Expression of the *gum* Operon Directing Xanthan Biosynthesis in *Xanthomonas campestris* and Its Regulation In Planta

Adrian A. Vojnov, Holly Slater, Michael J. Daniels, and J. Maxwell Dow

The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, U.K.

Submitted 20 November 2000; Accepted 1 March 2001.

The *gum* gene cluster of *Xanthomonas campestris* pv. *campestris* comprises 12 genes whose products are involved in the biosynthesis of the extracellular polysaccharide xanthan. These genes are expressed primarily as an operon from a promoter upstream of the first gene, *gumB*. Although the regulation of xanthan synthesis in vitro has been well studied, nothing is known of its regulation in planta. A reporter plasmid was constructed in which the promoter region of the *gum* operon was fused to *gusA*. In liquid cultures, the expression of the *gumgusA* reporter was correlated closely with the production of xanthan, although a low basal level of β -glucuronidase activity was seen in the absence of added carbon sources when xanthan production was very low. The expression of the *gumgusA* fusion also was subject to positive regulation by *rpjF*, which is responsible for the synthesis of the diffusible signal factor (DSF). The expression of the *gumgusA* fusion in bacteria recovered from inoculated turnip leaves was maximal at the later phases of growth and was subject to regulation by *rpjF*. These results provide indirect support for the operation of the DSF regulatory system in bacteria in planta.

Additional keyword: black rot.

Many plant pathogenic bacteria produce extracellular polysaccharides that contribute to the ability of these organisms to cause disease on plants (Denny 1995). In *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot disease of cruciferous crops, the extracellular polysaccharide is called “xanthan,” which is a polymer of repeating pentasaccharide units with the structure mannose-(β -1,4)-glucuronic acid-(β -1,2)-mannose-(β -1,3)-cellobiose (Sutherland 1993). The assembly of the pentasaccharide repeating unit, polymerization, and the export of xanthan are directed by a gene cluster comprising 12 genes, *gumB* to *gumM* (Harding et al. 1987; Vanderslice et al. 1990). Transcriptional analysis has revealed that the *gum* region is expressed mainly as an operon from a promoter located upstream of *gumB*, although weak promoters may exist upstream of *gumK* (Katzen et al. 1996) and *gumD* (Pollock et al. 1994; Vanderslice et al. 1990).

Corresponding author: J. M. Dow; Telephone: +44 1603 452571
Fax: +44 1603 250024; E-mail: max.dow@bbsrc.ac.uk

Current address of H. Slater: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

The production of xanthan is an energy-intensive process that is highly regulated in culture. A number of genes involved in positive or negative control have been identified (Barber et al. 1997; De Crcy-Lagard et al. 1990; Poplawsky et al. 1998; Slater et al. 2000; Tang et al. 1990; Tang et al. 1991). In our laboratory, we have shown that the biosynthesis of xanthan is coordinately regulated with the biosynthesis of extracellular enzymes by a cluster of genes called *rpj* (for regulation of pathogenicity factors). Several of the *rpj* gene products are implicated in control that is mediated by a small diffusible signal factor (DSF) molecule. The synthesis of DSF is directed by RpfB and RpfF (Barber et al. 1997). RpfG and RpfC encode elements of a two-component system that may couple the perception of DSF to the regulation of pathogenicity gene expression (Slater et al. 2000). *rpjF* mutants, which do not make DSF, can be phenotypically corrected for the production of enzymes and xanthan by the addition of DSF. In contrast, *rpjC* mutants, which also are down regulated for the production of xanthan and extracellular enzymes, overproduce DSF and are apparently unable to sense its presence.

Although synthesis of xanthan is implicated in the pathogenesis of *X. campestris* pv. *campestris* (Chou et al. 1997; Katzen et al. 1998; Newman et al. 1994), nothing is known of its regulation in bacteria within plants. In order to investigate this, a reporter construct was created by fusing a 290-bp region of the *gum* gene cluster that is immediately upstream of *gumB* with the coding sequence for β -glucuronidase (GUS) of *Escherichia coli* (*gusA*). This region of the *gum* gene cluster contains the transcriptional start site, as determined by Katzen et al. (1996). In initial experiments, we assessed the validity of the use of the reporter by analysis of *gusA* expression and xanthan production at different times and under different conditions of growth in culture. Subsequently, the expression of the reporter in bacteria in plants was examined. The results suggest considerable parallels between the regulation of xanthan biosynthesis in planta and in culture.

