Research Note

Synthesis of Extracellular Polysaccharide, Extracellular Enzymes, and Xanthomonadin in *Xanthomonas campestris*: Evidence for the Involvement of Two Intercellular Regulatory Signals

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Accepted 23 September 1997.

In previous work, the *pigB* transcriptional unit of *Xanthomonas campestris* was implicated in the generation of a diffusible factor (DF) that regulates xanthomonadin and extracellular polysaccharide (EPS) production. Similarly, the *rpfF* and *rpfB* genes were implicated in the generation of a diffusible factor (DSF) that regulates the production of extracellular enzymes and EPS. In this study, extracellular cross-feeding studies with *rpfF* and *pigB* mutant strains provided evidence for the existence of two separate intercellular signaling systems with overlapping roles in the regulation of EPS production.

Additional keywords: epiphytic survival, pathogenesis.

A great deal of interest has centered in recent years on the role of low-molecular-weight diffusible factors (pheromones) in the regulation of diverse physiological processes in bacteria (reviewed by Kell et al. 1995; Salmond et al. 1995). In phytopathogens, *N*-acyl homoserine lactones (*N*-AHLs) are known to regulate processes that contribute to virulence, such as synthesis of extracellular enzymes and capsular polysaccharide in *Erwinia* (Jones et al. 1993; Pirhonen et al. 1993; Beck von Bodman and Farrand 1995) and conjugal transfer in *Agrobacterium* (Piper et al. 1993; Zhang et al. 1993; Fuqua and Winans 1994). Molecules other than *N*-AHLs are also known to act as diffusible signals to regulate virulence gene expression in *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*) (Clough et al. 1994; Flavier and Denny 1994). Recently, our laboratories have independently obtained evidence for the involvement of low-molecular-weight diffusible factor(s) in the regulation of synthesis of extracellular enzymes, extracellular polysaccharide (EPS) and pigment (xanthomonadin) in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Poplawsky and Chun 1997; Barber et al. 1997). The production of these factors contributes to both epiphytic survival (Poplawsky et al. 1996; Chun et al. 1997) and pathogenesis (Barber et al. 1997) of *Xcc*. The chemical nature of the

active compound(s) remains uncertain, although they are probably not *N*-AHLs. Here we provide evidence that these factors are part of two distinct signaling systems in *Xcc* that have overlapping regulatory roles in EPS biosynthesis.

Two loci have been previously implicated in the biosynthesis of diffusible factors in *Xcc*: the *pigB* locus of strain B24 (Poplawsky and Chun 1997) and the *rpfF* and *rpfB* genes of strain 8004 (Barber et al. 1997). The *Xcc* strains carrying mutations in these different genes are described in Table 1. *pigB* mutants have reduced levels of EPS and xanthomonadin whereas *rpfF* and *rpfB* mutants show a reduction in the synthesis of extracellular enzymes and EPS (Table 2). The phenotypic defects in *pigB* and *rpfF* (but not *rpfB*) mutants can be corrected by addition of substances present in the culture supernatant of the cognate wild type. The factors responsible for these effects have been called DF and DSF, respectively (Poplawsky and Chun 1997; Barber et al. 1997).

As a basis for the experiments described in this paper, the phenotypes of *pigB* and *rpfF* mutants for extracellular enzyme production and pigmentation, respectively, were established. The synthesis of extracellular enzymes by *pigB* mutants was

Table 1. Strains of *Xanthomonas campestris* pv. *campestris* and plasmids

Strain or plasmid	Relevant characteristics	Reference
8004	Wild type	Turner et al. 1984
8523	<i>rpfF</i> ::Tn5 <i>lac</i> derivative of 8004	Tang et al. 1991
8550	<i>rpfB</i> ::Tn5 derivative of 8004	Barber et al. 1997
B24	Wild type	Poplawsky et al. 1993
B24-B1 ^a	<i>pigB</i> ::Tn3HoHo1 derivative of B24 (distal insertion)	Poplawsky and Chun 1997
B24-B9 ^a	<i>pigB</i> ::Tn3HoHo1 derivative of B24 (proximal insertion)	Poplawsky and Chun 1997
pIJ3020	<i>rpf</i> genes cloned in pLAFR1	Tang et al. 1991
pIG203	<i>pigB</i> locus cloned in pLAFR3	Poplawsky and Chun 1997

^a Since *pigB* apparently encodes at least two gene products (A. R. Poplawsky and W. Chun, *unpublished*), both proximal and distal mutations were included in this study.

