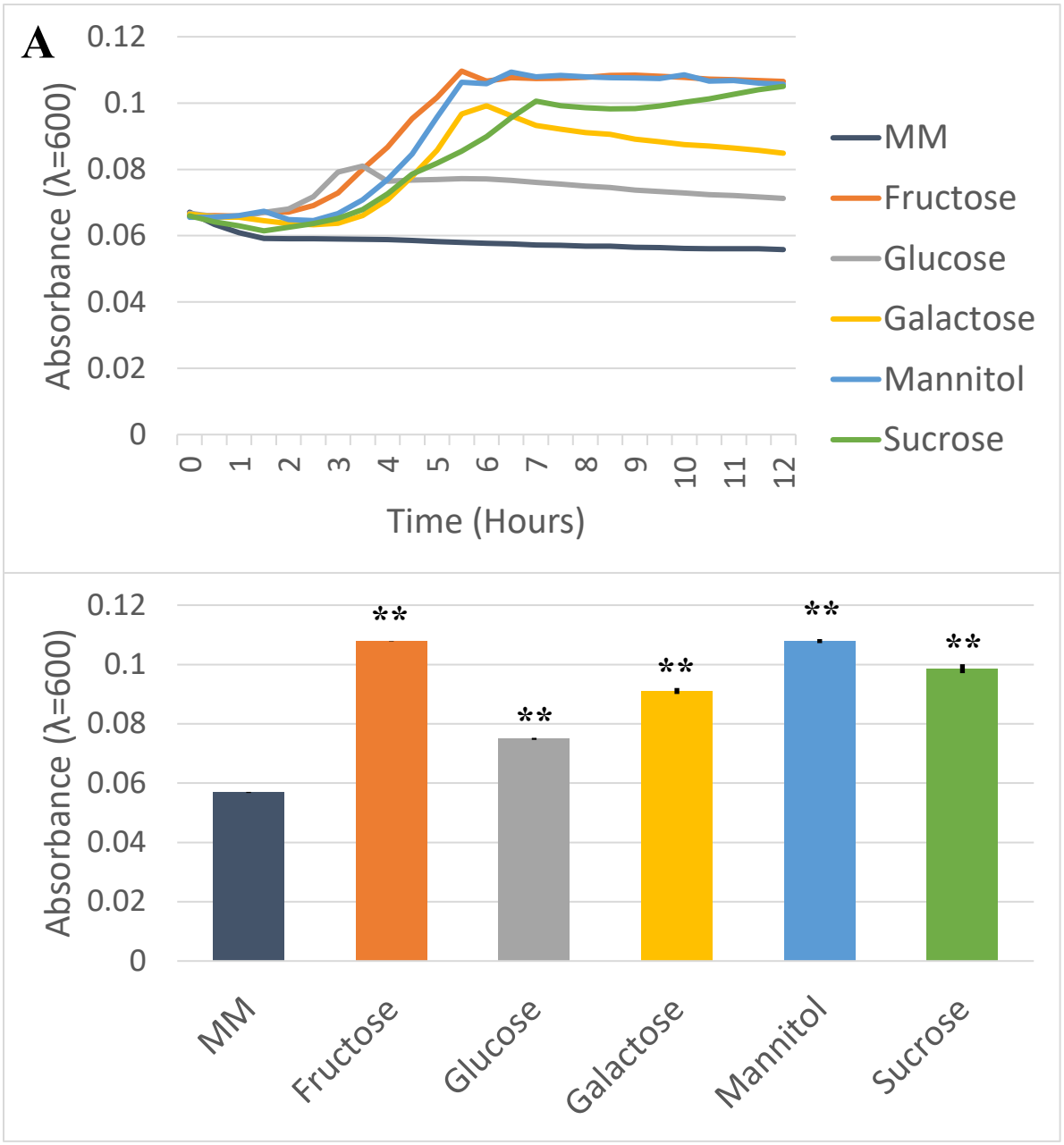


Figure 7 – Sugars are growth substrates for *P. agglomerans*. Growth of *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying the pPROBE-NT empty vector plasmid in a minimal medium supplemented with 25 mM of individual sugars as indicated. (A) Time course of *Pab* pPROBE-NT growth over 12 hours. Graphed are mean value of Absorbance values at λ=600 nm (Abs_{600}) from replicate wells in the same experiment, n=3. (B) Graphed are average Abs_{600} values ± SE at 8 hours post-inoculation, n=3. Data in panel B are from the same experiment shown in panel A. Asterisks indicate a statistically significant difference from MM only values based on a t-test comparison ($p<0.01$). Data in this figure are representative of three independent experiments.