RESULTS

Expression of the *gumgusA* fusion as a function of bacterial growth and carbon source.

The reporter plasmid pL6gumGUS, which carries the promoter region of the *gum* operon fused to the coding region for *gusA*, was constructed as described below. The relationship between the expression of the *gumgusA* fusion and the production of xanthan was first assessed as a function of the growth

time of wild-type strain 8004 carrying pL6gumGUS in peptone–yeast extract–malt extract (PYM) nutrient medium, with or without 2% (wt/vol) glucose. In medium containing glucose, xanthan production was initiated in the late-exponential phase of growth, with maximal production during the stationary phase (Fig. 1A). These kinetics are similar to those described previously by Harding et al. (1995). The expression of the *gumgusA* fusion mirrored the time course of xanthan production (Fig. 1B), with maximal expression per cell in stationary phase cultures. Except at the lowest level of expression, the two parameters were correlated (Fig. 1C). The value for r , the linear correlation coefficient of the sample, was 0.996. In the absence of glucose, very little xanthan was synthesized (0.11 ± 0.02 g of dry-weight bacteria per g at 38 h). There was, however, significant expression of the *gumgusA* reporter throughout the time course, and the activity per cell increased slightly at later time points (Fig. 1B). To further examine the relationship between the production of xanthan and the expression of the *gumgusA* reporter by strain 8004/pL6gumGUS, the effect of different carbon sources on the two parameters was tested. A number of carbon sources (glucose, sucrose, maltose, cellobiose, mannose, and xylose) induced a high level of expression of the *gumgusA* reporter, whereas others (mannitol and rhamnose) gave very low levels. The *gumgusA* expression level was, again, correlated closely ($r = 0.997$) with the level of xanthan production (Fig. 2).

Effects of mutation of *rpf* genes on the *gumgusA* expression.

As outlined above, the *rpf* genes of *X. campestris* pv. *campestris* regulate the synthesis of xanthan, and some of these genes are implicated in a regulatory mechanism involving the DSF molecule. We investigated whether DSF-dependent regulation was at the level of the promoter activity by comparing the expression of the *gumgusA* reporter in wild-type and *rpfF* mutant backgrounds during growth in PYM medium containing 2% (wt/vol) glucose. The results (Fig. 3) show that expression per cell was lower in the *rpfF* mutant background than in the wild type. The reductions were, however, fairly modest, and the levels of *gumgusA* expression in the *rpfF* mutant were 50 to 75% of the wild type. Xanthan production by *rpfF* mutants was typically 22 to 30% of the wild type throughout growth. Similar results were seen with *rpfC* mutants (data not shown). Although the effects are modest, the results indicate that the regulation of xanthan production by the *rpf* genes is, in part, at the level of transcription of the *gum* operon.

DSF can stimulate expression of the *gumgusA* reporter.

The synthesis of xanthan can be restored to the *rpfF* mutant (strain 8523) by the addition of DSF (Barber et al. 1997; Poplawsky et al. 1998). The effect of DSF on the expression of the *gumgusA* reporter in the *rpfF* mutant background was assessed in two ways. In the first set of experiments, DSF was added to cultures of the *X. campestris* pv. *campestris* strain 8523/pL6gumGUS. This led to an increase in the level of *gumgusA* expression, although even at the highest level of DSF used, expression was less than wild-type levels (Fig. 4A). The production of xanthan also was only partially restored under the same conditions (Fig. 4A). The second set of experiments were a variation of the plate bioassay for DSF

(Slater et al. 2000) with the use of strains with known DSF production as “donors” and 8523/pL6gumGUS as a recipient. GUS activity under the donor colonies was indicated with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-GlcA), which was incorporated into the nutrient agar. Blue haloes were seen