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compared with the wild-type strain by assessing the levels of extracellular endoglucanase (EngXca) of cultures grown in NYGB medium as described by Gough et al. (1988). The level of EngXca in wild-type strain B24 was lower than in wild-type strain 8004. Mutation of the *pigB* locus, however, had no effect on this level of EngXca production (Table 2). The level of xanthomonadin pigment in strain 8004 and in *rpjF* and *rpjB* mutants was assessed as described by Poplawsky et al. (1993). No differences were seen between wild-type strain 8004, the *rpjB* mutant derivative, and wild-type strain B24. However, pigment production in the *rpjF* mutant was increased (Table 2). Pigments extracted from the *rpjF* mutant and wild-type strain 8004 had similar absorption spectra in the range 320 to 600 nm (data not shown). Thus, *pigB* and *rpjF* mutants have distinct phenotypes for pigment and extracellular enzyme levels. These phenotypic differences do not necessarily imply that DF and DSF are different; it is possible that *rpjF* and the *pigB* locus are involved in the production of the same signal that regulates different phenotypes in the two *X. campestris* isolates used.

In order to obtain evidence that DF and DSF are not identical, the ability of culture supernatants from different mutants to correct the phenotypic defects of *rpjF* and *pigB* mutants was assessed. Culture supernatants from wild-type strain B24 and two *pigB* mutants grown for 30 h in NYGB medium (Turner et al. 1984) were extracted with ethyl acetate and the extracts were tested for their ability to restore EngXca production to the *rpjF* mutant as described by Barber et al. (1997). In this assay, a volume of ethyl acetate corresponding to 1 ml of the original culture supernatant was used (after drying down) in a 1-ml assay volume. All three extracts were active in the assay when used at this level (Table 3), although extracts from the two *pigB* mutants were up to 10-fold more active than that from the B24 wild type, which had only minimal activity. Even after concentration, ethyl acetate extracts from B24 did not show full activity (data not shown). Culture supernatants from wild-type strain B24 and *pigB* mutants were also tested for their ability to restore EPS production to *rpjF* mutants as described by Poplawsky and Chun (1997), in which conditioned media are diluted 1:1 with new medium. Culture supernatants of both *pigB* mutants restored EPS production in the *rpjF* mutant to levels seen in wild-type strain 8004 (Table 3). Unexpectedly, culture supernatants from wild-type strains 8004 and B24 showed no activity in this assay. However, after concentration of the material by extraction into ethyl acetate, both of the wild-type strains but not the *rpjF* or *rpjB* mutants did show full activity (Table 3). The

minimum concentration factor required was twofold. In reciprocal experiments, the ability of culture supernatants from wild-type strain 8004 and *rpjF* and *rpjB* mutants to restore pigment and EPS production to *pigB* mutants was assayed again as described by Poplawsky and Chun (1997). All three strains produced extracellular factors able to restore both EPS and pigment production to both *pigB* mutants tested, although *rpjF* and *rpjB* mutants were less active than the wild-type strains in pigment restoration (Table 3). These complementation studies were repeated with essentially the same outcome. Overall, the results are consistent with the contention that the diffusible factors DF and DSF are different and suggest that *pigB* and *rpjF* are involved in separate pathways leading to the synthesis of DF and DSF, respectively. In the absence of chemical characterization, however, we have no proof that the DF (or DSF) activity from the two *X. campestris* isolates resides in the same molecular species.

Direct physical evidence that DF and DSF are different was provided by thin-layer chromatography on silica gel G (Analtech, Newark, DE) in hexane/diethyl ether/glacial acetic acid (70:30:1 by volume). After chromatography, areas of gel were scraped off the plates and compounds extracted in methanol for bioassay. DSF, measured by endoglucanase restoration to the *rpjF* mutant, has an R_f of approximately 0.5 under these conditions (Barber et al. 1997). In contrast, DF, measured by restoration of pigment production to the *pigB* mutant B24-B9, had an R_f of approximately 0.2. Initial structural characterization of DF from B24 suggests that it is a butyrolactone (Chun et al. 1997); currently, we have no structural information on DSF.

