MOLECULAR PLANT PATHOLOGY (2008) 9(6), 819–824 DOI: 10.1111/J.1364-3703.2008.00495.X

The role of PilZ domain proteins in the virulence of *Xanthomonas campestris* pv. *campestris*

YVONNE MCCARTHY[1], ROBERT P. RYAN[1], KAREN O'DONOVAN[1], YONG-QIANG HE[2], BO-LE JIANG[2], JIA-XUN FENG[2], JI-LIANG TANG[2] AND J. MAXWELL DOW[1,*]

[1]*BIOMERIT Research Centre, Department of Microbiology, BioSciences Institute, National University of Ireland, Cork, Ireland*
[2]*The Key Laboratory for Subtropical Bioresources Conservation and Utilisation, Guangxi University, 75 Xiuling Road, Nanning, Guangxi, 530005 PRC*

SUMMARY

Cyclic di-GMP [(bis-(3′–5′)-cyclic di-guanosine monophosphate)] is an almost ubiquitous second messenger in bacteria that is implicated in the regulation of a range of functions that include developmental transitions, aggregative behaviour, adhesion, biofilm formation and virulence. Comparatively little is known about the mechanism(s) by which cyclic di-GMP exerts these various regulatory effects. PilZ has been identified as a cyclic di-GMP binding protein domain; proteins with this domain are involved in regulation of specific cellular processes, including the virulence of animal pathogens. Here we have examined the role of PilZ domain proteins in virulence and the regulation of virulence factor synthesis in *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot of crucifers. The *Xcc* genome encodes four proteins (XC0965, XC2249, XC2317 and XC3221) that have a PilZ domain. Mutation of *XC0965*, *XC2249* and *XC3221* led to a significant reduction of virulence in Chinese radish. Mutation of *XC2249* and *XC3221* led to a reduction in motility whereas mutation of *XC2249* and *XC0965* affected extracellular enzyme production. All mutant strains were unaffected in biofilm formation *in vitro*. The reduction of virulence following mutation of *XC3221* could not be wholly attributed to an effect on motility as mutation of *pilA*, which abolishes motility, has a lesser effect on virulence.

* *Correspondence*: Tel.: 353 21 4901316; Fax: 353 21 4275934; E-mail: m.dow@ucc.ie

The production of virulence determinants by pathogenic bacteria is strictly regulated and probably occurs as an adaptation to particular environmental changes. There is a great deal of interest in the signals that activate virulence gene expression and in the processes of signal perception and signal transduction. Cyclic di-GMP [bis-(3′–5′)-cyclic di-guanosine monophosphate] is a novel second messenger in bacteria that was first described as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (Ross *et al*., 1987). It is now established that this nucleotide regulates a range of functions in diverse bacteria, including the virulence of bacterial pathogens of animals and plants (for recent reviews see Jenal and Malone, 2006; Römling *et al*., 2005; Römling and Amikam, 2006; Ryan *et al*., 2006b). Cellular levels of cyclic di-GMP are controlled through synthesis, catalysed by the GGDEF protein domain, and degradation by EAL or HD-GYP domains (Christen *et al*., 2005; Paul *et al*., 2004; Ryan *et al*., 2006a; Ryjenkov *et al*., 2005; Schmidt *et al*., 2005; Tamayo *et al*., 2005). Proteins with GGDEF, EAL and HD-GYP domains are involved in the regulation of bacterial properties that include developmental transitions, aggregative behaviour, adhesion and biofilm formation in addition to synthesis of virulence factors and virulence.

Comparatively little is known regarding the mechanisms by which cyclic di-GMP exerts its diverse actions within the bacterial cell. Bioinformatics studies carried out by Amikam and Galperin (2006) identified the PilZ domain, named after the PilZ protein from *Pseudomonas aeruginosa*, as a candidate player in cyclic di-GMP signalling mechanisms. PilZ domain proteins from a number of bacteria have been shown to bind cyclic di-GMP and in some cases have been implicated in the regulation of specific cellular processes such as motility, polysaccharide synthesis, biofilm formation and virulence of animal pathogens (Benach *et al*., 2007; Christen *et al*., 2007; Merighi *et al*., 2007; Pratt *et al*., 2007; Ramelot *et al*., 2007; Ryjenkov *et al*., 2006). It is unlikely that PilZ domain proteins mediate all of the cellular effects of cyclic di-GMP however; PelD of *Pseudomonas aeruginosa* is a cyclic di-GMP receptor that mediates regulation of polysaccharide (PEL) biosynthesis but lacks a recognizable PilZ domain (Lee *et al*., 2007).

