A Metabolic Regulator Modulates Virulence and Quorum Sensing Signal Production in *Pectobacterium atrosepticum*

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Plant cell wall-degrading enzymes (PCWDE) are key virulence determinants in the pathogenesis of the potato pathogen *Pectobacterium atrosepticum*. In this study, we report the impact on virulence of a transposon insertion mutation in the *metJ* gene that codes for the repressor of the methionine biosynthesis regulon. In a mutant strain defective for the small regulatory RNA *rsmB*, PCWDE are not produced and virulence in potato tubers is almost totally abolished. However, when the *metJ* gene is disrupted in this background, the *rsmB*[−] phenotype is suppressed and virulence and PCWDE production are restored. Additionally, when *metJ* is disrupted, production of the quorum-sensing signal, *N*-(3-oxohexanoyl)-homoserine lactone, is increased. The *metJ* mutant strains showed pleiotropic transcriptional impacts affecting approximately a quarter of the genome. Genes involved in methionine biosynthesis were most highly upregulated but many virulence-associated transcripts were also upregulated. This is the first report of the impact of the *MetJ* repressor on virulence in bacteria.

Pectobacterium atrosepticum is a gram-negative phytopathogen that infects potato plants in temperate climates. *P. atrosepticum* infection causes two distinct diseases: soft rotting of tubers and blackleg disease of the plant stem. The pathology of both of these diseases is principally, but not exclusively, caused by the coordinated secretion of a barrage of plant cell wall-degrading exoenzymes (PCWDE) which degrade plant tissues (Toth and Birch 2005). Release of these exoenzymes, including isoenzymic forms of proteases (Prt), endoglucanases, polygalacturonases, and pectate lyases (Pel), is controlled by a variety of control systems, including quorum sensing (QS) and the RsmAB post-transcriptional regulation system (Barnard et al. 2007; Lapouge et al. 2008; Liu et al. 2008; Romeo 1998; Whitehead et al. 2002).

QS is an intercellular communication system widespread throughout many bacterial species. This mechanism relies on freely diffusible *N*-acyl homoserine lactone molecules produced throughout growth. At low cell densities, the concentration of the molecule is too low to elicit a response. However, at high

cell densities, the concentration of the signaling molecule reaches a threshold level and interacts with a cognate receptor protein causing transcriptional changes in the organism (Miller and Bassler 2001). In the paradigm model of QS in *Vibrio fischeri*, the signaling molecule is synthesized by the enzyme LuxI. The signaling molecule then interacts with its cognate receptor protein, LuxR (Fuqua et al. 1994). *P. atrosepticum* produces the signaling molecule *N*-(3-oxohexanoyl)-homoserine lactone (OHHL), synthesized by the enzyme ExpI. At high concentrations, this molecule binds to VirR, the primary QS transcriptional regulator. The current model is consistent with VirR acting as a global repressor protein that inhibits production of PCWDE and other virulence factors. When bound to OHHL, this repression is released and virulence determinants are expressed. Repression in the absence of OHHL also occurs via direct transcriptional activation of the global repressor gene, *rsmA*. A further LuxR homologue, ExpR, has also been identified in the genome of this strain of *P. atrosepticum*. However, in strain SCRI 1043, a mutant defective in this gene has no discernible phenotype in either a wild-type, *virR*[−], or *expI*[−] background and its function remains unknown (Burr et al. 2006; Monson et al. 2012).

Another key regulatory mechanism in *P. atrosepticum* is the regulator of secondary metabolism (Rsm) system. This involves a global repressor protein, RsmA, and a small regulatory RNA, *rsmB*. RsmA, a homologue of the *Escherichia coli* CsrA protein (Romeo et al. 1993), is a small protein of 6.8 kDa which binds to GGA motifs on target mRNAs, commonly around the Shine-Dalgarno sequence. This usually impairs transcript stability and translation of the message, although there is evidence that CsrA is also able to stabilize transcripts (Wei et al. 2001). The small RNA, *rsmB*, contains several stem-loop structures with GGA motifs on the loops which bind RsmA. This results in *rsmB* effectively out-competing the target transcripts for RsmA binding, increasing target mRNA stability and translation of Rsm-regulated messages (Lapouge et al. 2008). Together, RsmA and *rsmB* are major regulators of virulence in *Pectobacterium* spp. (Liu et al. 1998). The effect of this post-transcriptional regulation system is phenotypically dramatic, since an *rsmB* mutant strain is almost totally avirulent due to uninhibited repression of virulence factors by RsmA. Although a mutant defective in *rsmA* has not yet been isolated in this strain, homologous mutations in other closely related organisms confer a hypervirulent phenotype and growth impairment (Cui et al. 1995). Therefore, the stoichiometric relationship of this RNA and repressor protein is clearly vital to striking a balance between virulence and fitness of the organism.

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The initial aim of this study was to investigate the Rsm system in *P. atrosepticum* further through the creation of an *rsmA* mutant strain. The screen designed to identify such a mutant was to conduct a random transposon mutagenesis on a nonvirulent *rsmB* mutant, using the protease-negative (Prt^-) phenotype as a simple indicator assay. Mutant colonies could then be screened for a restored ability to produce Prt , the logic being that the lack of the RsmA repressor partner in an *rsmB* mutant background would result in restoration to an easily observable wild-type (or perhaps even “hyper”) phenotype, as seen in other species. However, this screening approach led to the unexpected discovery of *rsmB* suppression through mutations in a metabolic regulatory gene (*metJ*) not previously linked to pathogen virulence. The *MetJ* regulator in *P. atrosepticum* has now been shown to interact with the QS and Rsm systems and to impact on virulence in planta.