Fig. 1. Expression of the *gumgusA* reporter and production of xanthan in liquid cultures of wild-type *Xanthomonas campestris* pv. *campestris*. **A**, Time course of xanthan production in peptone–yeast extract–malt extract medium, with or without 2% (wt/vol) glucose. Filled bars represent medium with glucose. Measurements in medium without glucose (open bars) were made only at 4 and 38 h. Values are the means and standard deviations of triplicate measurements. Bacterial growth, estimated by an optical density (OD) at 600 nm, is very similar in the presence and absence of glucose. An OD measurement of 1.0 corresponds to a bacterial density of 5×10^8 CFU/ml. **B**, Expression of the *gumgusA* reporter in medium with (filled bars) or without (open bars) 2% (wt/vol) glucose. Values given are the means and standard deviations of triplicate measurements. **C**, Plot of the xanthan production against β -glucuronidase activity for the different time points. Data are from **A** and **B**; error bars are omitted for clarity.

under *X. campestris* pv. *campestris* strain 8557 (*rpfC*, a DSF overproducer) but not under 8523 (which makes no DSF) or 8004 (wild type) (Fig. 4B). The absence of an effect with the wild-type strain 8004 in this plate experiment is consistent with the relatively weak response of the *gumgusA* reporter to the addition of low levels of DSF (data not shown). Both experiments indicate that the activity of the *gum* operon promoter is responsive to DSF. The background levels of *gumgusA* expression in the reporter strain 8523/pL6gumGUS and the degree of responsiveness to DSF in the two experiments appear to be different. This may reflect the differences in the physiology of the reporter strain when grown in soft agar with glycerol as the carbon source compared with growth in broth culture with glucose as the carbon source.

The expression of the *gumgusA* reporter in bacteria in planta.

X. campestris pv. *campestris* is a vascular pathogen that normally colonizes xylem vessels in the early stages of black rot disease (Bretschneider et al. 1989). It is, however, capable of substantial growth in leaf mesophyll tissue (Kamoun et al. 1992; Newman et al. 1994). The *X. campestris* pv. *campestris* strain 8004/pL6gumGUS was introduced into the petioles of turnip cultivar Just Right, and bacteria recovered at 7 days postinoculation (dpi) from 2-cm sections of the petiole and mid vein. The bacteria had moved several centimeters up the petiole from the initial point of inoculation. Under these conditions, in stem sections distant from the site of inoculation, the bacteria were restricted to the xylem elements (M. J. Daniels, unpublished observations). The level of *gumgusA* expression in bacteria recovered from the plants was low, with a maximum of 2.0 nmoles of product per min per 10^8 CFU (Table 1). In a separate experiment, *X. campestris* pv. *campestris* strains 8004/pL6gumGUS and 8523/pL6gumGUS were introduced into the leaf mesophyll tissue by inoculating leaf panels. For both strains, the numbers of bacteria recovered

Fig. 2. Correlation between the expression of the *gumgusA* reporter and the production of xanthan in liquid cultures with different carbon sources. Bacteria were grown for 2 d in Y medium with 2% (wt/vol) of 1, mannitol; 2, rhamnose; 3, glycerol; 4, maltose; 5, cellobiose; 6, xylose; 7, sucrose; 8, mannose; and 9, glucose. Values are the average of three replicate measurements. Standard deviations (not shown for clarity) were less than 10% of the means.

from the leaves increased in a logarithmic fashion by two orders of magnitude in the first 2 dpi before reaching a plateau level at day 3. Additionally, the level of *gumgusA* expression in bacteria recovered from the plants at 1 dpi, for both strains, was higher than that seen in bacteria from the petiole (Fig. 5). At later stages of infection, *gumgusA* expression increased in both strains, although 8004/pL6gumGUS activity was significantly higher than 8523/pL6gumGUS. This indicates that in planta, the *gum* promoter was subject to similar regulation as in culture, where it was expressed maximally during the stationary phase and influenced positively by *rpfF*.