The aim of the present study was to expand our understanding of cyclic di-GMP signalling in plant pathogenesis by addressing the role of PilZ domain proteins in *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot in crucifers. We have previously examined the contribution of all proteins with GGDEF, EAL or HD-GYP domains to virulence and virulence factor production in *Xcc* (Ryan *et al.*, 2007). Genes with significant roles in virulence to plants include those encoding proteins whose probable function is in cyclic di-GMP synthesis as well as others, including the HD-GYP domain regulator RpfG, implicated in cyclic di-GMP degradation. Mutation of *rpfG* in *Xcc* has pleiotropic effects, leading to significant reduction in the synthesis of the extracellular cell-wall-degrading enzymes protease and endoglucanase, reduction in motility and enhanced biofilm formation in rich medium (Dow *et al.*, 2003; Ryan *et al.*, 2007; Slater *et al.*, 2000). Other GGDEF-EAL domain proteins influence motility but have no effect on virulence factor synthesis (Ryan *et al.*, 2007). Here we examine the role of the different PilZ domain proteins of *Xcc* in controlling these various phenotypes.

The *Xcc* genome encodes four proteins with a PilZ domain (Amikam and Galperin, 2006); XC0965, XC2249 and XC3221 comprise 'stand-alone' domains whereas XC2317 has an additional N-terminal YcgR domain. These proteins show no significant amino acid sequence similarity with each other on pairwise searches using BLASTP. Nevertheless, in all cases proteins with related amino acid sequence are found in other bacteria, although for XC2249 these homologues only occur in the proteomes of other xanthomonads (data not shown). Searches against a protein fold library using the FUGUE algorithm (Shi *et al.*, 2001) showed predicted structural homology of all *Xcc* proteins with the PilZ domain proteins VCA0042 from *Vibrio cholerae* (PDB 1yln) and PP4397 from *Pseudomonas putida* (PDB 2gjg) at above the 99% confidence level.

The functions of XC0965, XC2249, XC2317 and XC3221 were examined using mutant strains in which the genes were disrupted using either Tn*5gusA* (*XC2249*, *XC3221*), or pK18*mobsac* carrying an internal fragment of the gene amplified by PCR (*XC0965*, *XC2317*) (Table 1). *XC2317* and *XC2449* are predicted to be in single transcriptional units whereas *XC0965* and *XC3221* are the most downstream genes in the predicted *XC0960–XC0965* and *XC3224–XC3221* operons (Qian *et al.*, 2005). This indicates that mutation of the genes under investigation is unlikely to have polar effects. For complementation studies, genes were amplified using the PCR primers given in Table 1 (which also have sites for

Table 1 Strains, plasmids and primers used in this study.

Strain/plasmid	Genotype/Phenotype	Reference
X. campestris pv. *campestris* 8004	Wild-type Rif^R	Qian *et al.* (2005)
△*rpfG*	*rpfG* deletion mutant of 8004, Rif^R	Slater *et al.* (2000)
△*hrpG*	*hrpG* deletion mutant of 8004, Rif^R; Km^R	Xu *et al.* (2008)
△*hrpX*	*hrpX* deletion mutant of 8004, Rif^R; Km^R	Xu *et al.* (2008)
pilA	*XC3823*::Tn*5gusA5*; Rif^R; Tc^R	Ryan *et al.* (2007)
XC2317	pK18*mobkan*, *XC2317*; Rif^R; Km^R	This study
XC2249	*XC2249*::Tn*5gusA5*; Rif^R; Tc^R	This study
XC3221	*XC3221*::Tn*5gusA5*; Rif^R; Tc^R	This study
XC0965	pK18*mobkan*, *XC0965*; Rif^R; Km^R	This study
XC0965 (*pXC0965*)	Complemented mutant Cl^R; Rif^R; Km^R	This study
XC2317 (*pXC2317*)	Complemented mutant Cl^R; Rif^R; Km^R	This study
XC2249 (*pXC2249*)	Complemented mutant Cl^R; Rif^R; Km^R	This study
XC3221 (*pXC3221*)	Complemented mutant Cl^R; Rif^R; Km^R	This study
Primers sets used in construction of clones for complementation (5′–3′)		
Target gene	**Forward**	**Reverse**
XC0965	GGATCC ATGATCCAGGACACCCGCC	AAGGCTGAGCATCTGGTCTAG
XC2249	GGATCCGTGCAGCGCATGGACGCCAA	AAGGCTTTCAGCGCTGGCGACGGGCG
XC2317	GGATCCGTGCTTGTGCCCATGTCCGAA	AAGGCTTCAGAACACGCCGCTCTT
XC3221	GGATCCATGAGTGCAATGAATGCACG	AAGCTTTTACATCGTGTGGGTCGGCTTG
RT-PCR primer sets (5′–3′)		
Target gene	**Forward**	**Reverse**
XC0965	CTGTATCAGCTGCGGTTCG	AGATGCTCTTCCGCAATCC
XC2249	CCAAACTCGACCTCATCCTG	ACAATTCAGGAAGCCAGTCC
XC2317	CATCGAACAGGCCAAGTACC	GAATCGGTGATGGGTGTTTC
XC3221	GCGATGAGGTGTTTCTGCTT	GCCAGCAGTGTTTCGATCTT
16S rDNA	GTCAGGAGTCGGTGCTCAGT	CCTTCCAGATCGACCCAGTA