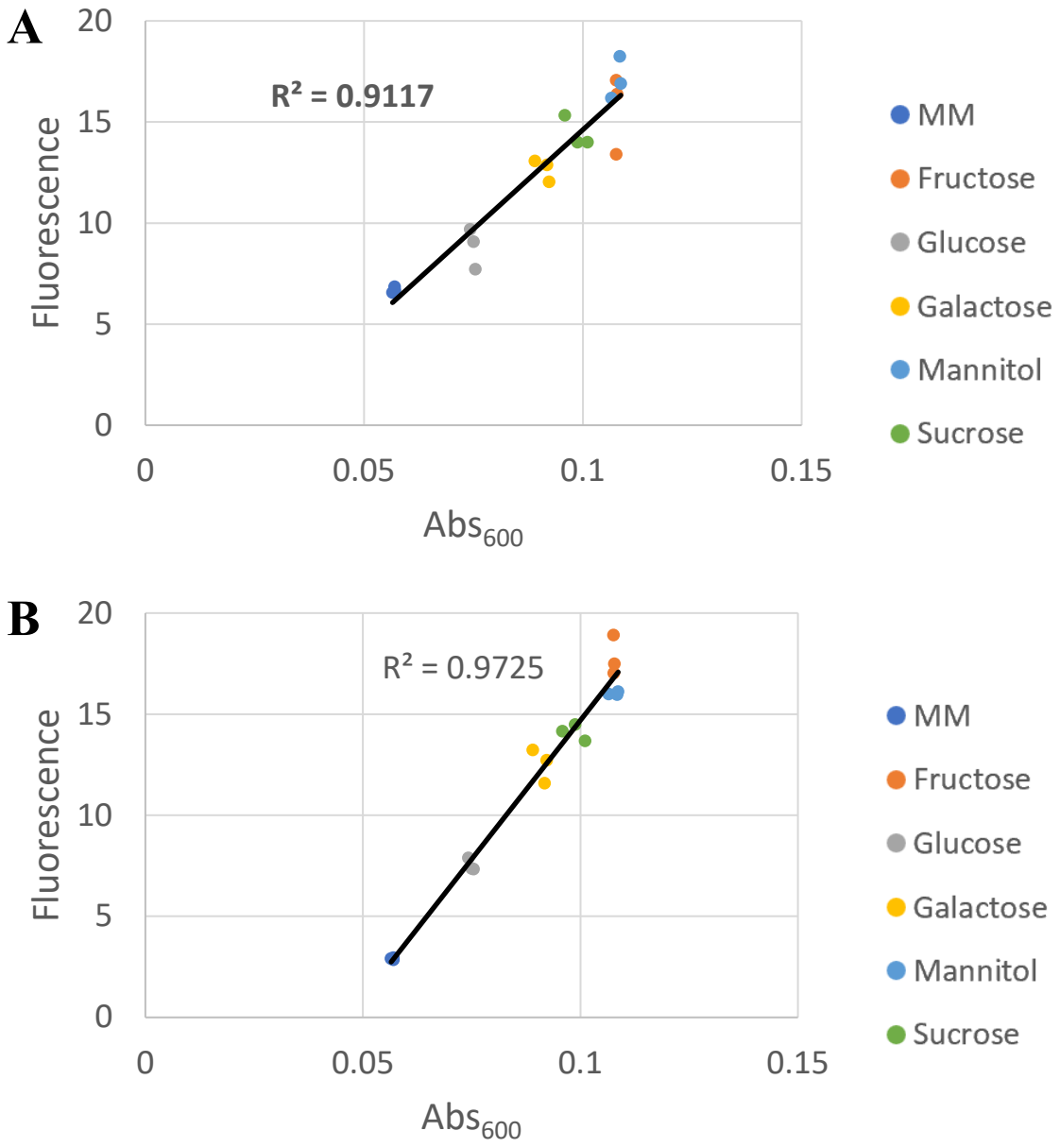


Figure 8 – A strong linear relationship is indicated between the ability of a sugar substrate to induce expression of *hrp* genes and to induce growth of *P. agglomerans*. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying $hrp_{promoter}$:*gfp* constructs on the vector pPROBE-NT were inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A) Scatterplot of pPROBE-NT empty vector Absorbance values (λ = 600) against $hrpL_{promoter}$:*gfp* fluorescence values for the same sugar treatments with linear fit shown. (B) Scatterplot of pPROBE-NT empty vector Absorbance values (λ = 600) against $hrpXY_{promoter}$:*gfp* fluorescence values for the same sugar treatments with linear fit shown.