DISCUSSION

In wild-type bacteria in liquid culture, a strong linear correlation exists between the expression of the *gumgusA* reporter and the production of xanthan. This correlation breaks down, however, under growth conditions that support very little xanthan synthesis, where there are still significant levels of GUS activity. The reasons for this are not known, but it may be that a basal level of *gum* gene expression is required for cell viability. The first two genes in the *gum* operon, *gumB* and *gumC*, encode proteins implicated in the polymerization and export of xanthan (Harding et al. 1995; Vojnov et al. 1998). It is not possible to obtain mutations in these genes in a wild-type background, although they can be generated in cells that lack UDPglucose pyrophosphorylase (Katzen et al. 1998). It has been suggested that mutation of *gumB* or *gumC* is lethal in a wild-type background because of toxic effects of accumulating lipid intermediates of xanthan biosynthesis (Katzen et al. 1998). Low-level expression of the *gum* operon may be required to prevent the accumulation of such toxic intermediates. In the *rpfF* mutant, the relationship between the level of

Fig. 3. Regulation of expression of the *gumgusA* reporter by *rpfF*. *Xanthomonas campestris* pv. *campestris* strains 8004/pL6gumGUS and 8523(*rpfF*)/pL6gumGUS were grown in peptone-yeast extract-malt extract medium with 2% (wt/vol) glucose. Values of β -glucuronidase (GUS) activity are the average and standard deviation of three replicate measurements. Filled and open bars represent GUS activity in the wild-type and *rpfF* mutant backgrounds, respectively. Bacterial growth, measured as optical density at 600 nm, is very similar for the wild-type and *rpfF* mutant.

expression of the *gumgusA* reporter and the production of xanthan was different than in the wild type (Fig. 4A), although the two parameters were still correlated. This suggests that *rpjF* may regulate the biosynthesis of xanthan at steps additional to the activation of the *gum* operon promoter. Less-preferred carbon sources such as rhamnose and mannitol are poor inducers of the gum operon compared with carbon sources such as mannose and sucrose that support substantial growth.

The expression levels in bacteria recovered from plants differed depending upon the plant tissue inoculated. Inoculation of petioles and subsequent bacterial growth approximates natural infections by *X. campestris* pv. *campestris*. In bacteria from petioles at 7 dpi, expression levels of the *gumgusA* reporter were basal, and the maximum value observed was close to the level of *gumgusA* expression in medium without added glucose. In contrast, higher levels of expression of *gumgusA* were seen in bacteria recovered from the leaf mesophyll tissue 1 dpi. By 5 dpi, the expression level in the wild-type background had increased sixfold and was equivalent to the levels of expression seen in cultures grown for 3 days in medium containing glucose (where substantial levels of xanthan were produced). This difference in the level of *gumgusA* expression between bacteria in mesophyll tissue and in xylem may, in part, reflect differences in the availability of nutrients in the two plant environments, although other factors, as yet unknown, could be involved. The expression of the *gumgusA* reporter at the later phases of growth in mesophyll tissue was reduced in the *rpjF* mutant compared with the wild type. This observation provides circumstantial evidence for the operation of the DSF regulatory system in planta. Currently, there is no direct biochemical evidence for the presence of DSF in diseased plants.

In terms of the time course of expression and regulation by *rpjF*, *gumgusA* expression in bacteria in leaves appear to be controlled in a similar fashion to bacteria in culture. These are similar results to those obtained by Kang et al. (1999), who studied the expression of the *eps* operon of *Ralstonia solanacearum*, which directs the biosynthesis of the acidic exopolysaccharide of this pathogen. The *eps* operon of *R. solanacearum* is activated only at the later stages of tomato infection. Although extrapolation of the level of *gumgusA* expression to the level of xanthan produced by bacteria in planta must be made with caution, the results strongly suggest that *X. campestris* pv. *campestris* produces copious amounts of xanthan only at the later phases of disease. Nevertheless, the production of xanthan does appear to be required at early stages of *X. campestris* pv. *campestris* infection in leaf mesophyll tissue (Newman et al 1994) and in vascular inoculation (M. J. Daniels, *unpublished data*). As discussed by Denny (1999), there are several possible reasons why bacteria would not wish to produce large amounts of extracellular polysaccharides (EPS) during the early phases of pathogenesis. The limited production of EPS may allow adherence of bacterial colonies to plant cell surfaces, promoting the establishment of biofilms or microcolonies or the proper functioning of type III secretion systems encoded by *hrp* gene clusters. These processes may be critical in the establishment of infections or in xylem colonization. Copious production of EPS early in pathogenesis also may interfere with the movement of bacteria through the plant (Beck von Bodman et al. 1998). Conversely, EPS production at later stages of pathogenesis in tissue undergoing necrosis may protect bacteria against various

stresses such as desiccation and damage by reactive oxygen species (Kiraly et al. 1997).

