# Final Report

# Low molecular weight inhibitors of (p)ppGpp-dependent virulence factor production by *Pectobacterium atrosepticum*

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# 1. PREFACE

This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) as an Industrial Partnership Award. Potato Council acted as the industrial partner and provided a 10% contribution to the total cost of the project.

The project involved studies of the bacterium *Pectobacterium atrosepticum* (Pba) which was previously referred to as *Erwinia carotovora* subspecies *atroseptica* (Eca). Its name was changed as a result of work to study the relatedness of different pectolytic bacteria (i.e. bacteria that can produce enzymes which break down pectin, a component of plant cell walls). The work leading to the reclassification of Eca was made possible as a result of the availability of DNA sequencing techniques. The same approaches lead to the reclassifying of *Erwinia chrysanthemi* as several different species of *Dickeya*.

The aim of the work described in this report was to provide a better understanding of the complex interactions which determine if Pba causes disease in potatoes. It was expected that a better understanding of the processes involved, and how they are governed at the molecular level, would facilitate the identification of new anti-rot agents.

The work has shown that colonies of Pba only produce plant cell wall degrading enzymes when they begin to run short of nutrients; and when they are present in sufficient numbers such that the production of enzymes is likely to result in disease. The process of causing disease in turn releases nutrients for the bacteria. The results have confirmed a particular signalling pathway, involved in the regulation of the production of the cell wall degrading enzymes, as a suitable target for the development of anti-rot agents. However, the identification of molecules that disrupt the pathway has not been possible within the lifetime of the project and will continue beyond the end of the project.

*Potato Council, 2013*

# 2. BACKGROUND

## *2.1. Pectobacterium atrosepticum*

*Pectobacterium atrosepticum* (Pba) causes blackleg symptoms (typically under cool, wet conditions) and soft rot of potato tubers. Tuber symptoms can range from slight vascular discoloration to complete decay. Although it does not easily penetrate the outer surface of healthy tissue, Pba readily infects tubers that have been damaged e.g., through mechanical abrasion during harvesting. Once an infection is established, Pba produces numerous secreted enzymes (exoenzymes) which break down the plant tissues, releasing nutrients that enable accelerated bacterial growth. Because of this, once rot sets in, it rapidly spreads. The exoenzymes produced by Pba include a range of pectate lyases, polygalcturonases, pectin methylesterases, proteases and, to a lesser extent, cellulases. Over the last decade or so it has become clear that synthesis and secretion of such plant cell wall (PCW) degrading enzymes by Pba is under the control of “quorum sensing” (see below). However, preliminary results available before the start of this project suggested that another regulatory pathway may also influence the production of the enzymes. The objectives of the project were to provide a better understanding of this other pathway; and to identify compounds that might be used to disrupt the pathway and therefore be potential anti-rot compounds for subsequent commercialisation.

## 2.2. Quorum Sensing

Bacteria use small molecule signals to communicate with each other and monitor their population density in a particular environment. This cell-to-cell signaling is referred to as “quorum sensing”. It provides a mechanism to synchronize the gene expression (ie which genes are “switched on”) of the bacteria and regulate activities such as the production of exoenzymes or secondary metabolites (e.g. antibiotics). Quorum sensing has been studied in Pba and it is known that the production of plant cell wall (PCW) degrading enzymes is regulated by a low molecular weight quorum sensing signal molecule, 3-oxo-hexanoyl-L-homoserine lactone (OHHL). The molecule is cell permeable and freely diffuses in and out of bacterial cells. Because of this, it accumulates in the local environment (e.g. in lenticels, wounds on the tubers) at levels which are in proportion to the number of Pba present. Once a critical threshold level of OHHL is reached at the wound site, the molecule interacts with a cytoplasmic Pba protein called VirR, which in turn, influences whether production of PCW degrading enzymes occurs. The interactions between the molecules in this system ensure that the PCW degrading enzymes are only produced at high cell densities, when Pba are sufficiently numerous to ensure that infection has a reasonable chance of success.

## 2.3. Other Regulatory Pathway

Preliminary results available before the start of this project suggested that the quorum sensing-dependent production of PCW degrading enzymes only becomes stimulated in Pba when it begins to run short of nutrients. From the perspective of the bacterium, this probably makes good economic sense; after all, why bother to synthesize and secrete PCW-degrading enzymes, which carry a substantial metabolic “price tag” if there is plenty of nutrient around anyway? Two key molecule(s) begin to accumulate in the cell when Pba becomes nutritionally limited; guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate pppGpp. Together these are abbreviated as (p)ppGpp. The established function of (p)ppGpp in most bacteria is to act as a stress “alarmone” – when the nutrients required for cell growth and division become limiting, (p)ppGpp causes the cell to shut down the production of proteins and shift to making amino acids instead. Crucially, preliminary work by the researchers indicated that (p)ppGpp was also required for the production of PCW degrading enzymes by Pba. They therefore set out to better understand the role of (p)ppGpp in regulating virulence, and to identify small molecules that would disrupt the signalling pathway used by (p)ppGpp to activate virulence. Such molecules may reasonably be expected to prevent the bacteria from being able to cause disease.

# 3. WORK COMPLETED & KEY OUTCOMES

In the original research proposal, the researchers planned to manipulate ppGpp levels by working with the bacterium’s own enzymes, in particular an enzyme referred to as RelA. This proved to be problematic. However, an alternative approach using an enzyme (RelV) from another bacterium, (the cholera pathogen, *Vibrio cholerae*) was shown to be effective. The enzyme was transferred to Pba and resulted in the enzymatic synthesis of ppGpp in Pba, which in turn led to complete and immediate cessation of Pba growth. This confirmed that the presence of ppGpp results in the shutting down of processes underpinning cell growth and division. In parallel, the researchers also succeeded in chemically synthesizing a derivative of ppGpp. Chemical synthesis (which does not involve enzymes from bacteria) is useful in that it allows researchers to produce a ppGpp derivative that could be bound to a solid

support, making it possible to screen for molecules that bind to the immobilised ppGpp. It was expected that molecules binding to ppGpp will be part of the (p)ppGpp signalling pathway and therefore would warrant further study.

Modified versions of a Pba strain were produced in the lab. These differed from the "wild-type" parent strain according to whether genes, hypothesised to be important in the (p)ppGpp regulatory pathway and/or quorum sensing, were present. Importantly, studies comparing these different strains led to the conclusion that the two regulatory pathways interact and that (p)ppGpp and OHHL are *simultaneously* required for the production of PCW degrading enzymes. If either signal is absent the enzymes are not produced. OHHL and (p)ppGpp achieve this outcome because they independently influence whether a small protein (RsmA), which binds to RNA, is produced. This is the protein which in turn influences whether production of PCW degrading enzyme takes place. Overall it seems that the two regulatorry pathways ((p)ppGpp and quorum sensing) act as a coordinated "belt-and-braces" mechanism to ensure that PCW degrading enzymes are only produced (i) when Pba becomes nutritionally restricted, and (ii) only when the Pba density is high enough to ensure that any attack on a plant stands a reasonable chance of success.