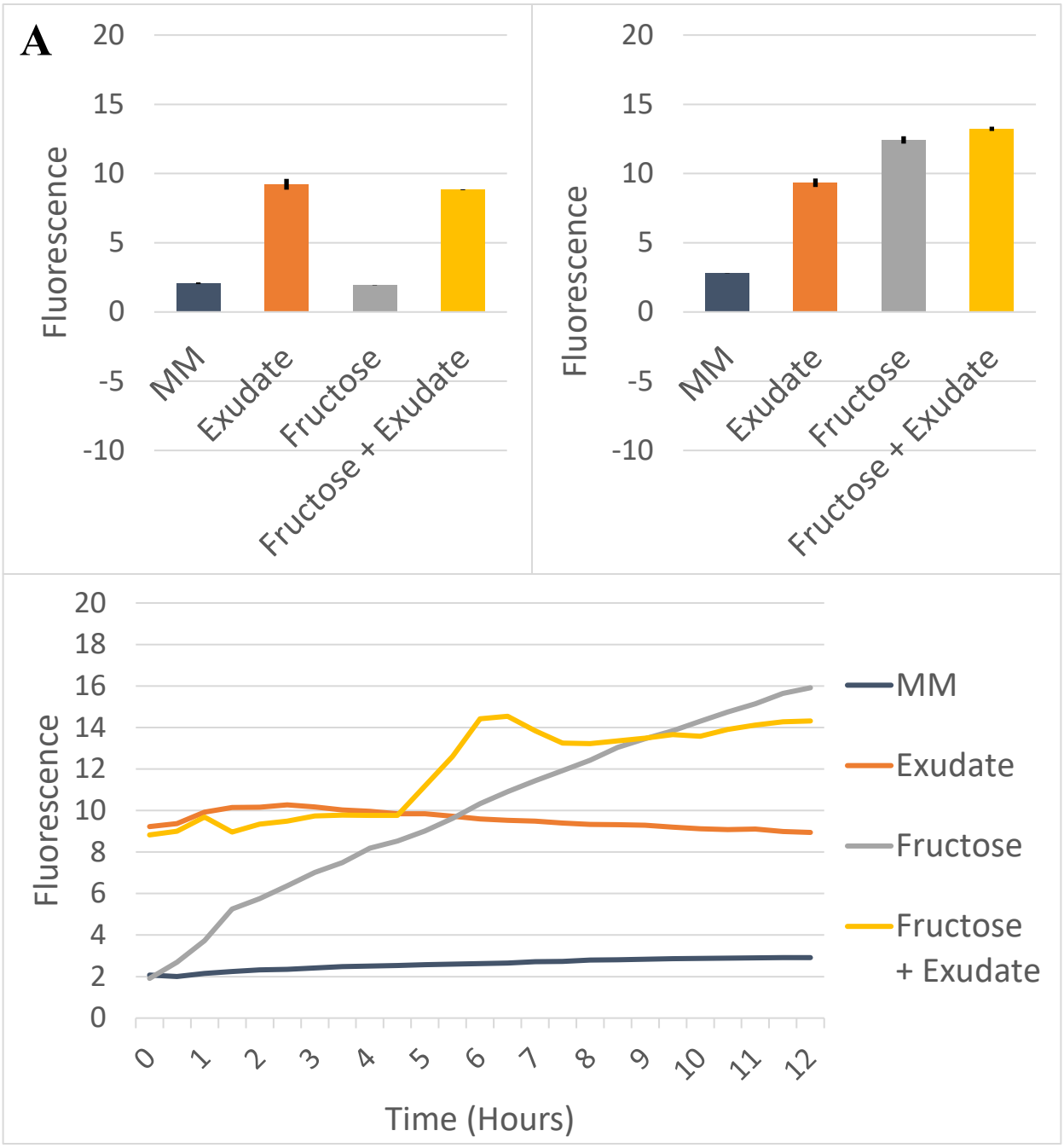


Figure 9 – Auto-fluorescence limits beet exudate's ability to induce expression of *P. agglomerans hrp*$_{promoter}$*:gfp* constructs. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying the pPROBE-NT empty vector plasmid was inoculated in minimal medium containing 30% beet exudate, 25 mM fructose, or both as indicated. (A) Average GFP fluorescence values ± SE at time 0. (B) Average GFP fluorescence values ± SE at 8 hours post inoculation. (C) Time course of fluorescence from *Pab* pPROBE-NT empty vector. Graphed are average fluorescence values from replicate wells in the same experiment, n=3. Data in each panel are from the same experiment. Data are representative of two independent replicates.