MATERIALS AND METHODS

Bacterial strains and culture conditions.

X. campestris pv. *campestris* strains 8004 (wild type), 8523 (*rpjF*::Tn5*lac*), and 8557 (*rpjC*::pUIRM504) are described in

Fig. 4. Stimulation of expression of the *gumgusA* reporter by the diffusible signal factor (DSF). **A**, DSF was added to cultures of *Xanthomonas campestris* pv. *campestris* strain 8523/pL6gumGUS, which were assayed for xanthan production and β -glucuronidase (GUS) activity after 3 d growth in Y medium with 2% (wt/vol) glucose. The values of GUS activity (open bars) and xanthan levels (in grams per gram of dry-weight of bacteria) are the average and standard deviation of three replicate measurements. 1, 8004/pL6gumGUS; 2, 8523/pL6gumGUS; and 3, 8523/pL6gumGUS with added DSF. **B**, Plate test for the effect of DSF on *gumgusA* expression. *X. campestris* pv. *campestris* strains producing different levels of DSF were replicated by pin inoculation onto nutrient agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-GlcA) with a layer of soft-top agar containing X-GlcA and seeded with 8523/pL6gumGUS. The expression of the *gumgusA* reporter, detected by the presence of a blue halo around the donor colony, was assessed after overnight incubation. *X. campestris* pv. *campestris* strains used as donors were 8004 (wild type); 8523 (*rpjF*), a nonproducer; and 8557(*rpjC*), an overproducer.

Turner et al. (1984), Tang et al. (1991), and Slater et al. (2000), respectively. *X. campestris* pv. *campestris* strains were cultured at 28°C with shaking in PYM (Cadmus et al. 1976) or in Y minimal medium (Sherwood 1970) supplemented where indicated with sugars at a final level of 2% (wt/vol). Bacterial growth was measured in a U-3200 spectrophotometer (Hitachi Instruments, San Jose, CA, U.S.A.) at 600 nm. Antibiotics (tetracycline [Tc] and rifampicin [Rif]) were used, as required, at the concentrations indicated. Xanthan was precipitated from culture supernatants by ethanol, dried, and weighed, as described by Vojnov et al. (1998).

Construction of pL6gumGUS.

The reporter plasmid pL6gumGUS carries the promoter region of the *gum* operon (located upstream of *gumB*) fused to the coding region for *gusA* at the ATG start codon. The 290-bp region upstream of, and including, the *gumB* ATG start codon was amplified by polymerase chain reaction (PCR) with cosmid clone pIZD15-261 (Katzen et al. 1996), as template, and primers Gum1 (5'-GGAATTCACGTCGAGGCGACGAAC-TTGAA-3') and Gum2 (5'-ACGGCCATGGAGCAGAGCAGAG-3'). Oligonucleotide primers were purchased from Genosys Biotechnologies (Cambridge, U.K.). The nucleotide sequence of Gum1 differs from the *gum* sequence by the incorporation of an *EcoRI* restriction (underlined) at the 5' end to facilitate cloning. The nucleotide sequence of Gum2 contains two alterations (also underlined), which modify the sequence to contain a *NcoI* site. The PCR product was cleaved with *EcoRI*-*NcoI* and ligated into the plasmid pIJ3099-1 (Smith et al. 1996), cut previously with *EcoRI*-*NcoI* to excise the *sodA* gene promoter. This generated the intermediate construct, pBSgumGUS. The construction was verified by sequence analysis. The 2.89-kb gumGUS cassette was cloned as an *EcoRI*-*HindIII* fragment into the broad-host-range vector pLAFR6 to create pL6gumGUS. This plasmid was transferred to wild-type and *rpfF* mutant strains of *X. campestris* pv. *campestris* by triparental mating with the *E. coli* DH5 α donor strain and helper strain HB101/pRK2013, as previously described (Turner et al. 1985).