RESULTS

Construction and phenotypic analysis of an *rsmB* mutant strain, NW155.

In order to investigate the phenotypes regulated by the RsmA/B system in *P. atrosepticum*, an *rsmB* mutant strain was constructed (as described in Materials and Methods). This strain (NW155) completely lacked the *rsmB* coding sequence, which had been replaced by a kanamycin resistance cassette. The mutant displayed no growth defect but Prt and Pel activity was undetectable in culture media and the strain showed strongly reduced virulence in plant infection (Figs. 1 and 2). This strain could be complemented with a wild-type copy of the *rsmB* gene supplied in trans (Apagyi 2012).

Transposon mutagenesis screen.

Due to the clear Prt^- phenotype displayed by NW155, this strain could be used as background to screen for mutations in genes able to bypass the Rsm system. This would potentially include the *rsmA* gene, because the repression caused by free RsmA should be relieved in an *rsmAB* double mutant. Therefore, NW155 was used as the recipient in a conjugation with donor strain β 2163 (pDS1028). This plasmid was loaded with a transposon conferring resistance to chloramphenicol and containing the *uidA* reporter gene. With the aim of identifying an insertion in *rsmA*, transconjugants were screened for restored Prt production. Over 27,000 colonies were screened and three Prt^+ “revertants” were shown to carry independent insertions in the gene *metJ*, a homologue of the gene coding for the *E. coli* repressor of the methionine regulon. A fourth Prt^+ revertant carried an insertion in the virulence regulator, *hexY*. It was not unexpected to find an insertion in *hexY*, because mutation of this gene has been shown previously to enhance PCWDE production (Shih et al. 1999). The three mutations in the *metJ* coding sequence were an unexpected finding, with insertions present at 38, 167, and 205 bp into the 318-bp open reading frame. The insertion at 38 bp was in the sense orientation, such that a *metJ::uidA* transcriptional fusion was formed; therefore, further work was carried out on this mutant, named strain MC2.1.

The *metJ* mutation was transduced using the generalized transducing phage Φ M1 from strain MC2.1 into wild-type *Pba*1043, to produce strain MC1. This MC1 strain gave a Prt^+ phenotype similar to the wild type (Fig. 1A). The functional wild-type *metJ* gene was cloned into the Qiagen vector pQE80L and, when present in the double-mutant strain MC2.1, restored this to a Prt^- phenocopy of the progenitor *rsmB* mutant strain (Fig. 1B). This demonstrated genetically that it was, indeed, the *metJ* mutation that caused the corresponding phenotypic changes. Both strains carrying the insertion in *metJ* displayed

a slight growth defect (compared with wild-type and NW155 strains) in pectate minimal medium. Although the shape of the growth curve was identical, the absolute optical density at 600 nm (OD_{600}) values were reproducibly lower, maintaining stationary phase at an OD_{600} of approximately 3.5 compared with a value of approximately 5 for the wild type.

Although the initial aim of the screen was to identify an *rsmA* mutant, this aspect of the screen was unsuccessful. The creation of such a mutant in this bacterial strain has been attempted many times previously by random mutagenesis and allelic exchange methods but met with no success. Studies in other γ -proteobacteria have indicated that, in some organisms, knockout of *rsmA* (or the homologous gene *csrA*) may be either lethal or conditionally lethal (Langridge et al. 2009; Timmermans and Van Melderden 2009), which could explain the lack of success in this aspect of the screen.

A *metJ* mutation partially restores virulence in an *rsmB* mutant strain.

Liquid assays for Pel and Prt activity were performed on culture supernatant from stationary phase cultures of wild-type cells and strains NW155, MC1, and MC2.1 (mutated in *rsmB*, *metJ*, or both *rsmB* and *metJ*, respectively). These assays showed that, in a *metJ* mutant, PCWDE production was slightly lower than wild-type levels whereas, in an *rsmB* mutant, PCWDE activity was almost totally abolished. However, in the *rsmB metJ* double mutant, exoenzyme activity was partially restored to the level equivalent to that seen in the *metJ* mutant strain (Fig. 2A). To test whether these changes were reflected

Fig. 1. A, Skimmed milk agar plate showing protease production in wild type and NW155 (*rsmB* mutant), MC1 (*metJ* mutant), and MC2.1 (*rsmB metJ* double mutant) strains. **B**, Skimmed milk agar plates showing the complementation of strain MC2.1 with a wild-type copy of *metJ* supplied in trans.

in virulence phenotypes, each of the three mutant strains was inoculated into potato tubers and the amount of rotting was measured and compared with that of the wild type (Fig. 2B). The degree of rotting seen in each test strain was compared with that seen in the wild-type controls from the same tubers and analyzed by Student's *t* test. NW155 was the only test strain to show significantly different rotting levels from those of the wild type ($P < 0.05$). In addition, each strain was inoculated into 20 potato plant stems and the degree of rotting measured over 14 days (Fig. 2C). This appeared to show the same trend as seen in the PCWDE and tuber assays, with the *rsmB* mutant strain displaying almost no rotting and the *rsmB metJ* double mutant showing a partial restoration toward wild-type levels. On further analysis of these data, it became apparent that, in the *rsmB metJ* double mutant, although a successful infection caused the same amount of rotting as the wild type, establishment of the infection failed more often (Fig. 2D). Taken together, these data on in planta infection strongly reflect the trend seen in the liquid PCWDE assays and confirm that the *metJ* mutation partially restores virulence to an *rsmB* mutant strain. These data also suggest that the *metJ* mutation increases virulence through both mediating the levels of PCWDE production and the process of infection establishment.