The phenotypes and cross-complementation studies of the mutants implicate both DF and DSF in the regulation of EPS biosynthesis but suggest that they act independently to regulate biosynthesis of pigment and EngXca, respectively. This interpretation was supported by the results of additional experiments. The *pigB* mutant B24-B9 has increased levels of DSF (measured by restoration of EngXca production to the *rpjF* mutant) over the parent wild-type strain B24. Introduction into B24-B9 of the cosmid clone pIJ3020 (Table 1), which carries *rpjF* and *rpjB* and other regulatory genes, further increased the level of DSF by approximately threefold and elevated levels of EngXca and EPS above those of the

Table 2. Production of extracellular polysaccharide (EPS), pigment, and endoglucanase by strains of *Xanthomonas campestris* pv. *campestris*

Strain	EPS ^a	Pigment ^a	Endoglucanase ^b
8004 (wild type)	25 to 75	100	100
8523 (<i>rpjF</i>)	<10	>125	<10
8550 (<i>rpjB</i>)	10 to 25	100	10 to 25
B24 (wild type)	100	100	25 to 75
B24-B1 (<i>pigB</i>)	10 to 25	10 to 25	25 to 75
B24-B9 (<i>pigB</i>)	<10	<10	25 to 75

^a Levels are expressed as a percentage of the level in strain B24, which is given as 100%.

^b Levels are expressed as a percentage of the level in strain 8004, which is given as 100%.

Table 3. Ability of culture supernatants from strains of *Xanthomonas campestris* pv. *campestris* to restore endoglucanase, pigment, and extracellular polysaccharide (EPS) production to *pigB* and *rpjF* mutants

Source of supernatant	Tester strain phenotype tested			
	B24-B9(<i>pigB</i>)		8523 (<i>rpjF</i>)	
	Pigment ^a	EPS ^a	Endoglucanase ^b	EPS ^b
None	<10	<10	<10	<10
8004 (wt)	75 to 125	75 to 125	75 to 125	75 to 125 ^c
8550 (<i>rpjB</i>)	25 to 75	75 to 125	<10	<10
8523 (<i>rpjF</i>)	25 to 75	75 to 125	<10	<10
B24 (wt)	75 to 125	75 to 125	10 to 25	75 to 125 ^c
B24-B1 (<i>pigB</i>)	<10	<10	75 to 125	75 to 125
B24-B9 (<i>pigB</i>)	<10	<10	75 to 125	75 to 125

^a Levels of pigment and EPS are expressed as a percentage of those of strain B24. Similar results were seen with B24-B1 as tester strain.

^b Levels of endoglucanase and EPS are expressed as a percentage of those of strain 8004.

^c Effects seen only after concentration of extracts.

B24 wild type, but did not restore pigment production (data not shown). This suggests that over-production of DSF can compensate for absence of DF in EPS but not pigment biosynthesis in *pigB* mutants, and supports the idea that DF affects the production of EPS and xanthomonadin via two separate regulatory circuits (Poplawsky and Chun 1997). Addition of extra copies of *pigB* into the *rpjF* mutant in the form of pIG203 (Table 1) had no effect on the levels of extracellular enzymes and EPS and, as expected, did not restore DSF production. Extracts from culture supernatants of *rpjF* mutants had no antagonistic or incremental effects on the restoration of EngXca biosynthesis to *rpjF* mutants by extracts from *pigB* strains. This suggests no direct interaction between the factors in regulation of enzyme biosynthesis. However, the fact that extracts from *pigB* mutants were more effective in restoring endoglucanase and EPS biosynthesis to *rpjF* mutants (Table 3) is consistent with the possibility that DF down-regulates the production of DSF.

The existence of multiple signaling pathways involving extracellular diffusible factors has now been demonstrated in a number of bacteria (Kaiser and Losick 1993; Kuo et al. 1994; Pearson et al. 1995; Latifi et al. 1996). The existence and interaction of different diffusible regulatory signals in *Xcc* is redolent of these regulatory circuits in other bacteria. The chemical nature of the diffusible factors in *Xcc* and the precise roles of the gene products of *rpjF* and *rpjB* and the *pigB* locus in their biosynthesis will be the subject of future work. The utilization of multiple signaling systems by *Xcc* may confer considerable flexibility to allow expression of specific sets of genes required for epiphytic or pathogenic survival in response to a changing bacterial environment.