Assay of *gumgusA* expression in culture.

Flasks (500 ml) containing 100 ml of media were inoculated with 1.0 ml of an overnight culture of *X. campestris* pv. *campestris* strains 8004/pL6gumGUS or 8523/pL6gumGUS diluted to an optical density at 600 nm of 0.5. The activity of GUS per

bacterial cell was measured as described by Wilson et al. (1992) with *p*-nitrophenol- β -D-glucuronide as substrate. The average and standard deviation of three replicate measurements were subsequently calculated.

Assay of *gumgusA* expression in planta.

For assay of the expression of the *gumgusA* reporter in bacteria in plant leaves, leaf panels of 4- to 5-week-old Just Right plants were inoculated by syringe infiltration with a suspension in water of *X. campestris* pv. *campestris* strains 8004/pL6gumGUS or 8523/pL6gumGUS at 5×10^7 CFU/ml, as described by Newman et al. (1994). Leaf discs (0.9 cm in diameter) were taken from the inoculated area at different time points after inoculation. These were homogenized in sterile distilled water, and the homogenates assayed for GUS activity with the fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide after permeabilization of the bacterial cells by the addition of sodium dodecyl sulfate (SDS) and chloroform (Wilson et al. 1992). Fluorescence was measured on a Fluoroskan II microtiter plate reader (Titertek, Huntsville, AL, U.S.A.), with reference to a standard curve of 4-methylumbelliferone in 0.4 M sodium carbonate. Bacterial numbers were measured after plating 10- μ l aliquots of serial tenfold dilutions of the homogenate (before addition of SDS and chloroform) on NYGA agar plates with 50 μ g of Rif per ml and 5 μ g of Tc per ml. For expression studies of the *gumgusA* reporter in bacteria in the vascular system, bacteria were inoculated into wounded petioles on attached leaves as follows. A 4- to 5-week-old pot-grown turnip plant was supported so that the petiole of a fully expanded leaf was horizontal with the uppermost concave side. Blu-Tak mastic adhesive (Bostik, Leicester, U.K.) was used, and a well of

Fig. 5. Expression of the *gumgusA* reporter in bacteria isolated from turnip leaves. Leaves of 4- to 5-week-old turnip plants were inoculated with a suspension in water of bacteria at 5×10^7 CFU/ml. Leaf samples were taken and analyzed for bacterial numbers and for β -glucuronidase (GUS) activity measured with the fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide. GUS activity values per bacterial cell are the means and standard deviations of three independent measurements. Circles: 8004/pL6gumGUS; squares: 8523/pL6gumGUS.

Table 1. Expression of *gumgusA* in bacteria recovered from infected turnip petioles

Petiole segment ^a	Bacterial numbers ^b	β -glucuronidase (GUS) activity per 10^8 cells (nmoles 4-MU per min) ^c
1	2.5×10^6	0.52
2	4.4×10^6	0.95
3	6.2×10^6	1.34
4	1.5×10^7	2.04
5	5.1×10^6	1.29
6	7.0×10^2	Not detected
7	8.0×10^2	Not detected

^a Segments (2 cm) of petiole from above the wound site were assayed, as described in text.

^b Values given are the means of triplicate measurements.

^c Values given are the means of triplicate measurements; 4-MU is 4-methyl umbelliferone.

approximately $1 \times 0.5 \times 0.5$ cm was formed on the petiole surface and filled with sterile water. A no. 11 scalpel was passed through the water into the petiole, and an incision was made to sever two to three of the vascular bundles, taking care that the epidermis was not penetrated. A concentrated, washed aqueous suspension of bacteria (approximately 10^9 CFU total) was added to the water in the well. When the water and bacteria had just been drawn into the petiole, the well was removed and the incision in the petiole surface was covered with Saran Wrap (Dow Chemical, Midland, MI, U.S.A.). The plant was returned to the growth chamber (25°C, 15 h of light) until sampling. At 7 dpi, 2-cm sections of petiole starting 5 mm above the wound site were excised, homogenized, and assayed for GUS activity and bacterial numbers, as described above. By replicate plating on NYGA agar plates containing 50 µg of Rif per ml, with or without 5 µg of Tc per ml, it was determined that less than 5% of the bacteria recovered from the plant no longer carried pL6gumGUS.