The work to identify small molecules that block the the ppGpp-dependent signaling pathway has not yet been completed. There were considerable technical difficulties in establishing a sytem to monitor the impact of potential inhibitors on the pathway and consequently on the ability of Pba to cause disease. Work has focussed on producing an assay based on sections of DNA that initiate the transcription (ie first stage of gene expression) of particular genes. Genes where an interaction with ppGpp is believed to occur have been chosen. The resulting "reporter assays" were designed so that any inhibitory/disruptive effect of small molecules/natural products on the ppGpp-dependent pathway would be detectable through changes in fluorescence. However, initial screening has shown that the reporter assays are not sensitive enough to be able to identify potential inhibitors. Recent "tweaking" of the reporter assay (done since the formal end of the grant period) has improved on this situation and the work is continuing.

In addition to the work described within the original proposal, the researchers have also identified and charcterized a previously unreported ppGpp-regulated virulence factor in Pba. During work to study modified versions of a Pba strain, some Pba colonies that show surface spreading were observed. These are characterised by the appearance of a surface "slime" layer. The genes involved have been identified and the researchers propose that this form of surface spreading may play a key role in promoting the spread of soft rot in tubers.

# 4. SUMMARY OF THE RESEARCH COMPLETED DURING THE PROJECT

## *4.1.* (p)ppGpp impacts on pectate lyase and OHHL production in *Pectobacterium atrosepticum*

### 4.1.1. Background

During starvation bacteria accumulate guanosine tetraphosphate and guanosine pentaphosphate ((p)ppGpp) which is able to arrest cell growth and activate the transcription of genes involved in biosynthesis of essential metabolites and virulence determinants. The biosynthesis of (p)ppGpp in most members of the Enterobacteriaceae is dependent on the enzymes RelA and SpoT. We predict that RelA and SpoT are responsible for (p)ppGpp biosynthesis in *Pectobacterium atrosepticum* strain SCRI1043 (hereafter, Eca1043) as it possesses *relA* and *spoT* genes but no other genes that can synthesise (p)ppGpp. Consistent with this, an engineered Eca1043 *relA spoT* double mutant is auxotrophic for (i.e. unable to synthesise) multiple amino acids including leucine, isoleucine, valine and phenylalanine suggesting that (p)ppGpp biosynthesis is abolished in this strain. The Eca1043 *relA spoT* mutant is also severely reduced for secreted protease activity and OHHL production (Figure 1). Indeed, even an Eca1043 *relA* single mutant is strongly reduced for pectate lyase activity during growth in Pel Minimal Broth (PMB) and is also reduced for production of the quorum sensing molecule, OHHL (Figure 2). [Pectate lyase and protease are the major secreted mediators of soft rot in potatoes.] Note that the impact of (p)ppGpp on secreted protease production is independent of quorum sensing since provision of exogenous synthetic OHHL cannot restore protease production (Figure 1).

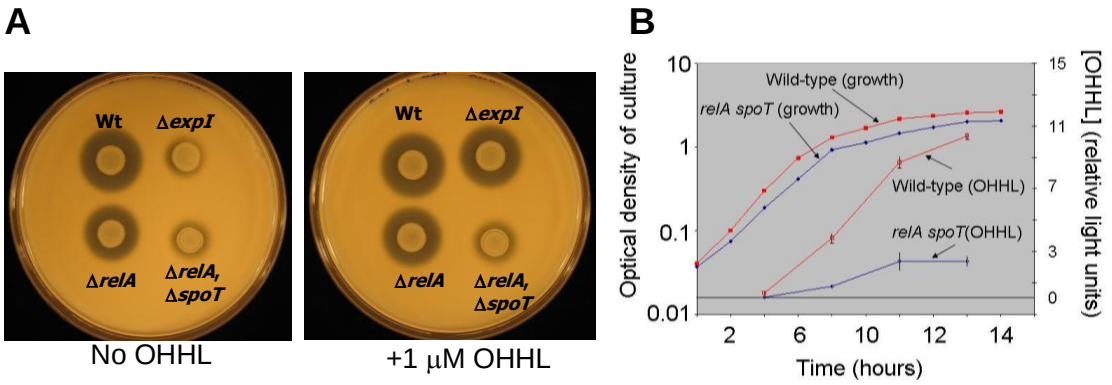



**Figure 1.** (A) Secreted protease production by wild-type, *relA*, *relA spoT* and *expI* mutants of Eca1043 on protease medium in the presence or absence of exogenous OHHL. OHHL can restore protease production to the *expI* mutant but not to the *relA spoT* mutant. Protease activity is indicated by a dark halo around each inoculated colony. (B) Growth and OHHL production by the wild-type and *relA spoT* mutant when grown in LB. The error bars represent the standard deviation from 3 biological replicates.

Unfortunately, the *relA spoT* mutant exhibits a large growth defect compared with the wild-type progenitor strain when grown in PMB. This defect is due to the low level of amino acids present in PMB; the growth rate of the *relA spoT* mutant can be restored to wild-type levels upon adding 1% w/v casamino acids to PMB media. However, the presence of casamino acids suppresses pectate lyase activity (but not OHHL) in the wild-type strain (Figure 3). Collectively these data are consistent with the notion that (p)ppGpp is required for wild-type levels of growth and pectate lyase activity in PMB media and that the presence of high concentrations of amino acids suppresses (p)ppGpp production leading to reduced pectate lyase activity.

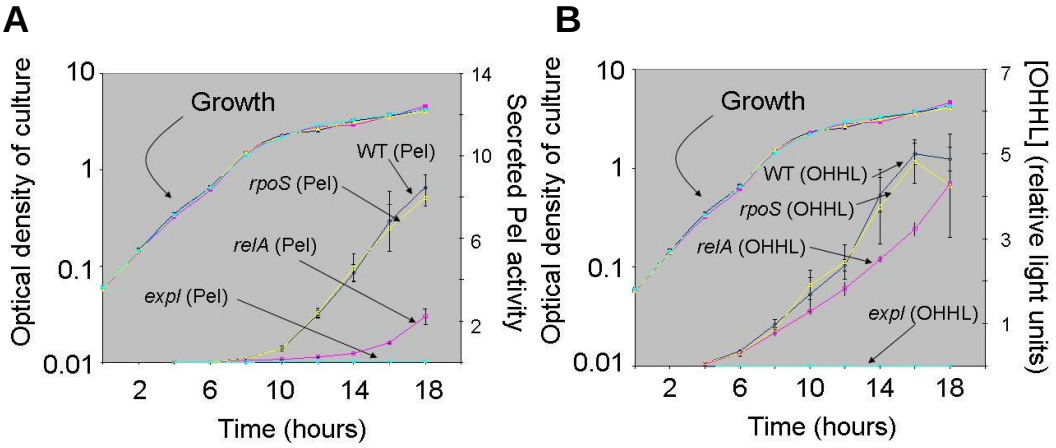



**Figure 2**. Growth of SB1004 (wild-type), SB1026 (*relA*), SB1046 (*rpoS*) mutant and SB1031 (*expI*) in PMB. Pectate lyase (Pel) activity (A) and OHHL production (B) are shown for each mutant. The error bars represent the standard deviation from 3 biological replicates.