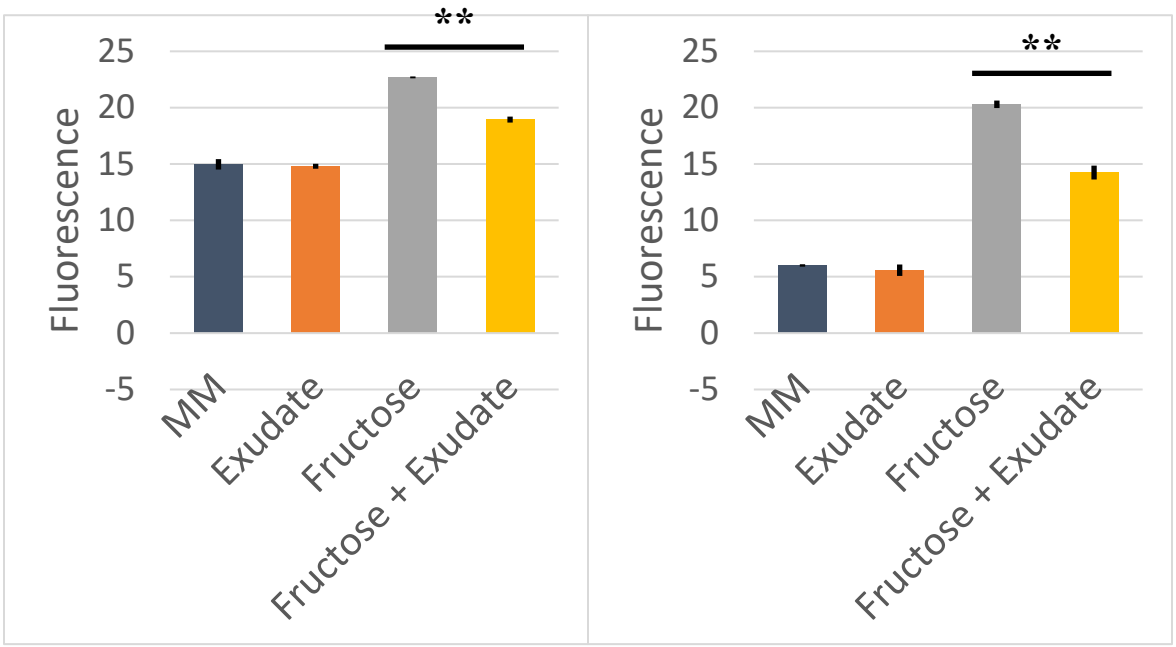


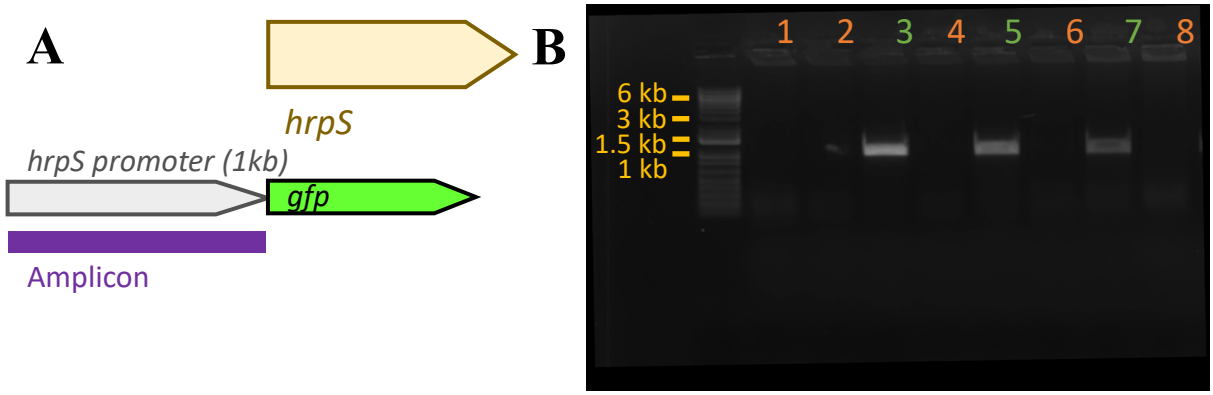
Figure 10 – Expression of *P. agglomerans hrpL* or *hrpXY* is not induced by beet exudate. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying the $hrpL_{promoter}$:*gfp* or $hrpXY_{promoter}$:*gfp* construct was inoculated in minimal medium containing 30% beet exudate, 25 mM fructose, or both as indicated. (A) Average GFP fluorescence values ± SE at 8 hours post-inoculation for $hrpL_{promoter}$:*gfp*. (B) Average GFP fluorescence values ± SE at 8 hours post inoculation for $hrpXY_{promoter}$:*gfp*. Graphed are average fluorescence values from replicate wells in the same experiment, n=3. Data in each panel are from the same experiment, as well as from the same experiment shown in Figure 8. Data are representative of two independent replicates. Asterisks indicate statistically significant values based on the results of a t-test ($p < 0.01$).

Primer	Sequence
A	actctagaggatccccGACGAATAATATTGCAGGTTTG
B	ttcgagctcggtacccAAGAAGTTACCCCTTTCC
C	gaattcctgcagcccGGCAGACCCTGCAGTTCG
D	TCAGGCGGGGTAAATAGCGCC