Key: Cl, chloramphenicol; Rif, rifampicin; Km, kanamycin; Tc: tetracycline.

Restriction enzyme sites for *Bam*HI or *Hin*dIII within the primer sequences are underlined.

*Bam*HI and *Hin*dIII) and cloned into the TOPO TA vector (Invitrogen). After verification by sequencing, inserts were excised with *Bam*HI and *Hin*dIII and were ligated into pBBR1MCS (Kovach *et al.*, 1995) cut with the same enzymes to create pXC0965, pXC2249, pXC2317 and pXC3221. This orientation allowed expression of the inserted gene in these constructs to be driven by the vector (*lac*) promoter. Constructs were introduced into *Xcc* strains by triparental mating.

The virulence of each mutant was tested by measurement of the lesion length after bacteria were introduced into the vascular system of Chinese radish by leaf clipping as previously described (Dow *et al.*, 2003; Ryan *et al.*, 2007). Each strain was tested on 60 leaves (20 leaves for each strain with three independent replicates). Although mutation of *XC2317* had no effect, strains with mutations in *XC0965*, *XC2249* and *XC3221* had significantly reduced virulence compared with the wild-type (Fig. 1A,B). Introduction of the cloned genes into the respective mutants restored virulence to wild-type levels (Fig. 1).

These studies were extended to examine the influence of mutation on specific factors associated with *Xcc* virulence including extracellular enzyme production, motility and biofilm formation. Levels of β-1,4-endoglucanase from culture supernatants were estimated by radial diffusion assays using carboxymethylcellulose as substrate. For these experiments bacteria were grown to an OD at 600 nm of 2.0 in NYGB medium. Relative protease production was measured by growth of colonies on NYG agar plates supplemented with skimmed milk (Ryan *et al.*, 2007; Slater *et al.*, 2000). Mutation of *XC2317* led to a small but reproducible reduction in protease production, although mutation of *XC0965*, *XC2249* or *XC3221* had no apparent effect (Fig. 2B). Mutation of *XC0965* and *XC2317* led to a reduction in endoglucanase levels, although mutation of *XC2249* or *XC3221* had no effect (Fig. 2C). These effects of *XC0965* and *XC2317* mutation on extracellular enzyme production were much less pronounced, however, than those seen after mutation of *rpfG* (Fig. 2C; Slater *et al.*, 2000). Introduction of the cloned *XC0965* or *XC2317* genes into the respective mutant restored production of extracellular enzymes to wild-type levels (Fig. 2C), whereas introduction of these constructs into the wild-type had no effect (data not shown). Motility of different strains was assessed on 0.6% Eiken agar plates as described by Ryan *et al.* (2007). Mutation of *XC2249* and *XC3221* but not *XC0965* and *XC2317* negatively affected motility (Fig. 3). Introduction of the cloned *XC2249* or *XC3221* gene into the respective mutant restored the wild-type phenotype (Fig. 3). However, introduction of the cloned *XC2249* gene into the *XC3221* mutant or the cloned *XC3221* gene into the *XC2249* mutant had no effect. Taken together these findings suggest that different PilZ domain proteins have independent action in the regulation of motility.