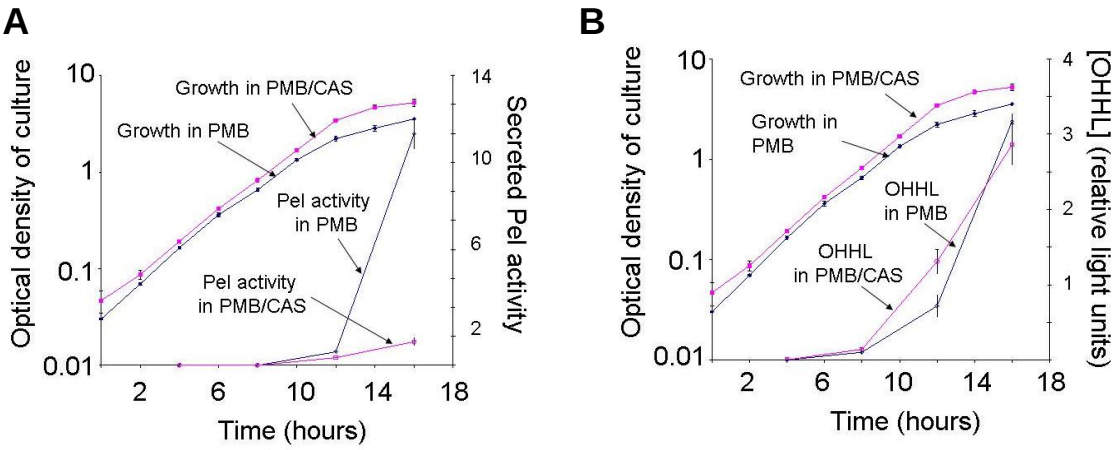



**Figure 3**. Growth of wild-type Eca1043 in PMB or PMB supplemented with 1% w/v casamino acids. Pectate lyase activity (A) and OHHL production (B) are shown. The error bars represent the standard deviation from 3 biological replicates.

To assess the global impact of (p)ppGpp on virulence in Eca1043 we carried out a microarray analysis. Given the complications introduced by the growth defect of the *relA spoT* double mutant when grown in PMB alone, and the exoenzyme suppressive effect of casamino acids on the wild-type, we decided to compare the *relA* mutant to the wild-type when grown in PMB media. This is a condition in which growth was comparable in both strains and virulence is clearly affected in a (p)ppGpp dependent way (Figure 1). We chose to compare cDNA from 6h and 14h time points, which correspond to the log phase and stationary phase of growth, respectively. Amino acid analysis of the PMB medium at these two time points indicated that after 14h growth, most amino acids were depleted from the medium (Table 1). For comparison we also included samples from an *expI* mutant (unable to synthesise OHHL) and an *rpoS* mutant (RpoS is an important regulator in *Escherichia coli*, where *rpoS* expression is dependent upon (p)ppGpp). RNA samples were extracted from 3 separate cultures of each strain to ensure the data was statistically robust.

| Sample | PMB | | Wildtype 6 hours | | *relA* 6 hours | | Wildtype 14 hours | | *relA* 14 hours | |
|---|---|---|---|---|---|---|---|---|---|---|
| | data | nmoles/ml | data | nmoles/ml | data | nmoles/ml | data | nmoles/ml | data | nmoles/ml |
| Asp | 2.4461 | 159 | 0.3126 | 21 | 0.2751 | 18 | 0.189 | 12 | 0.1574 | 10 |
| Thr | 2.4027 | 157 | 1.9638 | 129 | 2.0293 | 132 | 0.0291 | 2 | 0.0293 | 2 |
| Ser | 4.5439 | 296 | 0.1259 | 8 | 0.1308 | 8 | 0.0761 | 5 | 0.122 | 8 |
| Glu | 5.7897 | 377 | 3.6378 | 239 | 3.9187 | 254 | 0.0245 | 2 | 0.0354 | 2 |
| Gly | 2.6956 | 176 | 2.1863 | 144 | 2.2624 | 147 | 1.1305 | 72 | 4.7561 | 306 |
| Ala | 6.1036 | 398 | 5.6719 | 373 | 5.9456 | 386 | 0.3799 | 24 | 1.2985 | 84 |
| Val | 3.7199 | 243 | 2.9974 | 197 | 3.1321 | 203 | 0.0396 | 3 | 0.0385 | 2 |
| Met | 0.7964 | 52 | 0.5439 | 36 | 0.5776 | 37 | | | | |
| Ile | 2.8916 | 189 | 2.2417 | 147 | 2.2904 | 149 | 0.0295 | 2 | 0.032 | 2 |
| Leu | 4.9155 | 320 | 3.5754 | 235 | 3.7215 | 241 | | | | |
| Norleu std | 3.8346 | | 3.8042 | | 3.8548 | | 3.906 | | 3.8801 | 250 |
| Tyr | 0.5227 | 34 | 0.1971 | 13 | 0.216 | 14 | | | | |
| Phe | 2.0715 | 135 | 1.5714 | 103 | 1.6471 | 107 | | | | |
| His | 0.5071 | 33 | 0.3762 | 25 | 0.3972 | 26 | 0.0156 | 1 | 0.0113 | 1 |
| Lys | 2.4181 | 158 | 2.138 | 141 | 2.2381 | 145 | 0.0232 | 1 | 0.0192 | 1 |
| Arg | 1.4201 | 93 | 0.7049 | 46 | 0.7752 | 50 | | | | |
| Pro | 1.3295 | 87 | 1.1006 | 72 | 1.114 | 72 | 0.4812 | 31 | 1.4246 | 92 |

**Table 1**. Concentration of amino acids in PMB media and supernatant from wild-type and *relA* mutant cultures grown in PMB for 6 hours and 14 hours. Amino acids were detected by derivatization with ninhydrin followed by HPLC analysis.