A mutation in *metJ* fully restores production of the OHHL signaling molecule.

The three mutant strains were compared for their ability to produce the QS signaling molecule, OHHL. Culture superna-

tant samples were taken throughout growth and tested for OHHL production using a bioluminescent sensor strain. Induction of OHHL production began in late exponential phase but occurred at much lower levels in the *rsmB* mutant. However, in both strains carrying the *metJ* mutation, OHHL induction occurred earlier and to higher concentrations than in the wild type (Fig. 3B). The final concentrations of OHHL in the culture medium were between 5 and 8 μM for all four strains. This confirms that OHHL as well as PCWDE production in an *rsmB* mutant is restored by mutation of *metJ*.

To test whether it was simply restoration of the QS signaling molecule that was leading to PCWDE production in an *rsmB metJ* double mutant, strains were grown on specific media to test for exoenzyme production, with varying concentrations of OHHL. The *expI* mutant TB6 does not produce OHHL and, therefore, also does not produce PCWDE such as Prt. This mutant could be chemically complemented by 0.5 μM OHHL, whereas the *rsmB* mutant NW155 showed no increase in Prt production when supplied with up to 10 μM OHHL (Fig. 3C). The concentration of 10 μM was selected as a maximum because this is higher than levels measured in stationary phase cultures of wild-type *P. atrosepticum* (discussed above). This effect was also seen on cellulase and Pel production (Supplementary Fig. S1). In addition, providing exogenous OHHL to wild-type cultures at the start of growth did not induce early PCWDE production (Fig. 3B). Therefore, the restoration of OHHL production does not appear to be the direct cause of the restoration of virulence seen in this strain.

Fig. 2. A, Pectate lyase (Pel) and protease (Prt) activity in stationary phase cultures in wild type and NW155 (*rsmB* mutant), MC1 (*metJ* mutant), and MC2.1 (*rsmB metJ* double mutant) strains. Cultures were grown in pectate minimal media (PMM) at 25°C. Data for the mutant strains is shown relative to the wild type, which is represented as 1. B, Rotting in potato tubers as quantified by weighing. Data for the mutant strains is shown relative to the wild type, which is represented as 1. C, Rotting in potato plant stems as quantified by lesion length measured over 14 days. D, Distribution of rotting in the sample on the 14th day, with each point representing a successful infection.

In order to test whether MetJ was affecting OHHL production via transcriptional regulation of the OHHL synthase, levels of the *expI* transcript were measured by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Expression

of the *expI* transcript during late stationary phase was reduced by approximately twofold in an *rsmB* mutant but increased by 2.8-fold in the *metJ* mutant (Fig 3D). These changes in *expI* expression show the same pattern as for OHHL expression at

Fig. 3. A, Protease production by NW155 (*rsmB* mutant; top left), wild type (middle right), and TB6 (*expI* mutant; bottom left) strains grown on protease agar supplemented with varying concentrations of *N*-(3-oxohexanoyl)-homoserine lactone (OHHL) or a dimethyl sulfoxide (DMSO) control. **B**, OHHL production in culture supernatants measured throughout growth. Cultures were grown in peptone minimal media (PMM) at 25°C. **C**, Pectate lyase (Pel) activity (bars) in wild-type culture supernatants measured throughout growth (line graphs) in the presence of either 1 μ M OHHL or a DMSO control. **D**, Expression of the OHHL synthase gene, *expI*, in wild type and NW155 (*rsmB* mutant), MC1 (*metJ* mutant), and MC2.1 (*rsmB metJ* double mutant) strains. Quantitative reverse-transcriptase polymerase chain reaction was carried on cDNA generated from RNA samples taken after 18 h of growth in PMM.

18 h (Fig. 3B). This suggests that modulation of ExpI levels is at least partly responsible for the changes observed in OHHL production.

Bioinformatic analysis of the MetJ regulon.

Alignment using the National Center for Biotechnology Information BLAST tool (Altschul et al. 1997) revealed a high degree of similarity between the *E. coli* and *P. atrosepticum* MetJ proteins, with only one of a total of seven amino acid changes occurring in a predicted functional domain (Fig. 4A). Due to the closeness in predicted structure and, therefore, function of these two proteins, genes homologous to those reported to be in the *E. coli* met regulon (Weissbach and Brot 1991) were predicted as likely targets for MetJ binding in *P. atrosepticum*. Therefore, the promoter regions upstream of these genes would be likely to contain a MetJ binding site.

To identify the *P. atrosepticum* MetJ binding site, the 500-bp regions preceding genes annotated as homologues of genes in the *E. coli* MetJ regulon were analyzed using the “multiple em for motif elicitation” (MEME) tool. The predicted motif from this output was TAGACGTCTA, identical in sequence to the published *E. coli* MetJ “met box” AGACGTCT (Belfaiza et al. 1986), flanked by an additional thymine and adenine (underlined). This was not surprising given that the DNA binding domains of *E. coli* and *P. atrosepticum* MetJ are identical (Fig. 4A). This motif was entered into the “find individual motif occurrences” (FIMO) program to identify locations of this motif in the 500-bp upstream sequences of all annotated genes in the *P. atrosepticum* genome. This search identified 32 genes with *P* values lower than that for the *metK* promoter, which is known to be bound by MetJ in *E. coli*. Therefore, these 32 genes, including several *met* genes such as *metN*, *metF*, *metB*, *metR*, and *metE*, as well as *metJ* itself, together with several genes not annotated as being involved in methionine metabo-

lism, were provisionally predicted to be regulated directly by MetJ in this organism (Table 1).