Wild-type *Xcc* 8004 does not form biofilms in liquid L medium although the *rpfG* mutant does (Dow *et al.*, 2003). Ectopic expression in wild-type *Xcc* of *wspR19*, which encodes a GGDEF domain protein from *Pseudomonas fluorescens* (Aldridge *et al.*, 2003), gives a phenocopy of the *rpfG* mutant with increased cellular levels of cyclic di-GMP, increased biofilm formation and aggregation

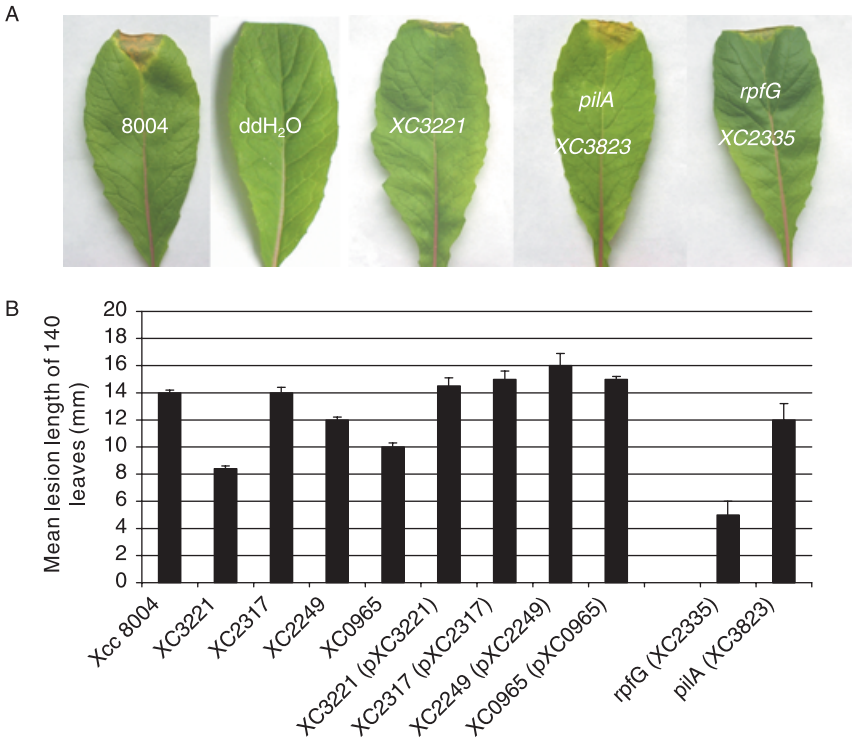

Fig. 1 The effects of mutation of genes encoding the PilZ domain proteins XC0965, XC2249, XC2317 and XC3221 on the virulence of *Xcc* to Chinese radish. The virulence of each mutant was tested by measurement of the lesion length after bacteria were introduced into the vascular system of Chinese radish by leaf clipping. Values given are the means and SD of 60 measurements. Also shown are the effects of mutation of *rpfG*, which encodes an HD-GYP domain regulatory protein and *pilA* encoding the major pilin. (A) Representative virulence assays for (from left to right) *Xcc* wild-type strain 8004, negative control (H_2O), *XC3221*::Tn*5gusA5* mutant, *pilA* mutant (*XC3823*::Tn*5gusA5*) and *rpfG* deletion mutant. (B) Mutation of *XC3221*, *XC2249* and *XC0965* gave a significant reduction in virulence in repeated tests, although mutation of *XC2317* had no effect. Introduction of the cloned genes restores virulence of these mutants to wild-type levels, but had no influence on the *XC2317* mutant, which retained wild-type virulence.