The data from the microarray was analysed using GeneSpring software. This revealed that at 6h the *expI* mutant had the greatest impact on gene expression with 110 genes down-regulated compared with the wild-type strain (Table 2). These results suggest that quorum sensing impacts on gene expression even at relatively low cell densities e.g., during exponential phase growth, when OHHL levels are still very low. In contrast, the *relA* and *rpoS* mutants did not have much effect at 6h growth, as may be expected for these stationary phase regulators. During stationary phase 412 genes were down-regulated in the *expI* mutant and 333 genes were up-regulated, demonstrating that the role of quorum sensing in regulating gene expression is more dramatic during the later stages of growth, again, as would be predicted. However, the predominant role of (p)ppGpp in regulating gene expression during stationary phase became clear when comparing the wild-type and *relA* mutant 14h time point samples. From a total of 4472 ORFs to which probes were present for on the microarray, 771 were down-regulated and 1950 were up-regulated in this mutant. Therefore, (p)ppGpp production by RelA is required to modulate expression of over half of the genes in the chromosome during entry into stationary phase.

| | 6 hours | 6 hours | 14 hours | 14 hours |
|---|---|---|---|---|
| | No. of genes down regulated | No. of genes up regulated | No. of genes down regulated | No. of genes up regulated |
| *expI* mutant | 110 | 0 | 412 | 333 |
| *relA* mutant | 2 | 7 | 771 | 1950 |
| *rpoS* mutant | 1 | 0 | 1 | 0 |

**Table 2**. Number of genes whose expression is significantly altered (compared with the wild-type strain) during growth in PMB media.

In contrast to expectation, the *rpoS* mutant was not altered in any genes apart from the *rpoS* gene itself, demonstrating that its role in *E. coli* cannot be extrapolated to *Pectobacterium atrospeticum*. However, this does not preclude the possibility of RpoS playing a role in gene expression during growth in other media. Alternatively, the *rpoS* gene may be non-functional in Eca1043. If this is the case, the strain has obviously acquired mechanism(s) to bypass this defect.

By comparing the number of genes altered in different functional categories it seems that the *relA* mutant is up-regulated for genes required for active growth including DNA synthesis, translation and cell division (Figure 4A). This is to be expected given the known role(s) of (p)ppGpp in suppressing these physiological processes. Notably, the

only class of genes that are predominantly down-regulated in the *relA* mutant are those relating to cell motility and secretion (which includes virulence genes - Figure 4A and 4B). We speculate that when Eca1043 has sufficient nutrients for active growth it has no requirement to activate expression of its virulence genes. One of the roles of (p)ppGpp maybe to control the switch from active growth to virulence; this has been observed in a few other bacterial species.

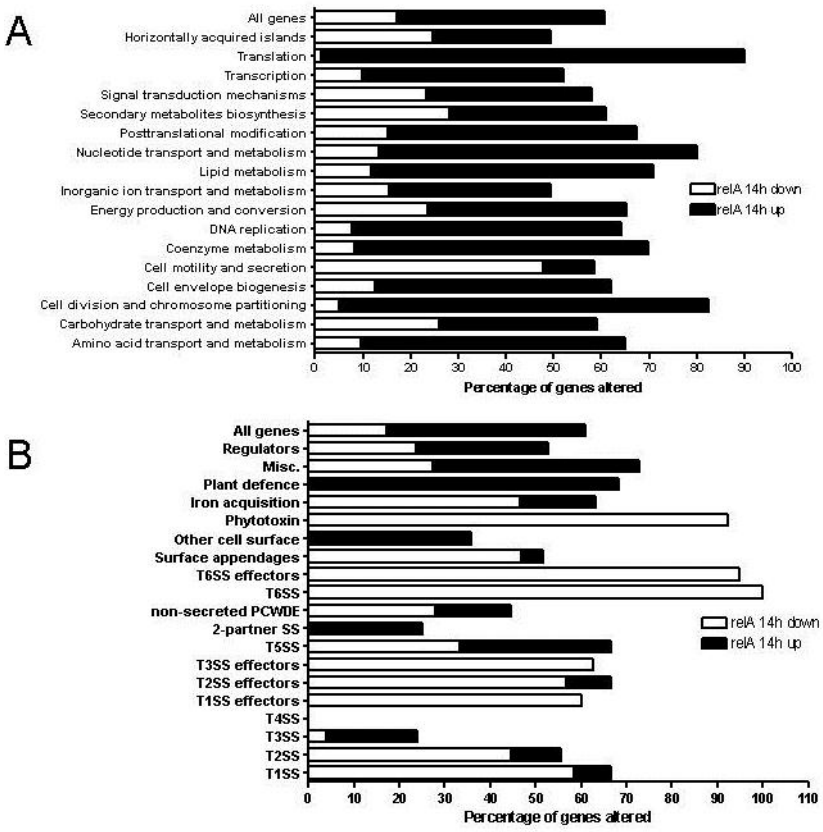



**Figure 4**. Percentage of genes down-regulated or up-regulated in the *relA* mutant (compared with the wild-type) from different functional categories (A) and functional categories specifically related to virulence (B).

Consistent with the notion that (p)ppGpp plays an important role in regulating virulence, when comparing the genes that are altered in the *relA* mutant and in the *expI* mutant during stationary phase, it is clear that there is a strong overlap (Figure 5). For example 70% of genes down-regulated in the *expI* mutant are also down-regulated in the *relA* mutant and 48% of genes up-regulated in the *expI* mutant are also up-regulated in the *relA* mutant. The correlation between those genes that are down-regulated is particularly striking and includes many of the predicted virulence genes present in Eca1043. Examples of virulence genes altered in the *relA* and *expI* mutants include the Type-1, Type-2 and Type-6 secreted protein genes and the putative phytotoxin biosynthesis genes (see also Figure 4B).

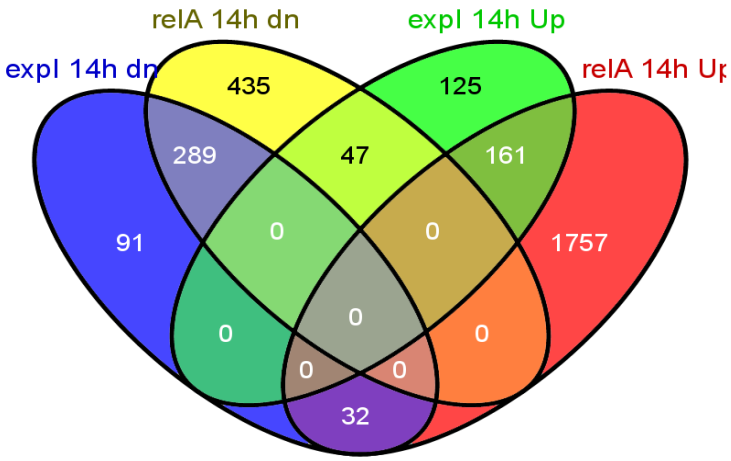
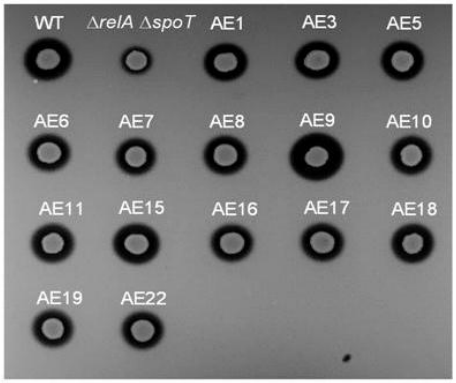



**Figure 5**. Venn diagram comparing the genes that are down-regulated or up-regulated in the *relA* and *expI* mutants (relative to the wild-type strain) at 14h growth in PMB.