ACKNOWLEDGMENTS

The Sainsbury Laboratory is supported by a grant from the Gatsby Charitable Foundation. This work was carried out according to the provisions of the Ministry of Agriculture, Fisheries and Food license PHF1185/8(48) issued under the Plant Health (Great Britain) Order 1987 (Statutory Instrument 1758). This work was also supported in part by award no. 9601190 from USDA/NRICGP to A.R.P. and W.C.

LITERATURE CITED

Barber, C. E., Tang, J.-L., Feng, J.-X., Pan, M.-Q., Wilson, T. J. G., Slater, H., Dow, J. M., Williams, P., and Daniels, M. J. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol.* 24:555-566.

Beck von Bodman, S., and Farrand, S. K. 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J. Bacteriol.* 177:5000-5008.

Chun, W., Cui, J., and Poplawsky, A. R. 1997. Purification, characterization and biological role of a pheromone produced by *Xanthomonas campestris* pv. *campestris*. *Physiol. Mol. Plant Pathol.* 51:1-14.

Clough, S. J., Schell, M. A., and Denny, T. P. 1994. Evidence for involvement of a volatile extracellular factor in *Pseudomonas solanacearum* virulence gene expression. *Mol. Plant-Microbe Interact.* 7:

621-630.

Flavier, A. B., and Denny, T. P. 1994. Purification and characterization of a volatile endogenous factor that restores expression of virulence-associated genes in *Pseudomonas solanacearum*. (Abstr.) *Phytopathology* 84:1134.

Fuqua, W. C., and Winans, S. C. 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J. Bacteriol.* 176:2796-2806.

Gough, C. L., Dow, J. M., Barber, C. E., and Daniels, M. J. 1988. Cloning of two endoglucanase genes in *Xanthomonas campestris* pv. *campestris*: analysis of the role of the major endoglucanase in pathogenesis. *Mol. Plant-Microbe Interact.* 1:275-281.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1993. The *lux* autoinducer regulates production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J.* 12:2477-2482.

Kaiser, D., and Losick, R. 1993. How and why bacteria talk to each other. *Cell* 73:873-885.

Kell, D. B., Kaprelyants, A. S., and Grafen, A. 1995. Pheromones, social behaviour and the function of secondary metabolism in bacteria. *Trends Ecol. Evol.* 10:126-129.

Kuo, A., Blough, N. V., and Dunlap, P. V. 1994. Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine bacterium *Vibrio fischeri*. *J. Bacteriol.* 176:7558-7565.

Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* 21:1137-146.

Pearson, J. P., Passador, L., Iglewski, B. H., and Greenberg, E. P. 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Nat. Acad. Sci. USA* 92:1490-1494.

Piper, K. R., Beck von Bodman, S., and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti-plasmid transfer by autoinduction. *Nature* 362:448-450.

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* 12:2467-2476.

Poplawsky, A. R., and Chun, W. 1997. *pigB* determines a diffusible factor needed for extracellular polysaccharide slime and xanthomonadin production in *Xanthomonas campestris* pv. *campestris*. *J. Bacteriol.* 179:439-444.

Poplawsky, A. R., Cui, J., and Chun, W. 1996. A *Xanthomonas campestris* pheromone with a role in epiphytic survival and host plant infection. In: *Int. Congr. [on] Mol. Plant-Microbe Interact.*, 8th. (Abstr. Bk. G47).

Poplawsky, A. R., Kawalek, M. D., and Schaad, N. W. 1993. A xanthomonadin-encoding gene cluster for the identification of pathovars of *Xanthomonas campestris*. *Mol. Plant-Microbe Interact.* 6:545-552.

Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P. 1995. The bacterial 'enigma': Cracking the code of cell-cell communication. *Mol. Microbiol.* 16:615-624.

Tang, J.-L., Liu, Y.-N., Barber, C. E., Dow, J. M., Wootton, J. C., and Daniels, M. J. 1991. Genetic and molecular analysis of a cluster of *rpj* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. *Mol. Gen. Genet.* 226:409-417.

Turner, P., Barber, C. E., and Daniels, M. J. 1984. Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pathovar *campestris*. *Mol. Gen. Genet.* 195:101-107.

Zhang, L. H., Murphy, P. J., Kerr, A., and Tate, M. E. 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature* 362:446-448.