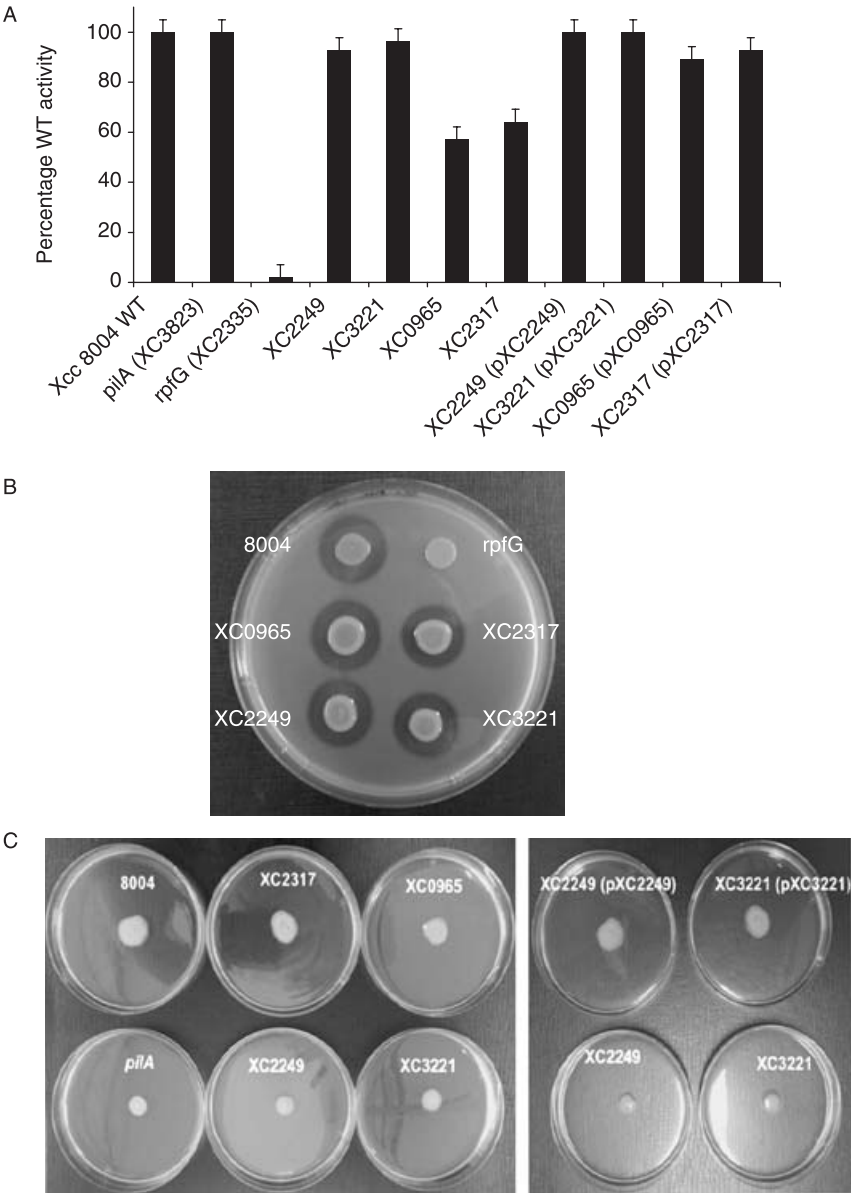

Fig. 2 Mutation of genes encoding PilZ domain proteins has effects on motility and the synthesis of extracellular enzyme virulence factors by *Xcc*. (A) *XC0965* and *XC2317* mutants have lower levels of extracellular endoglucanase than wild-type; the complemented strains *XC0965*/pXC0965 and *XC2317*/pXC2317 have wild-type levels. The effects of mutation of *XC0965* and *XC2317* are much less than those seen after mutation of *rpfG* (which is *XC2335*), whereas mutation of *pilA* (which is *XC3823*) has no effect on endoglucanase activity. (B) Mutation of *XC2317* led to a small reduction in protease production whereas mutation of *XC0965*, *XC2249* and *XC3221* had no apparent effect. (C) Mutation of *XC2249* and *XC3221* influence motility on 0.5% Eiken agar-NYG plates. These effects are reversed by complementation with the cloned *XC2249* and *XC3221* genes respectively.

in liquid culture, decreased motility and decreased production of extracellular enzymes (Ryan *et al.*, 2006a; and data not shown). To examine any potential role of PilZ domain proteins in biofilm formation, *wspR19* cloned in pVSP61 (Table 1) was introduced into the wild-type and panel of mutants. Expression of *wspR19* promoted biofilm formation equally in the wild-type and all the mutant strains (data not shown). As expected, expression of *wspR19* reduced endoglucanase levels and motility in wild-type *Xcc*. Similar effects on endoglucanase production were seen in all of the mutant backgrounds, and all strains carrying *wspR19* showed no motility. These findings suggest that none of the PilZ domain proteins individually has a significant role in the repression of extracellular enzyme production and motility by elevated cellular levels of cyclic di-GMP.