Following the microarray analyses, we carried out a transposon bypass screen to try and identify mutants that would restore the production of plant cell wall degrading exoenzymes to the *relA spoT* mutant. Of ca. 12,500 Tn mutants screened, 15 were found to restore protease production (an assay which formed the basis of our initial screening strategy) to the mutant (Figure 6). We mapped the insertions in these and found that the mutants were disrupted in 14 unique loci. The most highly-restored Tn mutant (AE9) contained an insertion in *rsmA*. [This is the first time such a mutant has been obtained in Pba.] RsmA is able to bind to specific mRNA transcripts – e.g., virulence-related transcripts - and reduce their stability, therefore behaving as a post-translational inhibitor of gene expression. This suggested that (p)ppGpp might be acting by modulating RsmA levels in the cell. Indeed, *rsmA* transcription is known to be promoted by the LuxR-type central quorum sensing regulator, VirR. In the current model for quorum sensing, as the OHHL concentration increases, VirR interacts with the OHHL signal molecule and its affinity for the *rsmA* promoter is reduced leading to reduced expression of *rsmA*. The reduced level of RsmA protein leads to derepression of the virulence genes and therefore increased production of virulence factors. To test the possible involvement of (p)ppGpp in regulating RsmA levels, we raised an antibody against RsmA and used this to determine RsmA expression levels in the wild-type, *expI* mutant and *relA* mutant. However, although RsmA levels were elevated in the *expI* mutant (as expected, based on the current model), they appeared to be very similar in both the wild-type and *relA* mutant (Figure 7B, lower panel). This suggested that (p)ppGpp is unlikely to act by modulating *rsmA* expression. However, RsmA activity is known to be antagonised by the regulatory RNA, rsmB, which is able to sequester the RsmA protein from its target mRNA transcripts. We therefore began to wonder whether (p)ppGpp might regulate *rsmB* expression instead. Consistent with this, Q-RT-PCR analyses showed that in the (p)ppGpp-deficient *relA* mutant, *rsmB* expression does not increase upon entry into the stationary phase (as it does in the wild-type and *expI* mutant – Figure 7B (upper panel)). It therefore seems that quorum sensing influences virulence by impinging upon RsmA levels, while (p)ppGpp counteracts this by activating expression of the RsmA antagonist, rsmB.

A



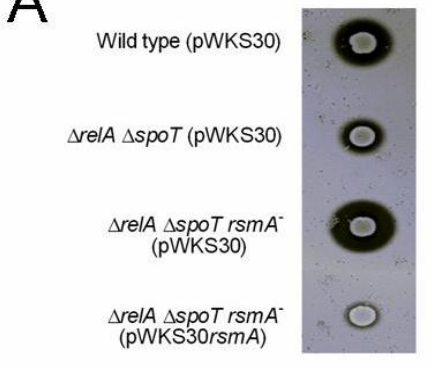

B

| Strain | Insertion site | Description/comments |
|---|---|---|
| **AE1** | 158727 (*eca0137*) | ABC transporter operon homologous to *yliABCD* glutathione transporter from *E. coli*. |
| **AE3** | 4073419 (*eca3624*) | 3' end of methyl-accepting chemotaxis protein. Potentially polar effect on downstream *eca3625* encoding for a GGDEF protein. |
| **AE5** | 5001636 (*eca4456*) | 3' end of GGDEF pseudogene. Potentially operonic with downstream *smvA* gene. |
| **AE6** | 4913785 (*eca4381*) | Homolog of *yjjM* transcriptional regulator from *E. coli* that is essential for growth on L-galactonate. |
| **AE7** | 3879933 (*eca3460*) | Phage integrase gene. Operonic with phage hypothetical and endonuclease genes. |
| **AE8** | 1569515 (*eca1385*) | Outer-membrane efflux protein. Operonic with copper efflux genes. |
| **AE9** | 3782056 (*rsmA*) | Insertion within promoter region of *rsmA*. |
| **AE10** | 682226 (*eca0610*) | Putative transcriptional regulator divergently transcribed from coronofacic acid biosynthesis operon. |
| **AE11** | 1328193 (*eca1172*) | Hypothetical gene 3' of *priC* encoding primosomal replication protein N'' |
| **AE15** | 897662 (*hexY*) | Within 5' UTR of *hexY* mRNA transcript, a small protein that interacts with FlhDC and represses PCWDEs. |
| **AE16** | 457053 (*eca0400-0401*) | Intergenic insertion within a probable small hydrophobic transmembrane peptide. |
| **AE17** | 197208 (*eca0177*) | Hypothetical gene. Operonic with hypothetical gene encoding a transmembrane protein. |
| **AE18** | 897668 (*hexY*) | Within 5' UTR of *hexY* transcript, encoding a small protein that interacts with FlhDC and represses PCWDEs. |
| **AE19** | 3388896 (*hexA2*) | Within *hexA2*, a transcriptional regulator known to repress PCWDEs in *Pectobacterium* sp. |
| **AE22** | 640458 (*eca0577*) | Within putative tra locus that could be involved in conjugal DNA transfer. |

Location of transposon insertions that restore secreted protease production in the *ΔrelAΔspoT* mutant.

**Figure 6**. Transposon mutants that bypass the exoenzyme defect in the *relA spoT* mutant. (A) A panel of protease assays for each of the mutants displaying restored protease production. Protease activity is indicated by a dark halo around each inoculated colony. (B) Location of the mapped transposon insertions in each of the indicated mutants.

A




B

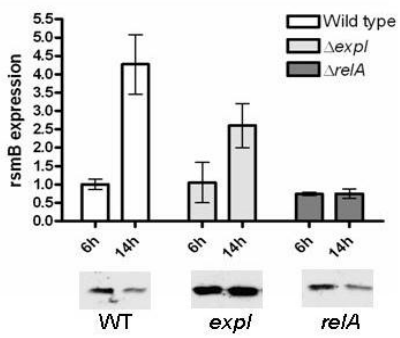



**Figure 7**. Disruption of *rsmA* restores protease production to the *relA spoT* mutant. (A) A panel of protease assays showing that disruption of *rsmA* leads to restoration of protease production in the *relA spoT* mutant. pWKS30 is a low copy plasmid vector. pWKS30*rsmA* contains a cloned copy of the *rsmA* gene. Protease activity is indicated by a dark halo around each inoculated colony. (B) Q-RT-PCR analysis of rsmB expression (upper panel) and western blot analysis of RsmA levels (lower panel) in the indicated strains

To further test this model, we introduced *rsmB* on a plasmid *in trans* into each of the virulence-defective strains (*expI*, *relA spoT* etc etc) and examined the impact of this on virulence factor production. As shown in Figure 8, unregulated *rsmB* expression was able to robustly bypass the virulence defect in all of the strains examined.