Transcriptomic analysis of metJ mutant strains.

To try to define a mechanism for the impact of *metJ* on virulence phenotypes, a global transcriptomics approach was selected. A β-glucuronidase reporter gene study had shown that *metJ* was most highly expressed later in growth (Fig. 4B). This was also investigated by qRT-PCR in a wild-type background and indicated that *metJ* expression was increased by 1.31- and 2.23-fold compared with exponential phase cells during early and late stationary phase, respectively (Supplementary Fig. S2).

Therefore, a microarray study was carried out on late-stationary-phase RNA samples from wild type and *rsmB*, *metJ*, and *rsmB metJ* mutant cultures to examine global changes in transcription across the different strains. Each mutant was compared with the wild type and probes were filtered to include only those with a *t* test significance of *P* ≤ 0.01 and a minimum twofold change. This left 254 (160 up, 94 down), 1,136 (812 up, 324 down), and 1,199 (793 up, 406 down) significantly differentially expressed probes between the wild type and the *rsmB* and *metJ* single mutants and the double mutant strains, respectively (Supplementary Tables S1, S2, and S3).

The transcriptional changes seen in strains carrying the *metJ* mutation were numerous and widespread across genes involved in diverse cellular processes. The most striking gene expression changes were among the three genes *metA*, *metE*, and *metR*, which exhibited increases in expression of 90- to 100-fold in the *metJ* mutant and 110- to 130-fold in the *rsmB metJ* mutant. Another gene showing similar levels of upregulation in these mutants was *ECA1113*, a putative methionine synthase identified as having a *met* box in its promoter region in both this study (Table 1) and by Augustus and Spicer (2011). Taken with the evidence that it is differentially regulated to a degree

Fig. 4. A, Amino acid sequence alignment of MetJ proteins from *Pectobacterium atrosepticum* (Pba) and *Escherichia coli* (Eco). Boxes indicate an amino acid difference and lines indicate dimerization, DNA binding, and S-adenosylmethionine (SAM) binding domains. **B**, Expression of *metJ* throughout growth in the MC1 strain of *P. atrosepticum* grown in pectate minimal media at 25°C. β-Glucuronidase expression from the reporter insertion in the *metJ* gene was measured by fluorescence. OD₆₀₀ = optical density at 600 nm.

similar to other *met* genes, these data suggest that the transcription of this gene is under direct repression by MetJ.

The expression of genes listed in Table 1 as having a predicted *met* box was of particular interest as a measure of the reliability of the searching method. In addition to the expected upregulation of *met* genes, five other genes from Table 1 were shown to be differentially regulated in the *metJ* mutants (Table 2). Of particular interest are the genes *ECA1405* and *ECA3955*, which show a very large degree of up- and downregulation, respectively, in the *metJ* mutants. Both genes encode putative ABC transporters but the precise substrates of these are unknown. Additional trends seen in these data include the upregulation of exoenzyme and secretion-associated genes and differential expression of the regulators *rsmA* and *expR* (Table 2). The differential expression of *expR* was somewhat expected, because it follows the same pattern of expression as *expI* (Fig. 3D). These genes are divergently transcribed; therefore, it is likely that their expression would be co-regulated. Expression of the *virR*, *hexY*, and *kdgR* regulatory genes was not significantly different from the wild type in any of the three mutant strains.

qRT-PCR analysis of an independent set of RNA samples confirmed many results seen in the microarray study (Table 2). Virulence-associated genes, such as those involved in secretion and those coding for exoenzymes, were downregulated in the *rsmB* mutant but upregulated in both strains carrying the *metJ* mutation. However, motility-associated genes were not significantly affected in the *rsmB* mutant compared with the *metJ* mutant strains. The expression of the small regulatory RNA *rsmB* was unchanged in the *metJ* mutant (data not shown). These results suggest that somehow *metJ* modulates virulence, at least in part, through increased exoenzyme secretion but inde-

pendently of motility. A likely mechanism for this phenomenon could be the release of exoenzyme repression through downregulation of *rsmA* expression, which is seen in the *metJ* mutants.

DISCUSSION

The initial aim of this study was to isolate a transposon insertion mutant defective in the *rsmA* gene by genetic suppression of an *rsmB* mutant which had a clear Prt⁻ phenotype. However, using the Prt restoration assay, we defined a new role for a known metabolic regulator, MetJ, in suppression of the *rsmB* phenotype. Thus far, the *metJ* gene has not been linked with virulence or exoenzyme regulation in *Pectobacterium* spp., and most studies on MetJ have been carried out in nonpathogenic *E. coli* strains on the structure and function of MetJ as a DNA-binding protein and repressor. In *E. coli*, MetJ is known to bind to *met* boxes upstream of target genes involved in methionine biosynthesis. These *met* boxes are scattered around the chromosome and have a consensus sequence AGACGTCT. It is now well recognized that MetJ, when bound to its cofactor S-adenosylmethionine, binds to these sequences and inhibits target gene transcription. In a *metJ* mutant, this leads to constitutive expression of methionine biosynthesis genes (Weissbach and Brot 1991).

The MetJ regulon has been well studied in *E. coli* (Marincs et al. 2006; Old et al. 1993; Shoeman et al. 1985), and more recently in a variety of other γ -proteobacteria (Augustus and Spicer 2011). These studies show that the direct targets of MetJ regulation are almost exclusively involved in some stage of methionine biosynthesis or recycling, or that of related molecules such as S-adenosylmethionine or homocysteine.