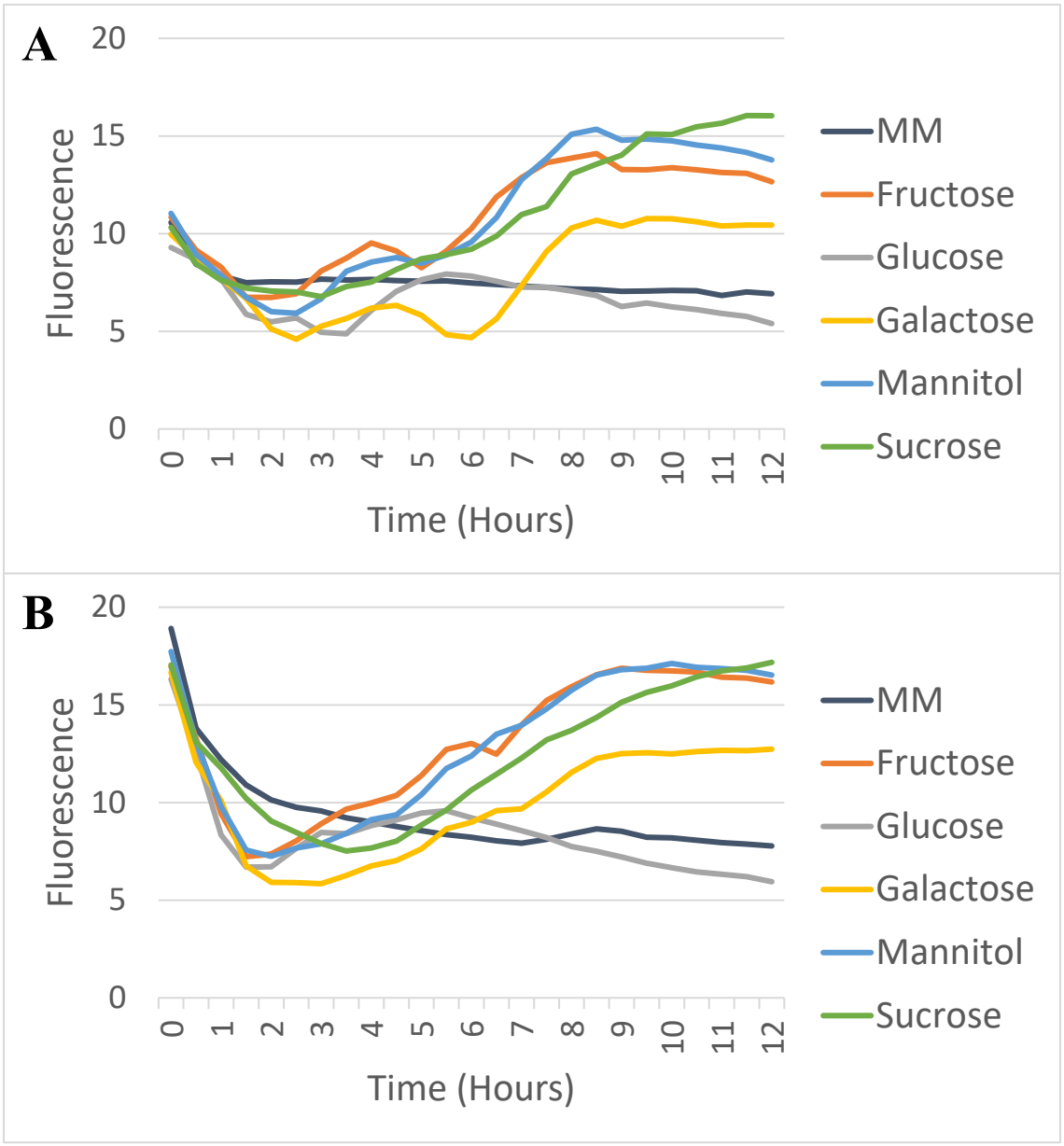
Table S1 – Oligonucleotide sequences used.

Organic Acids	Amino Acids
L-(+) lactic acid	L-leucine
citric acid	L-valine
oxalic acid	L-serine
D-malic acid	DL-isoleucine
fumaric acid	L-alanine
succinic acid	L-mimosine
	L-threonine
	L-methionine
	B-alanine
	phenylalanine
	tyrosine