In some cases genes encoding cyclic di-GMP signalling systems with a role in disease may only be expressed within the eukaryotic host (Osorio *et al.*, 2005). We have previously shown that HrpG, the master regulator of the *hrp* regulon (for hypersensitive response and pathogenicity), significantly influences expression of *pdeA*, encoding a GGDEF-EAL domain protein that contributes to *Xcc* virulence. The *hrpG* gene is expressed in minimal media, shows increased expression in the plant environment but is repressed in rich media (Wengelnik *et al.*, 1996, 1999). HrpG also affects the expression of *hrpX*, which encodes an AraC-type transcriptional activator that regulates expression of a subset of genes within the *hrp* operon. Comparison was made of the expression levels of *XC0965*, *XC2249*, *XC2317* and *XC3221* in wild-type, *hrpG* and *hrpX* deletion mutants (Xu *et al.*, 2008) grown in MME minimal

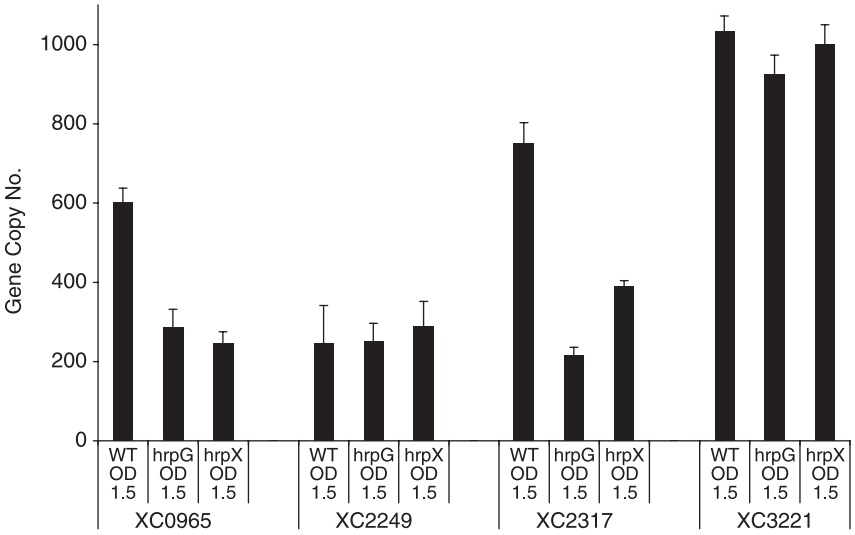

Fig. 3 Effect of mutation of *hrpG* and *hrpX* on the expression of genes encoding PilZ domain proteins. Transcript levels of all genes were measured by RT-PCR. Bacterial strains were grown to an OD at 600 nm of 1.5 in MME medium before RNA extraction. All RT-PCR results were normalized using the *Ct* values obtained for the 16S rRNA amplifications run in the same plate. The relative amounts of selected genes and relative levels of gene transcripts were determined by a standard curve (i.e. *Ct* values plotted against logarithm of the DNA copy number). The results shown are averages of three repetitions.

medium using RT-PCR. Primers for RT-PCR are given in Table 1 and procedures and medium composition are given in Ryan *et al.* (2007). The findings indicated that both HrpG and HrpX significantly influenced expression of *XC0965* and *XC2317* (Fig. 3). This raises the possibility that expression of these particular genes may be elevated in the plant environment.

Mutation of *XC3221*, *XC2249* and *XC0965* affects the virulence of *Xcc*, suggesting that there is no redundancy of action between these 'stand-alone' PilZ domain proteins, which also do not show any significant amino acid sequence homology (see above). As mutation of *XC2249* had a substantial effect on motility, we examined the relationship between motility and virulence in *Xcc*. The form of motility observed on Eiken agar plates is pilus-dependent and is abolished by mutation of *pilA* (Ryan *et al.*, 2007). Furthermore, wild-type *Xcc* 8004 does not show swimming motility, which is flagellum-dependent, when assessed in 0.1% (w/v) agar. Mutation of *pilA* led to a reduction in virulence similar to that seen after mutation of *XC2249* (Fig. 1A,B). This indicates the possibility that the effects of mutation of *XC2249* on virulence could be due to an effect on motility. In contrast, mutation of *XC3221*, which has only a small effect on motility *in vitro*, has a larger effect on virulence than mutation of *pilA* (Fig. 1B). This suggests that XC3221 influences further virulence functions that remain obscure. Mutation of *XC0965* and *XC2317* did not affect motility but each had a modest effect on production of extracellular enzymes *in vitro*. It is plausible that this effect may be more pronounced *in planta*, as both of these genes are under the regulation of HrpG and HrpX (Fig. 3). However, despite this co-regulation and apparent similarities in the phenotypes of the mutants, only mutation of *XC0965* had an effect on virulence.