Table 1. Genes preceded by a predicted “*met* box” sequence^a

Gene name ^b	Gene function	P value	Matched sequence
ECA3523*	Similar to <i>Yersinia pestis</i> hypothetical protein	7.3×10^7	CTAGACGGCTAA
<i>metN</i>	D-methionine transport ATP-binding protein	7.3×10^7	TTAGCCGTCTAG
ECA1113	Putative methionine synthase	1.4×10^6	TTAGACGTCCAG
ECA3955	ABC transporter membrane protein	1.4×10^6	TTGGACGTCTAA
<i>metB</i>	Cystathionine γ -synthase	2.3×10^6	TTAGATGTCTAG
<i>metF</i>	5,10-methylenetetrahydrofolate reductase	2.3×10^6	ATAGACGTCTAA
<i>metJ</i>	Repressor of the methionine regulon	2.3×10^6	CTAGACGTCTAA
<i>metE</i>	5-Methyltetrahydropteroyltryglutamate-homocysteine methyltransferase	3.1×10^6	CTGGACGGCTAA
<i>metR</i>	Transcriptional activator protein	3.1×10^6	TTAGCCGTCCAG
<i>uhpC</i>	Putative regulatory protein	4.7×10^6	TTAGCCATCTAA
ECA1014*	Putative membrane protein	5.9×10^6	TTAGCTGGCTAA
<i>gcvA</i>	Glycine cleavage system transcriptional activator	5.9×10^6	TTAGCCAGCTAA
<i>yfeD</i>	Chelated iron transport system membrane protein	5.9×10^6	TTAGCTGGCTAA
ECA2069	TetR-family transcriptional regulator	7.5×10^6	TTGGACGTCTAT
<i>metA</i>	Homoserine O-succinyltransferase	7.5×10^6	CTGGATGTCTAA
ECA1430	Putative dTDP-glucose 4-6-dehydratase	8.6×10^6	TTAGACATCTAT
ECA1014	Protein-export membrane protein	8.6×10^6	ATAGACGTCTAT
<i>panE</i>	2-Dehydropanoate 2-reductase	8.9×10^6	CTGGACGGCCAG
<i>rpmJ</i>	50S ribosomal protein	1.5×10^5	TTAGCCAGCCAA
<i>rpsM</i>	30S ribosomal subunit protein S13	1.5×10^5	TTAGCCAGCCAA
ECA3980	Unknown function	1.6×10^5	CTGGACGTCCAT
ECA1912	Putative periplasmic substrate-binding protein	1.8×10^5	ATAGCTGGCTAG
ECA0767	Similar to <i>Xanthomonas</i> hypothetical protein	2.1×10^5	ATAGACGTCCAT
ECA1405	Putative ABC sugar transporter protein	2.1×10^5	ATAGACATCCAG
ECA2199	Similar to <i>Xanthomonas</i> hypothetical protein	2.1×10^5	ATAGACGTCCAT
ECA065	t-RNA glycine	2.1×10^5	ATAGACGTCCAT
<i>galE</i>	Putative UDP-glucose 4-epimerase	2.6×10^5	TTACACGTCTAA
ECA3519	Putative acetyltransferase	2.8×10^5	TTGGACGGCCAT
ECA3683	Similar to <i>Y. pestis</i> hypothetical protein	2.8×10^5	CTGGACGGCCAT
<i>pehN</i>	Putative polygalacturonase precursor	2.8×10^5	CTGGCCATCCAG
ECA2661	Putative membrane protein	3.3×10^5	TTGGCTATCTAG
<i>metK</i>	S-adenosylmethionine synthetase	3.3×10^5	ATAGCCATCCAG

^a Genes identified by a search of the *Pectobacterium atrosepticum* SCRI1043 genome using the MEME tool for sequences resembling the consensus *P. atrosepticum* *met* box TAGACGTCTA.

^b Asterisk (*) indicates upstream of subsequent gene in table.

One exception is *metR*, which encodes a transcriptional regulator that controls expression of a subset of the *met* genes, including itself (Maxon et al. 1989). Additionally, there have been recent discoveries of MetJ-regulated genes with alternative functions, such as the gene for the cobalamin transporter, *btuB*, in *E. coli* (Augustus and Spicer 2011). In addition to these findings, this study reports the first link between the MetJ repressor and bacterial virulence.

The high degree of similarity between the *E. coli* and *P. atrosepticum* MetJ proteins, including their identical DNA binding domain, allows parallels to be drawn in the regulation of genes by MetJ in these two organisms. This is reinforced by the identification of a *met* box sequence nearly identical to that of the *E. coli* consensus. Predicted *met* boxes were identified in the promoter regions of a number of *met* genes involved in methionine biosynthesis in *P. atrosepticum*. Furthermore, the promoters of *E. coli* homologues of all *met* genes listed in Table 1 were shown to bind MetJ protein in gel shift assays (Augustus and Spicer 2011). As well as the predictions of *met* boxes in annotated *met* genes, several other genes were identified as potential regulatory targets for MetJ. Six of these genes (*ECA0767*, *ECA1113*, *ECA1405*, *ECA2661*, *ECA3683*, and *ECA3955*) were differentially expressed in the *metJ* mutants (Table 2). Overall, the computational method used for searching for *met* boxes is effective at identifying MetJ targets. However, there are many genes identified by this method which do not appear to be differentially regulated in a *metJ* mutant, implying a high false discovery rate. In spite of this, the transcriptomics data presented in this study better allows true targets of MetJ binding to be identified.

A particularly interesting finding from these data is that the gene *ECA3955* is predicted to have a close-to-consensus *met* box in its promoter region (Table 1). However, microarray and qRT-PCR data suggest that the expression of this gene is down-regulated by approximately 70- and 400-fold, respectively, in the *metJ* mutants (Table 2). The extent of this difference in expression strongly suggests that this effect is being mediated

directly by MetJ, which implies a role in activation rather than repression of *ECA3955*. Because MetJ is not known to function as an activator, this is certainly an observation that warrants further study.