Effects of DSF on gum operon expression.

DSF for addition to cultures of *X. campestris* pv. *campestris* was derived from culture supernatants of *X. campestris* pv. *campestris* strain 8557 by extraction into ethyl acetate, as described by Barber et al (1997). The ethyl acetate extract was dried, and the sample redissolved in methanol before use. The DSF level used was sufficient to cause maximal restoration of endoglucanase activity to *X. campestris* pv. *campestris* strain 8523 (Barber et al 1997). For tests on plates, *X. campestris* pv. *campestris* strains were replicated by pin inoculation onto NYGA nutrient agar plates (Turner et al. 1984) containing 50 µg of Rif per ml and 40 µg of X-GlcA per ml with a layer of soft-top agar containing X-GlcA and seeded with 8523/pL6gumGUS. The expression of the *gum* operon, detected by the presence of a blue halo around the donor colony, was assessed after overnight incubation.

ACKNOWLEDGMENTS

A. A. Vojnov was supported by a fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. 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, Agriculture, Fisheries and Food, licence PHF 1185/8(48) issued under the Plant Health (Great Britain) Order 1987 (Statutory Instrument 1758).

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., Majerczak, D. R., and Coplin, D. L. 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Nat. Acad. Sci. USA* 95:7687-7692.

Bretschneider, K. E., Gonella, M. P., and Robson, D. J. 1989. A comparative light and electron microscopical study of compatible and incompatible interactions between *Xanthomonas campestris* pv. *campestris* and cabbage (*Brassica oleracea*). *Physiol. Mol. Plant Pathol.* 34:285-297.

Cadmus, M. C., Rojovin, S. P., Burton, K. A., Pittsley, J. E., Knutson, C. A., and Jeanes, A. 1976. Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide of a

variant strain. *Can. J. Microbiol.* 22:942-948.

Chou, F.-L., Chou, H.-C., Lin, Y.-S., Yang, B.-Y., Lin, N.-T., Weng, S.-F., and Tseng, Y.-H. 1997. The *Xanthomonas campestris gumD* gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. *Biochem. Biophys. Res. Comm.* 233:265-269.

De Crècy-Lagard, V., Glaser, P., Lejeune, P., Sismeiro, O., Barber, C. E., Daniels, M. J., and Danchin, A. 1990. A *Xanthomonas campestris* pv. *campestris* protein similar to catabolite activation factor is involved in regulation of phytopathogenicity. *J. Bacteriol.* 172:5877-5883.

Denny, T. P. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:173-197.

Denny, T. P. 1999. Autoregulator-dependent control of extracellular polysaccharide production in phytopathogenic bacteria. *Eur. J. Plant Pathol.* 105:417-430.

Harding, N. E., Cleary, J. M., Cabañas, D. K., Rosen, I. G., and Kang, K. S. 1987. Genetic and physical analysis of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. *J. Bacteriol.* 169:2854-2861.

Harding, N. E., Cleary, J. M., and Ielpi, L. 1995. Genetics and biochemistry of xanthan gum production by *Xanthomonas campestris*. Pages 253-262 in: *Food Biotechnology: Microorganisms*. Y. H. Hui and G. G. Khachatourians, eds. ASM Press, Washington.

Kamoun, S., Kamdar, H. V., Tola, E., and Kado, C. I. 1992. Incompatible interactions between crucifers and *Xanthomonas campestris* involve a vascular hypersensitive response: Role of the *hrpX* locus. *Mol. Plant-Microbe Interact.* 5:22-33.