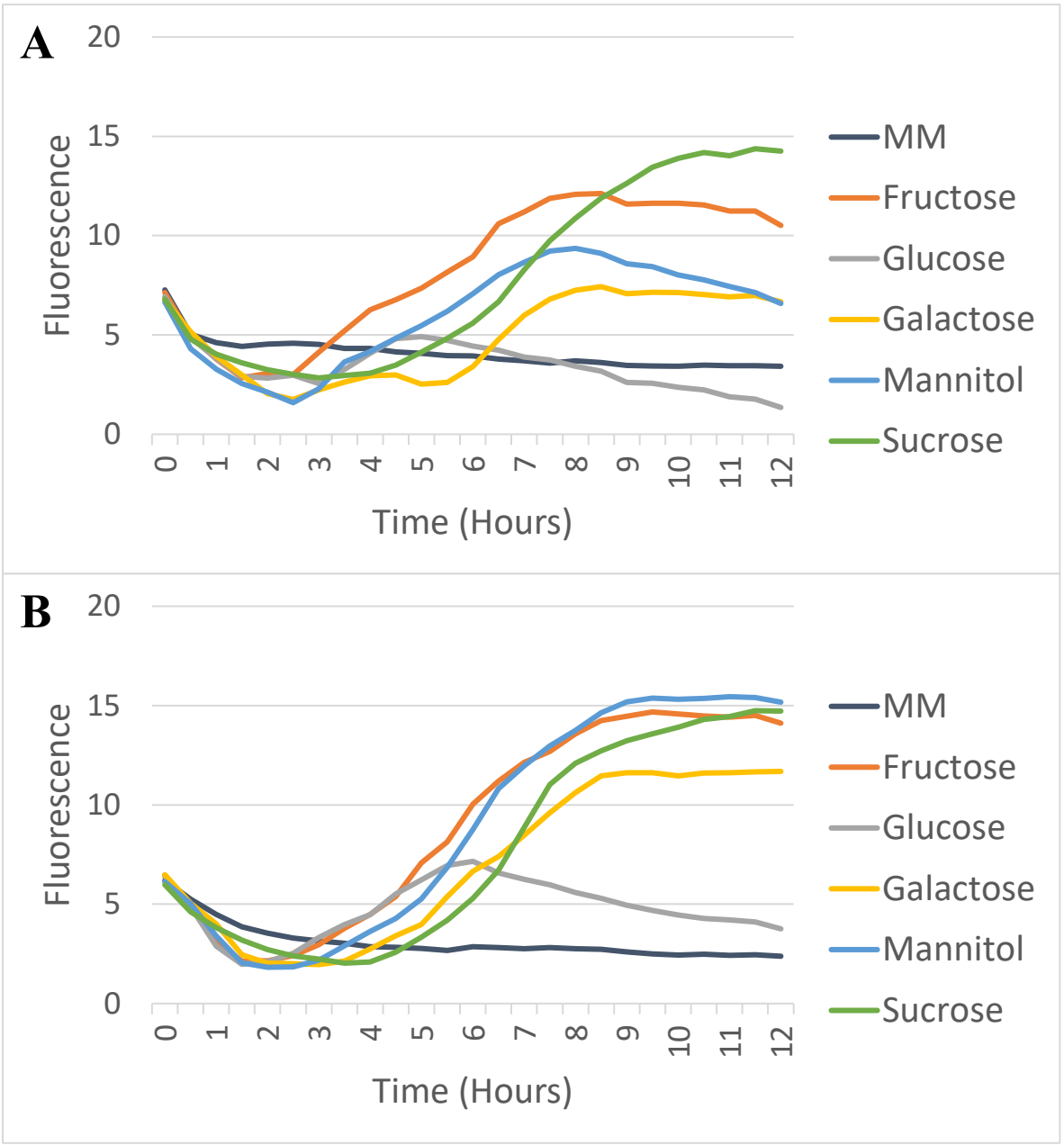
Table S2 – Sample of beet exudate composition.



Supplemental Figure 1 – Colony PCR results confirm transformation of pPROBE-NT *hrpS*$_{promoter}$:*gfp* (1 kb) into *E. coli*. DH10β *E. coli* competent cells were transformed with pPROBE-NT *hrpS*$_{promoter}$:*gfp* (1 kb) using heat shock. (A) *hrpS*$_{promoter}$:*gfp* scheme with PCR amplicon (1kb) labeled. (B) Positive colonies from colony PCR run labeled.



Supplemental Figure 2 – Expression of *hrpL*$_{promoter}$:*gfp* is induced by various sugar substrates across multiple independent replicates. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpL*$_{promoter}$:*gfp* construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A), (B) Time course of *Pab hrpL*$_{promoter}$:*gfp* expression. Graphed are average GFP fluorescence values from replicate wells in a single experiment, n=3. Data shown from two independent replicates.



Supplemental Figure 3 – Expression of *hrpXY*$_{promoter}$:*gfp* is induced by various sugar substrates across multiple independent replicates. *P. agglomerans* pv. *betae* 4188 (*Pab*) carrying a *hrpXY*$_{promoter}$:*gfp* construct on the vector pPROBE-NT was inoculated into a minimal medium supplemented with 25 mM of individual sugars as indicated. (A), (B) Time course of *Pab hrpXY*$_{promoter}$:*gfp* expression. Graphed are average GFP fluorescence values from replicate wells in a single experiment, n=3. Data shown from two independent replicates.

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