Although a number of reports have described the role of PilZ domain proteins in regulation of factors associated with bacterial virulence, only in a few cases has the role of these proteins been directly examined in virulence models. Pratt and colleagues have shown that a subset of PilZ domain proteins has been shown to contribute to colonization of infant mouse small intestine by *Vibrio cholerae* (Pratt *et al.*, 2007). Of particular relevance to the present work, Wang *et al.* (2007) identified through a transposon mutant screen that the homologue of XC3221 in the rice pathogen *Xanthomonas oryzae* pv. *oryzicola* contributes to virulence. Our findings thus contribute to a growing body of work that establishes a role in virulence for cyclic di-GMP signal transduction via PilZ domain proteins. The mechanisms by which these various PilZ domain proteins exert their action, however, remains largely unknown and this topic, together with other aspects of cyclic di-GMP signal transduction, are likely to be the focus of a considerable future research effort.

ACKNOWLEDGMENTS

This work was supported by Principal Investigator Awards from the Science Foundation of Ireland (to J.M.D.), grants from the National Natural Science Foundation of China Key Programme (30130010) and The National High Technology Research and Development Programme of China (2001AA223051 and 2004AA223060) (to J.L.T.) and by the China-Ireland Science and Technology Research Collaboration Programme (to J.M.D. and J.L.T.).

REFERENCES

Aldridge, P., Paul, R., Goymer, P., Rainey, P. and Jenal, U. (2003) Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. *Mol. Microbiol.* **47**, 1695–1708.

Amikam, D. and Galperin, M.Y. (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics*, **22**, 3–6.

Benach, J., Swaminathan, S.S., Tamayo, R., Handelman, S.K., Folta-Stogniew, E., Ramos, J.E., Forouhar, F., Neely, H., Seetharaman, J., Camilli, A. and Hunt, J.F. (2007) The structural basis of cyclic diguanylate signal transduction by PilZ domains. *EMBO J.* **26**, 5153–5166.

Christen, M., Christen, B., Folcher, M., Schauerte, A. and Jenal, U. (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J. Biol. Chem.* **280**, 30829–30837.

Christen, M., Christen, B., Allan, M.G., Folcher, M., Jeno, P., Grzesiek, S. and Jenal, U. (2007) DgrA is a member of a new family of cyclic diguanosine monophosphate receptors and controls flagellar motor function in *Caulobacter cresentus*. *Proc. Natl Acad. Sci. USA*, **104**, 4112–4117.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X., and Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc. Natl Acad. Sci. USA*, **100**, 10995–11000.

Jenal, U. and Malone, J. (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu. Rev. Genet.* **40**, 385–407.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M. 2nd and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, **166**, 175–176.

Lee, V.T., Matewish, J.M., Kessler, J.L., Hyodo, M., Hayakawa, Y. and Lory, S. (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol. Microbiol.* **65**, 1474–1484.

Merighi, M., Lee, V.T., Hyodo, M., Hayakawa, Y. and Lory, S. (2007) The second messenger bis-(3–5)-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **65**, 876–895.

Osorio, C.G., Crawford, J.A., Michalski, J., Martinez-Wilson, H., Kaper, J.B. and Camilli, A. (2005) Second-generation recombination-based *in vivo* expression technology for large-scale screening for *Vibrio cholerae* genes induced during infection of the mouse small intestine. *Infect. Immun.* **73**, 972–980.

Paul, R., Weiser, S., Amiot, N.C., Chan, C., Schirmer, T., Giese, B. and Jenal, U. (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev.* **18**, 715–727.

Pratt, J.T., Tamayo, R., Tischler, A.D. and Camilli, A. (2007) PilZ domain proteins bind cyclic diguanylate and regulate diverse processes in *Vibrio cholerae*. *J. Biol. Chem.* **282**, 12860–12870.