Kang, Y., Saile, E., Schell, M. A., and Denny, T. P. 1999. Quantitative immunofluorescence of regulated *eps* gene expression in single cells of *Ralstonia solanacearum*. *Appl. Environ. Microbiol.* 65:2356-2362.

Katzen, F., Ferreira, D. U., Oddo, C. G., Ielmini, M. V., Becker, A., Puhler, A., and Ielpi, L. 1998. *Xanthomonas campestris* pv. *campestris* *gum* mutants: Effects on xanthan biosynthesis and plant virulence. *J. Bacteriol.* 180:1607-1617.

Katzen, F., Becker, A., Zorreguieta, A., Puhler, A., and Ielpi, L. 1996. Promoter analysis of the *Xanthomonas campestris* pv. *campestris* *gum* operon directing biosynthesis of the xanthan polysaccharide. *J. Bacteriol.* 178:4313-4318.

Kiraly, Z., El Zahaby, H. M., and Klement, Z. 1997. Role of extracellular polysaccharide (EPS) slime of plant pathogenic bacteria in protecting cells to reactive oxygen species. *J. Phytopathol.* 145:59-68.

Newman, M.-A., Conrads-Strauch, J., Scofield, G., Daniels, M. J., and Dow, J. M. 1994. Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. *Mol. Plant-Microbe Interact.* 7:553-563.

Pollock, T. J., Thorne, L., Yamazaki, M., Mikolajczak, M., and Armentrout, R. W. 1994. Mechanisms of bacitracin resistance in Gram-negative bacteria that synthesise exopolysaccharides. *J. Bacteriol.* 176:6229-6237.

Poplawsky, A. R., Chun, W., Slater, H., Daniels, M. J., and Dow, J. M. 1998. Synthesis of extracellular polysaccharide, extracellular enzymes, and xanthomonadin in *Xanthomonas campestris*: Evidence for the involvement of two intercellular regulatory signals. *Mol. Plant-Microbe Interact.* 11:68-70.

Sherwood, M. T. 1970. Improved synthetic medium for the growth of *Rhizobium*. *J. Appl. Bacteriol.* 33:708-713.

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.

Smith, S. G., Wilson, T. J. G., Dow, J. M., and Daniels, M. J. 1996. A gene for superoxide dismutase from *Xanthomonas campestris* pv. *campestris* and its expression during bacterial-plant interactions. *Mol. Plant-Microbe Interact.* 9:584-593.

Sutherland, J. W. 1993. Xanthan. Pages 363-388 in: *Xanthomonas*. J. G. Swings and E. L. Civerolo, eds. Chapman and Hall, London.

Tang, J.-L., Gough, C. L., and Daniels, M. J. 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pathovar *campestris*. *Mol. Gen. Genet.* 222:157-160.

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 *rpf* 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., and Daniels, M. J. 1984. Behavior of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 195:101-107.
- Turner, P., Barber, C., and Daniels, M. J. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 199:338-343.
- Vanderslice, R. W., Doherty, D. H., Capage, M. A., Betlach, M. R., Hassler, R. A., Henderson, N. M., Ryan-Graniero, J., and Tecklenburg, M. 1990. Genetic engineering of polysaccharide structure in *Xanthomonas campestris* Pages 145-156 in: Biomedical and Biotechnological Advances in Industrial Polysaccharides. V. Crescenzi, I. C. M. Dea, S. Paoletti, S. S. Stivala, and I. W. Sutherland, eds. Gordon and Breach, New York.
- Vojnov, A. A., Zorreguieta, A., Dow, J. M., Daniels, M. J., and Dankert, M. A. 1998. Evidence for a role for the *gumB* and *gumC* gene products in the formation of xanthan from its pentasaccharide repeating unit by *Xanthomonas campestris*. Microbiology 144:1487-1493.
- Wilson, K. J., Hughes, S. G., and Jefferson, R. A. 1992. The *Escherichia coli* GUS operon. Induction and expression of the GUS operon in *E. coli* and the occurrence and use of the GUS operon in other bacteria. Pages 7-22 in: GUS protocols: Using the *gus* gene as a reporter of gene expression. S. Gallagher, ed. Academic Press, New York.