The large number of statistically significant changes in gene expression seen in the microarray study for strains carrying the *metJ* mutation indicates the pleiotropic effects of MetJ in this organism. Many of these changes, such as the upregulation of methionine biosynthesis enzymes, were expected given what is known in related bacteria. Furthermore, other genes such as those coding for major virulence regulators such as *expI* and *rsmA* were differentially regulated as a cause of this mutation. There were no *met* box sequences identified in the upstream regions of these genes, which suggests that these changes in gene expression are likely to be mediated indirectly. Regardless of how the differential expression of these genes arises, this could be one reason for the changes observed in PCWDE and OHHL expression.

The effect of the *metJ* mutation on transcription of genes involved in a wide variety of cellular processes is not unexpected, because methionine metabolism is involved in the production of several metabolically important compounds, such as methylthioadenosine and *S*-adenosylmethionine (SAM). These molecules are involved in various stages in the methionine recycling pathway and feed directly into the pathways for producing adenine, polyamines, and OHHL, the QS signaling molecule (Sekowska et al. 2004). OHHL is produced by the ExpI enzyme from the precursors SAM and hexanoyl-acyl carrier protein, and several studies have demonstrated that it is SAM which is the limiting factor in this reaction. As a result, increasing the levels of SAM in the cell through upregulation of *metK* could cause an increase in homoserine lactone production (Hanzelka and Greenberg 1996; Parsek et al. 1999; Val and Cronan 1998). Therefore, this could contribute to the increase in OHHL production seen in the *metJ* mutants.

Another important function of SAM in this context is its role as the co-repressor for MetJ. SAM is produced when the

Table 2. Selected genes differentially regulated in *rsmB*, *metJ*, and *rsmB metJ* mutants^a

Gene name	Microarray			qRT-PCR		
	<i>rsmB</i>	<i>metJ</i>	<i>rsmB, metJ</i>	<i>rsmB</i>	<i>metJ</i>	<i>rsmB, metJ</i>
Met biosynthesis						
<i>metE</i> [†]	0.47	94.1	130.6	4.61	597	596
<i>metA</i> [†]	0.84	93.5	129.0	...	...	...
<i>metR</i> [†]	0.81*	96.6	112.3	...	...	...
<i>metK</i> [†]	0.29	2.64	3.38	0.22	5.63	3.38
<i>ECA1113</i> [†]	0.77	84.4	100.0	...	...	...
PCWDE						
<i>pelB</i>	0.091	3.42	2.46	0.14	11.4	5.09
<i>pelC</i>	0.57	3.00	2.54	0.90	15.0	10.1
<i>pelW</i>	1.09	4.55	4.98	...	...	...
<i>celA</i>	1.99	5.24	5.11	...	...	...
Secretion						
<i>prtD</i>	0.25	17.8	12.9	0.45	12.8	4.70
<i>outD</i>	0.42	4.47	4.50	0.24	2.63	1.93
<i>ECA2105</i>	0.77*	18.1	10.8	0.35	5.95	2.78
<i>outL</i>	1.13*	9.39	11.2	...	...	...
<i>tatC</i>	1.17*	3.05	3.35	2.86	2.61	2.05
Regulators						
<i>rsmA</i>	0.92*	0.21	0.26	0.40	0.52	0.45
<i>expR</i>	0.39*	6.16	5.91	0.63	4.04	2.85
Other						
<i>ECA1405</i> [†]	1.25*	40.79	73.82	...	...	...
<i>ECA2661</i>	2.15	9.83	6.75	...	...	...
<i>ECA3683</i>	1.47	4.27	6.20	...	...	...
<i>ECA0767</i> [†]	0.98*	2.51	2.31	...	...	...
<i>ECA3955</i> [†]	0.91*	0.015	0.013	0.0048	0.0022	0.0026

^a Fold changes compared with wild type of selected genes as determined by microarray and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). PCWDE = plant cell wall-degrading enzymes, * indicates $P > 0.05$, and † indicates gene is preceded by a “met box” sequence.

enzymes of the MetJ regulon, such as *metK*, are expressed (i.e., when MetJ is unable to bind to *met* boxes due to either its absence or the fact that it has not formed its functional dimeric complex bound to SAM). In this way, SAM forms a link between MetJ and the production of molecules made by the enzymes that it regulates. This ties in with the pattern seen in *metJ* expression (Fig 4B) where, as amino acids in the growth medium become limiting in the entry to stationary phase, less SAM would be present, preventing MetJ from repressing the genes involved with methionine biosynthesis. The other transcriptional regulator within the MetJ regulon, MetR, also has a product of the methionine salvage pathway, homocysteine, as a co-repressor (Urbanowski and Stauffer 1987). Together, these co-repressors link environmental conditions to the expression of the genes in the methionine regulon.

In *P. atrosepticum*, the sensing of external conditions is vital for regulation of virulence. The QS signal OHHL provides a measure of population density, while systems such as the KdgR regulatory pathway indicate the presence of plant tissue breakdown products (Nasser et al. 1991). Therefore, it seems logical that a regulatory system which feeds in information concerning the state of nutrient availability could be of use in regulating a transition to virulence factor production.