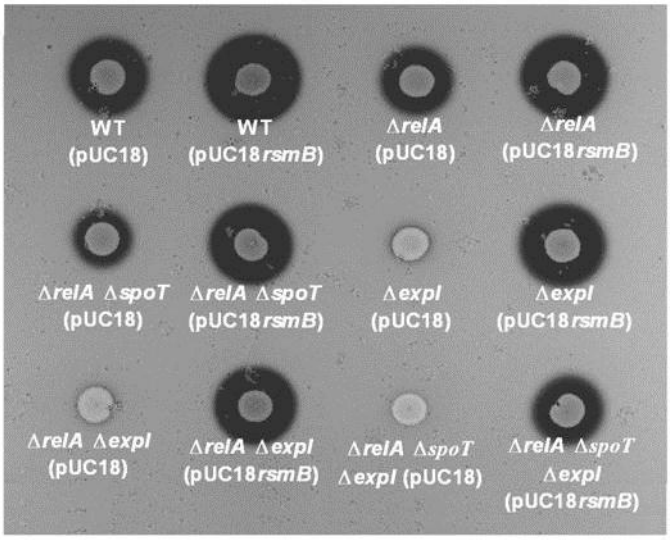



**Figure 8**. Expression of rsmB robustly restores protease production to virulence-defective mutants. The *rsmB* gene was expressed from the high copy number vector pUC18 in each of the indicated strains. Protease activity is indicated by a dark halo around each inoculated colony.

These observations supply a clear rationale behind the significant shared impact of RelA and ExpI on virulence gene expression (Figure 5) and indicate that these factors regulate virulence through a common mechanism (Figure 9). Anthropocentrically, it seems logical for quorum sensing and (p)ppGpp to regulate virulence together. When nutrients are freely available for bacterial growth on a plant surface there is no requirement to activate virulence gene expression and risk being killed by the plant hypersensitive response. However, should the nutrients available for *Eca1043* growth become limiting, then the bacteria face starvation and therefore cell death. Therefore, to circumvent this, virulence factors are only elaborated once the cell has run out of other sources of nutrient.

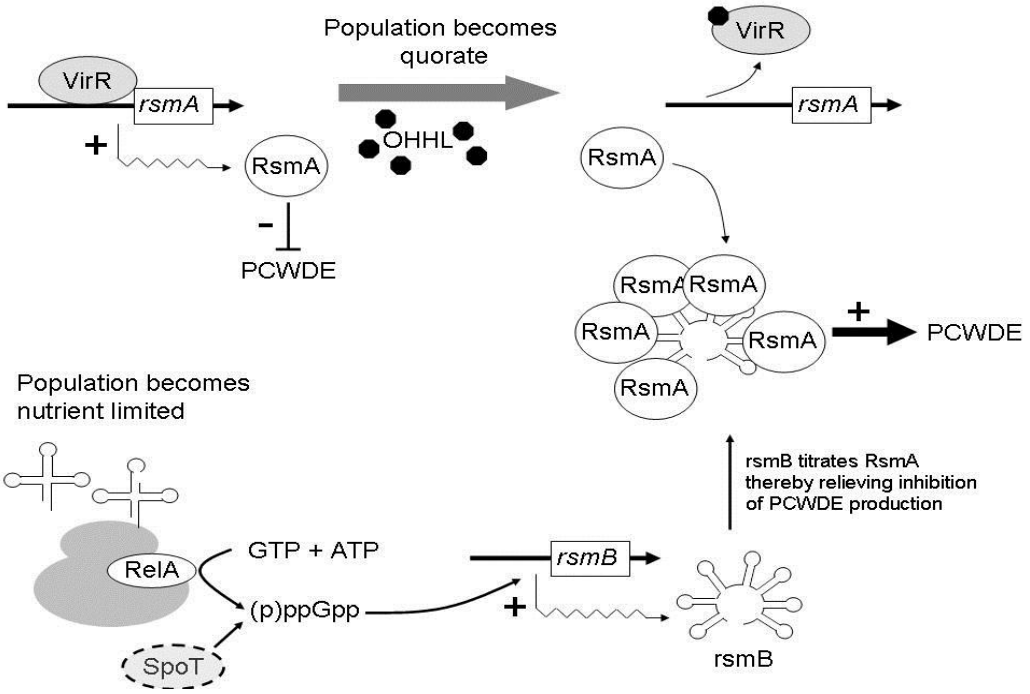



**Figure 9**. Model for the regulation of virulence factor production by quorum sensing and (p)ppGpp. **Upper arm.** At low cell densities (i.e., in the pre-quorate period) the LuxR homolog, VirR, activates transcription of *rsmA*. The RsmA protein targets PCWDE-encoding transcripts for degradation, thereby suppressing virulence. However, when the cell density is high, OHHL binds to VirR causing it to undergo a conformational change. In the ligand-bound conformation, the affinity of VirR for the *rsmA* promoter is diminished, and *rsmA* transcription decreases. This is why RsmA levels are constitutively high in the *expI* mutant (Figure 7B). **Lower arm.** RelA and SpoT synthesize (p)ppGpp when the culture becomes nutrient limited. (p)ppGpp directly or indirectly activates *rsmB* transcription. The rsmB binds and sequesters RsmA, thereby de-repressing PCWDE production. In the absence of rsmB production (i.e., in a *relA* or *relA spoT* mutant) PCWDE synthesis remains suppressed by RsmA, even in the presence of excess OHHL (Figure 1). The reciprocal effects of QS and (p)ppGpp on the RsmA/rsmB node would be expected to lead to a highly cooperative switch that is sensitive to both the physiological state of the cells and their population cell density.

## 4.2. Artificial modulation of ppGpp levels in the cell and the impact of this upon virulence

In the original grant proposal, we aimed to generate ppGpp *in vitro* using a RelA/ribosome-dependent system. However, we have now found a better way forward by exploiting a ribosome-independent ppGpp synthesizing enzyme from *Vibrio cholerae*, RelV. We have cloned *relV* and shown that, when expressed, it does indeed synthesize ppGpp which leads to the complete cessation of Eca growth (**Figure 8**) in liquid culture. This is expected because ppGpp acts to shut down ribosome synthesis. Addition of arabinose had no effect on the growth of cultures containing the empty vector (pBAD30), nor did addition of OHHL.