Qian, W., Jia, Y., Ren, S.X., He, Y.Q., Feng, J.X. Lu, L.F., Sun, Q.H., Ying, G., Tang, D. J., Tang, H., Wu, W., Hao, P., Wang, L., Jiang, B.L., Zeng, S., Gu, W.Y., Lu, G., Rong, L., Tian, Y., Yao, Z., Fu, G., Chen, B., Fang, R., Qiang, B., Chen, Z., Zhao, G.P., Tang, J.L. and He, C. (2005) Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Res.* **15**, 757–767.

Ramelot, T.A., Yee, A., Cort, J.R., Semesi, A., Arrowsmith, C.H. and Kennedy, M.A. (2007) NMR structure and binding studies confirm that PA4608 from *Pseudomonas aeruginosa* is a PilZ domain and a c-di-GMP binding protein. *Proteins*, **66**, 266–271.

Römling, U. and Amikam, D. (2006) Cyclic di-GMP as a second messenger. *Curr. Opin. Microbiol.* **9**, 1–11.

Römling, U., Gomelsky, M. and Galperin, M.Y. (2005) C-di-GMP: the dawning of a novel bacterial signaling system. *Mol. Microbiol.* **57**, 629–639.

Ross, P., Weinhouse, H., Aloni, Y., Michaeili, D., Weinberger-Ohana P. and Mayer, P. (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature*, **325**, 279–281.

Ryan, R.P., Fouhy, Y., Lucey, J.F., Crossman L.C., Spiro, S., He, Y.W., Zhang, L.H., Heeb, S., Camara, M., Williams, P. and Dow, J.M. (2006a) Cell–cell signalling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc. Natl Acad. Sci. USA*, **103**, 6712–6717.

Ryan, R.P., Fouhy, Y., Lucey, J.F. and Dow, J.M. (2006b) Cyclic di-GMP signalling in bacteria: recent advances and new puzzles. *J. Bacteriol.* **188**, 8327–8334.

Ryan, R.P., Fouhy, Y., Lucey, J.F., Jiang, B.-L., He, Y.-Q., Feng, J.X., Tang, J.-L. and Dow, J. M. (2007) Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. *Mol. Microbiol.* **63**, 429–442.

Ryjenkov, D.A., Simm, R., Römling, U. and Gomelsky, M. (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J. Biol. Chem.* **281**, 30310–30314.

Ryjenkov, D.A., Tarutina, M., Moskvin, O.M. and Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J. Bacteriol.* **187**, 1792–1798.

Schmidt, A.J., Ryjenkov, D.A. and Gomelsky, M. (2005) Ubiquitous protein domain EAL encodes cyclic diguanylate-specific phosphodiesterase: enzymatically active andinactive EAL domains. *J. Bacteriol.* **187**, 4774–4781.

Shi, J., Blundell, T.L. and Mizuguchi, K. (2001) FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. *J. Mol. Biol.* **310**, 243–257.

Slater, H., Alvarez-Morales, A., Barber, C.E., Daniels, M.J., and Dow, J.M. (2000) A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. *Mol. Microbiol.* **38**, 986–1003.

Tamayo, R., Tischler, A.D. and Camilli, A. (2005) The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J. Biol. Chem.* **280**, 33324–33330.

Wang, L., Makino, S., Subedee, A. and Bogdanove, A.J. (2007) Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. *Appl. Environ. Microbiol.* **73**, 8023–8027.

Wengelnik, K., Rossier, O. and Bonas, U. (1999) Mutations in the regulatory gene *hrpG* of *Xanthomonas campestris* pv. *vesicatoria* result in constitutive expression of all *hrp* genes. *J. Bacteriol.* **181**, 6828–6831.

Wengelnik, K., Van den Ackerveken, G. and Bonas, U. (1996) HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. *Mol. Plant–Microbe Interact.* **9**, 704–712.

Xu, R.Q., Blanvillain. S., Feng, J.X., Jiang, B.L., Li, X.Z., Wei, H.Y., Kroj, T., Lauber, E., Roby, D., Chen, B., He, Y.Q., Lu, G.T., Tang, D.J., Vasse, J., Arlat, M. and Tang, J.L. (2008) AvrAC$_{Xcc8004}$, a type III effector with a leucine-rich repeat domain from *Xanthomonas campestris* pathovar *campestris* confers avirulence in vascular tissues of *Arabidopsis thaliana* ecotype Col-0. *J. Bacteriol.* **190**, 343–355.