Ultimately, the impact of generating a *metJ* mutation in an *rsmB* mutant is the restoration of virulence. Given the modulation of *rsmA* and *expI* expression seen in *metJ* mutants, it is possible that a decrease in RsmA-mediated repression and an increase in QS signal production could lead to increased production of PCWDE and other virulence factors. However, because this study has shown that a restoration of the QS signal is not the sole reason for restoration of PCWDE production, there must be additional factors contributing to this effect. These factors are likely to include interplay with the Rsm system and could possibly include transcriptional regulator proteins or altered levels of metabolic products such as SAM. However, while further investigation into the precise mechanism is clearly needed, it is evident that methionine metabolism plays a hitherto unrecognized role in virulence regulation in the genus *Pectobacterium* and, potentially, other related species also.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains used in this study are given in Table 3. Strains were grown with good aeration in Luria-Bertani (LB) or peptate minimal medium (PMM) consisting of 7 g of K₂HPO₄, 2 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 1 mM MgSO₄, 5 ml of glycerol, and 5 g of polygalacturonic acid per liter. *E. coli*

strains were grown at 37°C and *P. atrosepticum* strains were grown at 25°C. Where appropriate, the following antibiotics were added to the media: ampicillin (Ap) to 50 µg/ml, chloramphenicol (Cm) to 50 µg/ml, kanamycin (Km) to 50 µg/ml, spectinomycin to 50 µg/ml, and tetracycline to 10 µg/ml.

Strain and plasmid construction.

To make the *rsmB* mutant strain NW155, the 5' flanking region of *rsmB* was amplified with primers NW281 and NW283. The 3' region was amplified with primers NW284 and NW282. These regions were joined using overlap-extension PCR with the primers NW281 and NW282. Cloning into pBluescriptKSII was via the *Bam*HI and *Sal*I sites to create the construct pNRW180. The Km^r gene was PCR amplified from pACYC177 with primers NW279 and NW280 and subcloned into the *Sph*I-digested and dephosphorylated construct pNRW180, creating the construct pNRW181. All primer sequences are listed in Supplementary Table S4. The *rsmB*::Km^r site was PCR amplified with primers NW281 and NW282, and the linear DNA was transformed by electroporation into *Pba*1043 (pKD46) using the method published by Datsenko and Wanner (2000). Stable integrants were selected on LB agar supplemented with Km at 50 µg/ml. One *rsmB*::Km^r *Pba*1043 colony was selected for further study and the *rsmB*::Km marker was transduced into a clean genetic background using the generalized transducing phage ΦM1. One Km^r Ap^r colony was selected and renamed NW155.

Additional strains were either made by random transposon mutagenesis or constructed by generalized transduction. Transposon mutagenesis was achieved via a conjugative plasmid loaded with the transposon TnDS1028, Cm^r *uidA* (Smith 2005). Overnight cultures were grown of the donor (β2163 [pDS1028]) and recipient (NW155) strains. Cells were washed and resuspended in LB. Equal volumes of each culture were mixed and 30 µl of the mixture was immediately spotted onto LB agar + 30 mM 2,3-diaminopimelic acid. After incubation overnight at 25°C, mating patches were resuspended in LB and serial dilutions were plated onto skimmed milk agar (nutrient broth agar, 10 g of Marvel skimmed milk powder per liter). Haloes indicating Prt production were visible after incubation at 25°C for 3 days. The transposon insertion sites were mapped using random prime PCR (Fineran et al. 2005). The generalized transducing phage ΦM1 was used to move mutations between different strains of *P. atrosepticum* (Toth et al. 1997). Plasmids used to complement the *metJ* mutation were constructed using the pQE80L vector (Qiagen, Crawley, U.K.). Briefly, the *metJ* coding sequence was cloned using primers with restriction sites and inserted into the cut vector using the restriction enzymes *Eco*RI and *Pst*I (New England Biosciences, Ipswich,

Table 3. Bacterial strains used in this investigation

Strain	Genotype ^a	Reference
<i>Escherichia coli</i>		
DH5α	<i>endA1, hsdR17, supE44, thi-1, recA1, gyrA96, ΔlacU169, relA1</i>	
β2163	(F ⁻), RP4-2-Tc::Mu, <i>dapA</i> ::(<i>erm-pir</i>) [Km ^r Em ^r]	Demarre et al. 2005
JM109 (pSB401)	OHHL sensor strain (pSB401 [Tc ^r , <i>luxR</i> , <i>luxCDABE</i>])	Winson et al. 1998
<i>Pectobacterium atrosepticum</i>		
SCRI1043	Wild type	JHI bacterial collection
EMS6.1	Lac ⁻ SCRI1043	Hinton et al. 1989
NW155	<i>rsmB</i> ::Km ^r	This study
TB6	EMS6.1 <i>expI</i> :: <i>lacZ</i>	Corbett et al. 2005
MC1	<i>metJ</i> ::TnDS1028 <i>uidA</i> , Cm ^r	This study
MC2.1	MC1, <i>rsmB</i> ::Km ^r	This study
MC4	TB6, <i>metJ</i> ::TnDS1028 <i>uidA</i> , Cm ^r	This study
MC5	TB6, <i>rsmB</i> ::Km ^r	This study
MC6	TB6, <i>metJ</i> ::TnDS1028 <i>uidA</i> , Cm ^r , <i>rsmB</i> ::Km ^r	This study

^a Km^r, Em^r, Tc^r, and Cm^r = resistant to kanamycin, erythromycin, tetracycline, and chloramphenicol, respectively; OHHL = *N*-(3-oxohexanoyl)-homoserine lactone.

MA, U.S.A.). Plasmids were introduced by electroporation as described by Evans (2009) and maintained with Ap. Artificially induced expression of cloned genes in vectors was not required, as leaky expression was sufficient for complementation.