Ideally, we would like to purify RelV to enable facile preparation of ppGpp *in vitro* (this ppGpp would be used for chemical immobilization to define the cellular “ppGpp-ome”). However, efforts to clone RelV for purification with suitable affinity tags have proven problematic. Addition of a C-terminal hexa-histidine tag and expression from the tightly-controlled (but still leaky – see Figure 10) arabinose-inducible vector, pBAD30, revealed that the tagged protein retained function i.e., caused immediate cessation of

cell growth upon induction, but we were unable to purify any expressed protein. Clearly, the expression of even tiny amounts of RelV causes the cells to stop growing. We tried to circumvent this problem through (*i*) transient high-level induction of *relV* and (*ii*) by targeting the synthesized RelV to the periplasm by incorporating a cleavable N-terminal signal sequence/MBP tag, but again, this failed to prevent growth cessation and yielded no purifiable protein. We therefore turned to using Yeast as an expression system. Yeast does not synthesize or respond to ppGpp. Expression of C-terminally histidine-tagged *relV* from a suitable Yeast vector slowed growth but did not stop it. Anti-$His_6$ antibodies were used to monitor RelV expression in the Yeast system. Yields are low and the high levels of proteolytic activity associated with expression in a eukaryotic system are proving [very] problematic. However, we now have some purified RelV and are currently characterizing this further. [Note that even small amounts of catalytically-active RelV offer the potential to generate large amounts of well-defined product – hopefully, pppGpp.]

In parallel, and as a backup, the chemists in Dr Spring's group at Cambridge are chemically synthesizing ppGpp for immobilization. Yields are currently low but the advantage here is that the positions upon the molecule that carry the immobilization linkers can be tightly-controlled.

## 4.3. Development of a facile ppGpp reporter assay

Our own microarray and RT-PCR analyses as well as work done by other teams on ppGpp, strongly suggest that ppGpp represses transcription from the promoter of *rpsM* (a ribosomal gene) and enhances expression from the *ilvG* promoter (which encodes an amino acid biosynthetic cluster). We therefore wondered whether we could develop a plasmid-borne mCherry/GFP dual reporter in which mCherry is expressed under the control of the *rpsM* promoter and GFP is expressed from the *ilvG* promoter. Compounds that antagonize ppGpp synthesis or reception would therefore increase the mCherry:GFP signal ratio by concomitantly up-regulating mCherry transcription and down-regulating GFP.

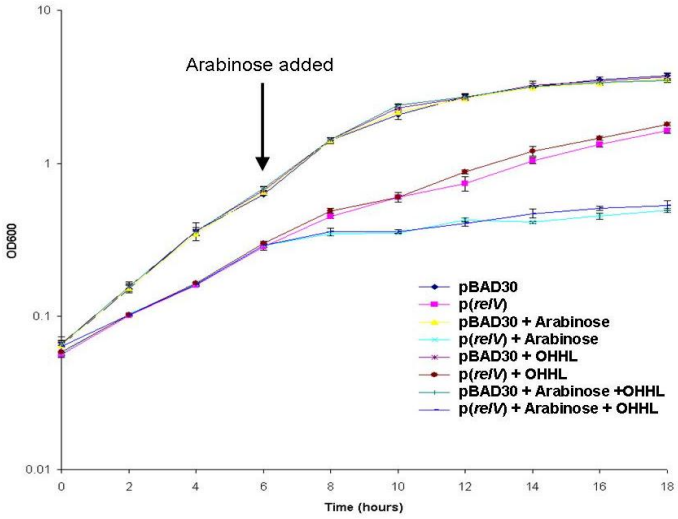



**Figure 10.** RelV is a ppGpp synthase. The *relV* gene from *V. cholerae* was cloned into a low copy number arabinose-inducible expression vector, pBAD30. The resulting construct and the empty vector were introduced separately into wild-type Eca strain 1043. Growth in Pel minimal broth (PMB) was monitored spectrophotometrically. Arabinose was introduced into the indicated cultures at t = 6h of growth. Where indicated, synthetic quorum sensing molecule, OHHL, was exogenously added to the cultures at t = 0h. Note that even in this tightly-repressed pBAD30 expression system, there is sufficient "leaky" expression from the p(*relV*)-containing strains that growth is slowed down. Growth ceases altogether when the p(*relV*)-containing strains are induced with arabinose.

Given the strength of the *rpsM* promoter, we initially decided to (presumptively) attach an ssrA tag to the mCherry protein. The ssrA tag targets the encoded protein for degradation by ClpXP and should (in principle) prevent the mCherry from accumulating to very high levels. [To maximize the sensitivity of the assay, we did not want the mCherry signal to dominate.] However, none of the resulting transformants produced detectable mCherry fluorescence. We therefore removed the ssrA tag from the mCherry ORF. This led to transformants showing detectable but still very low red fluorescence signals. To improve on this, we tried cloning the *rpsM* promoter along with a slightly larger region of upstream DNA (incorporating a small ribosomal gene called *rpsJ*) in front of the ssrA-less mCherry. This strategy yielded several transformants with a stronger mCherry signal. Finally, and to further boost expression of the protein, we replaced the sub-optimal mCherry ribosome binding site (RBS) with a more powerful RBS. This further improved the fluorescence response. Current efforts (in collaboration with Dr Paivi Tamela, Helsinki University, Finland) are aimed at (i) inserting the promoter for the *cfa* cluster of genes in place of the *ilvG* promoter (the *cfa* cluster is more strongly induced by ppGpp than the *ilvG* gene) and (ii) transferring the whole mCherry/GFP cassette onto a high copy vector (to further enhance the fluorescence signal).

## 4.4. Surface spreading phenotype

A new virulence-associated phenotype has been discovered during the course of the current study; ppGpp-dependent surface spreading by Pba (Figure 11). We have deciphered much about the mechanism(s) regulating this important new phenotype. Firstly, transposon mutagenesis screens were carried out to identify the structural genes that encode the secreted polymer. Remarkably, we found that these biosynthetic genes localize to a horizontally-acquired pathogenicity island (HAI-5) encoding a pathway for O-antigen production. We further established that the HAI-5

cluster encodes the only O-antigen synthesizing pathway in the cell (Figure 12) and is required for LPS biosynthesis. Mutants in the HAI-5 genes did not display surface spreading. Further mutagenesis experiments identified a number of additional regulators of surface spreading, including *hexY* and the surface "wetness" sensor, *rcsD*. This has led to the model of surface spreading shown in Figure 13.

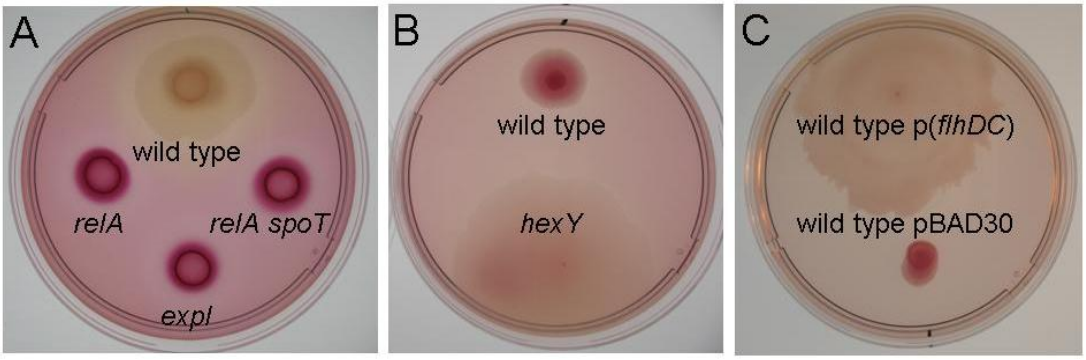



**Figure 11.** (**A**) ***Surface spreading is abolished in the absence of ppGpp or OHHL***. The panel shows surface spreading (manifested through the appearance of a surface "slime" layer) by the wild-type progenitor strain (Eca1043) is abolished in a *relA* mutant (deficient in ppGpp) and in a *relA spoT* double mutant (null for ppGpp). Slime is also absent in an *expI* mutant (unable to make OHHL, the quorum sensing molecule). (**B**) ***Surface spreading is up-regulated in a hexY mutant***. The HexY protein normally functions to sequester and inactivate a master regulator of virulence called $FlhD_2C_2$. The $FlhD_2C_2$ protein independently stimulates exoenzyme (virulence factor) production *via* an effect on *gacA*/*rsmB* and motility (*via* an effect on *fliA*), so mutants in *hexY* are normally "hyper" for both of these phenotypes. Surface spreading might plausibly involve one or both mechanisms of $FlhD_2C_2$ action. Indeed, ***(C) artificial over-expression of $FlhD_2C_2$*** leads to inappropriate surface spreading. However, when we expressed $FlhD_2C_2$ *in trans* in a *fliC* mutant, we did not observe copious surface spreading (*data not shown*). This suggests that $FlhD_2C_2$ promotes *fliA*-dependent surface spreading.

In the model, surface spreading is driven my motility. Mutations in the O-antigen synthesizing genes lead to altered cell surface properties, and concomitantly, altered surface "wetness". This altered wetness is sensed (through mechanisms not yet clear) by the RcsCDB system in the inner membrane. Consistent with the involvement of the Rcs signalling system, one

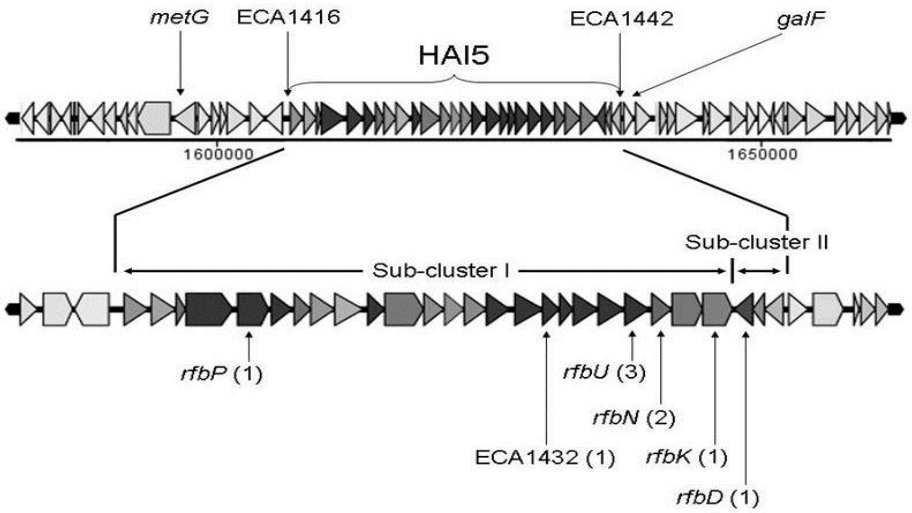



**Figure 12**. ***Location of insertions in HAI-5 that disrupt surface spreading (swarming) by Pba***. NB: We are currently chemically-analysing the EPS associated with surface spreading to confirm that it corresponds to HAI-5-encoded LPS O-antigen.

of the hyper surface spreader mutants that we isolated contained a transposon in *rcsD*. When activated, the RcsCD proteins phosphorylate RcsB, which leads to decreased expression of the master regulator $FlhD_2C_2$. The decreased $FlhD_2C_2$ level leads to decreased *fliA* transcription (see legend to Figure 11) and therefore, diminished surface spreading. This model accounts for all of the observations made so far. For example, a mutant in *fliC* does not display surface spreading. Inappropriate over-expression of $FlhD_2C_2$ in the wild-type enhances surface spreading. Mutation of *hexY* leads to higher "free" $FlhD_2C_2$ levels in the cell and consequently, increased surface spreading. Finally, over-expression of $FlhD_2C_2$ in the surface spreading-defective O-antigen mutants (in HAI-5) promotes surface spreading independent of the Rcs phospho-relay system.

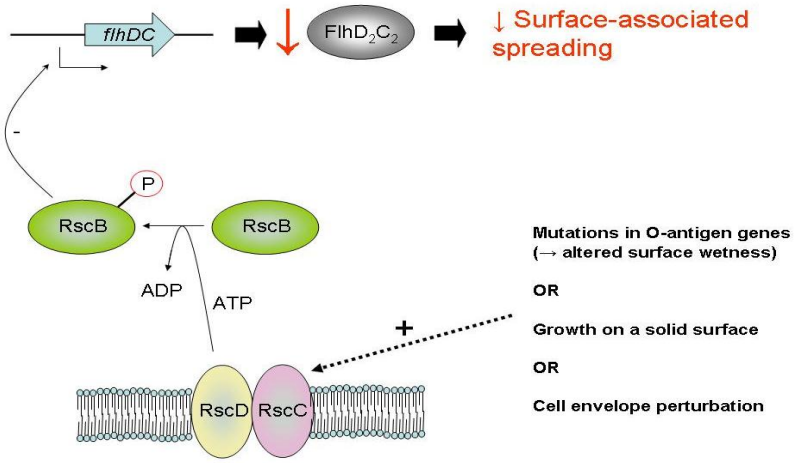



**Figure 13. Model accounting for how EPS ("slime") production is regulated in Pba**. See body text for details.

Given the regulation of this new phenotype by the major known "players" involved in virulence (quorum sensing, ppGpp, HexY, $FlhD_2C_2$, HNS and Fis, among others), we strongly suspect that this form of surface spreading may play a key role in promoting the spread of rot in tubers.