Phenotypic assays.

Assays for PCWDE and OHHL were carried out on culture supernatant samples from cells grown in PMM. Samples of culture were taken at specified times and cells were pelleted by centrifugation. Culture supernatants were removed to a separate tube (and filter sterilized in the case of the OHHL assay) and frozen at -80°C until analysis. Pel activity was measured using the method described by Coulthurst and associates (2006). Prt activity was measured using the Sigma-Aldrich Protease Fluorescent Detection Kit PF0100 according to the manufacturer's instructions, with a 4-h incubation step. OHHL was measured using the method described by Coulthurst and associates (2008). To test the effect of OHHL on exoenzyme production, 5 μl of a culture of $\text{OD}_{600} = 1$ cells were spotted onto appropriate assay indicator plates incubated at 25°C for 48 h.

Virulence assays.

Tuber virulence assays were performed on Maris Piper potato tubers according to Walker and associates (1994). Tubers were surface sterilized in 1% Virkon and stab inoculated with 10^3 CFU using a sterile pipette tip. Rotting was measured by scraping out and weighing the rotted tissue. The level of rotting was expressed relative to wild-type rotting in that tuber set. Plant stem pathogenicity tests were performed in potato stems ('Estima') by inoculating 20 replicate stems with 10^3 CFU per inoculation site. The plants were maintained in a controlled environment room at 21°C for 14 days. Disease symptoms were assessed daily by measuring the length of blackleg lesion produced (Holeva et al. 2004).

Transcriptional fusion assays.

Strains containing *uidA* transcriptional fusions were grown in PMM for 18 h and culture samples taken every 3 h and frozen at -80°C . At the time of assay, samples were thawed and 10 μl of each were pipetted into a round-bottomed 96-well plate. The assay was conducted according to the method of Ramsay and associates (2011) using 4-methylumbelliferyl- β -D-glucuronide at 250 $\mu\text{g}/\text{ml}$.

Computational analysis using the MEME suite.

The 500-bp regions upstream from annotated genes were obtained from the published sequence of *P. atrosepticum* SCRI1043 (Bell et al. 2004) using the Artemis software (Rutherford et al. 2000). To identify the MetJ-binding motif using MEME (Bailey and Elkan 1994), the 500-bp upstream sequences of *metA*, *metC*, *metF*, *meth*, *metJ*, *metN*, *metR*, *metB*, *metE*, *metG*, *metI*, *metK*, and *metQ* were submitted to the FIMO program (Grant et al. 2011) with the following parameters: distribution of motif occurrences = any number of repetitions, number of different motifs = 3, minimum number of sites = 2, maximum number of sites = 7, minimum motif width = 6, maximum motif width = 12, and looking for = palindromes only. To identify *met* boxes in the chromosome, the output from the MEME analysis was submitted to FIMO comparing against the database "Erwinia_carotovora_atroseptica_SCRI1043_upstream.na" with a *P* value display threshold of 1×10^{-4} .

RNA extraction.

Solutions for RNA work made in-house or their constituent parts were made RNase-free by treatment with 0.1% diethylpyrocarbonate (DEPC) at 37°C overnight, then autoclaving. For analysis throughout growth, cells were grown in PMM and

1-ml samples were taken. Cells were grown in PMM for 18 h and either 1-ml samples were taken throughout growth or 2-ml samples were taken at the end point. Samples were mixed with two volumes of 95% ethanol and 5% phenol and stored at -80°C until extraction. RNA was isolated from samples taken throughout growth using the Qiagen RNeasy mini kit according to the manufacturer's instructions. RNA was isolated from end point samples by hot phenol extraction as described by Mattatall and Sanderson (1996). Pellets were dissolved in 100 μl of DEPC-treated water. Residual DNA was removed using the Ambion TURBO DNA-free kit according to the manufacturer's instructions. Removal of DNA was confirmed by PCR on diluted RNA samples using *Pba1043* 16S RNA gene primers TBOL87 and TBOL88.

qRT-PCR.

RT and qRT-PCR were performed as by Burr and associates (2006). The gene *ffh* was used as a reference gene (Takle et al. 2007) and data were normalized to wild-type expression of *ffh*. Relative gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ (cycle threshold) method (Livak and Schmittgen 2001). Independently isolated RNA templates were used in the microarray and qRT-PCR analyses.

Microarray.

RNA from stationary-phase cells was processed and hybridized to 12 custom Agilent *P. atrosepticum* microarrays (ArrayExpress accession A-MEXP-1482) using three biological replicates on separate arrays for each of the four strains. Probes were 60 mers representing each of the 4,521 *P. atrosepticum* SCRI1043 predicted protein coding genes, plus 23 Lucidea Universal ScoreCard controls (GE Healthcare, Piscataway, NJ, U.S.A.), and each probe was printed twice. For each array, 12 μg of RNA was labeled as cDNA with Cy-3 dye (GE Healthcare) and hybridized to the array as described (Venkatesh et al. 2006). Arrays were scanned using the Agilent G2505B Scanner with 10- μm resolution. Data were extracted from each scanned image using Feature Extraction (FE) software (v. 10.7; Agilent Technologies, Santa Clara, CA, U.S.A.) with default settings, prior to import into Genespring (v. 7.3; Agilent Technologies) for analysis. Complete datasets along with the array experimental design are available at ArrayExpress (accession number E-MTAB-1167). Data were normalized using default settings for Agilent one-color arrays and unreliable data removed using flags derived from FE. Significantly, differentially expressed genes between the wild type and each mutant were identified using volcano plot filtering (Students *t* test, *P* value ≤ 0.01 and a minimum twofold change).

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