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Research Notes

Identification of a *dsp* DNA Region Controlling Aggressiveness of *Pseudomonas solanacearum*

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Tn*5*-induced mutations carried by three nonpathogenic mutants of *Pseudomonas solanacearum* were found to be located in different regions of the genome. Localized mutagenesis of one of these DNA regions showed that it covers approximately 15 kilobases and contains genes simultaneously modulating aggressiveness toward tomato and sensitivity to acidic pH.

Additional keywords: pathogenicity, transposon.

Pseudomonas solanacearum E. F. Sm. causes a lethal wilt disease on more than 200 different plant species worldwide (Buddenhagen and Kelman 1964). In strain GMI1000, which is pathogenic toward tomato, use of Tn*5* mutagenesis allowed us to isolate nine Hrp$^-$ mutants altered both for the production of disease symptoms on tomato plants and the induction of the hypersensitive response (HR) on nonhost plants and three (d)isease (sp)ecific (Dsp$^-$) mutants impaired in the ability to wilt tomato (Boucher *et al.* 1985). Previous data have shown that eight of the nine Hrp$^-$ mutations are clustered (Boucher *et al.* 1987). Here we show that the Tn*5* insertions of the three Dsp$^-$ mutants are scattered in the genome, and we describe the genetic study of the DNA region altered in one of these mutants.

To clone the genes altered in the Dsp$^-$ Tn*5* mutants GMI1299, GMI1314, and GMI1330 (Boucher *et al.* 1985), we screened separately a genomic bank of the wild-type strain GMI1000 constructed in pLAFR3 (Staskawicz *et al.* 1987) with probes corresponding to cloned *Eco*RI fragments containing Tn*5* and the flanking sequences of the three Dsp$^-$ mutants (Boucher *et al.* 1985). Each probe led to the isolation of a set of clones carrying overlapping inserts, none of which overlapped with cosmids corresponding to the two other sets. This result shows that the *dsp* genes altered in mutants GMI1299, GMI1314, and GMI1330 are different and are scattered in the genome of strain GMI1000.

Figure 1 shows the restriction map of the four cosmids, pB1E6, pD1D4, pA1C3, and pI1F4, obtained by hybridization with the *Eco*RI fragment corresponding to mutant GMI1299. Hybridization experiments using the four cosmids as probes against genomic DNA of strains GMI1000 or GMI1299 digested with *Eco*RI or *Bam*HI showed that the inserts carried by the four cosmids are colinear with genomic DNA (data not shown).

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The four cosmids, pB1E6, pD1D4, pA1C3, and pI1F4, were individually introduced into the mutant GMI1299, and transconjugants were then tested for restoration of pathogenicity on axenic tomato plants (Boucher *et al.* 1985). Whereas cosmids pA1C3, pD1D4, and pB1E6 restored pathogenicity, cosmid pI1F4, which does not overlap the transposon insertion site of mutant GMI1299, was unable to complement the mutation.

Localized mutagenesis of pD1D4 was performed in *Escherichia coli* with Tn*5lac* (Kroos and Kaiser 1984) or the MuDII*lac*1734 miniphage (Castilho *et al.* 1984) as described previously (Boucher *et al.* 1987). We observed that in some cases MuDII*lac*1734 insertions induced deletions in the cosmid, whereas Tn*5lac* did not. We marker-exchanged (Boucher *et al.* 1987) miniphage and transposon insertions as well as three of the deletions generated by MuDII*lac*1734 into the genome of strain GMI1000. For each mutant, the exchange was verified by Southern hybridization. The position of each insertion and deletion marker-exchanged in the genomic region corresponding to the pD1D4 insert is presented Figure 2.

The pathogenicity of each mutant was tested on tomato plants (cv. Marmande) that were 15 to 20 cm tall and grown in a soil-peat mixture in 5-cm-diameter pots. Inoculation was performed by watering each pot with 50 ml of a suspension containing 5×10^8 *P. solanacearum* cells. Inoculated plants were placed in a growth chamber (Boucher *et al.* 1985). Each strain was tested in triplicate on sets of six plants, and disease development was scored daily using a disease index ranging from 0, no leaves wilted, to 4, 76 to 100% of leaves wilted (Roberts *et al.* 1988). The cumulative disease index (CDI) of the six plants inoculated with each mutant was calculated.

The tests distinguished three classes of mutants as illustrated in Figure 3:

1) Mutants that were not affected in pathogenicity, giving a CDI rating similar to the one induced by the wild-type strain. Tomato plants inoculated with these mutants were completely wilted 16 to 18 days after inoculation and reached the maximal CDI value of 24.

2) Deletion mutants MD34 and MD82 that, like the original mutant GMI1299, failed to induce any symptoms.

3) Mutants that induced delayed wilting of a limited number of plants, with the others remaining symptomless. Plants inoculated with such mutants reached CDI values ranging from 7 to 14, 17 days after the inoculation.

All of the insertions that affected pathogenicity, including the Tn*5* insertion of the original mutant GMI1299, are clustered on a stretch of DNA of approximately 15 kilobases (Fig. 2). However, all of the Tn*5lac* and MuDII*lac*1734 insertions in this region induced a hypoaggressive phenotype on tomato plants, while the original Tn*5* mutant, GMI1299, induced no symptoms. The only other nonpathogenic mutants carried deletions D34 and D82, which remove either all or the right part of the region controlling pathogenicity (Fig. 2).

None of the mutants generated in this region (including mutants GMI1299, MD34, and MD82) were affected in their HR-inducing ability on tobacco (data not shown). The DNA region was thus considered to be a *dsp* rather than a *hrp* region.

All of the mutants affected in the region corresponding to pD1D4 were prototrophic. The generation times in minimal (MM) and rich (B) media (Boucher *et al.* 1985) of five randomly chosen insertion mutants, of mutant GMI1299, and of deletion mutant MD73 were indistinguishable as compared to that of the wild-type strain (data not shown), suggesting that the decreased aggressiveness of the Dsp$^-$ mutants was not due to an alteration in their growth rate. However, deletion mutants MD34 and MD82 showed a significant decrease in their growth rates in both B and MM media. The doubling times in B and MM media were, respectively, 1.6 and 4.7 hr for the wild-type strain and 2.7 and more than 8 hr for the two mutants. The nonpathogenic phenotype of these two mutants might result from the drastic alteration in their growth rate. However, the nonpathogenic phenotype of mutant GM1299 cannot be explained by the same hypothesis, because the growth rate of this mutant *in vitro* is indistinguishable from that of the wild-type strain.

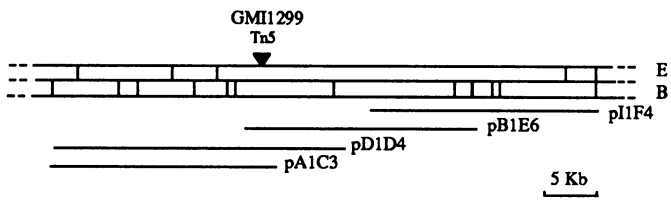

Fig. 1. Physical map of the region flanking the insertion site of Tn*5* (▼) in mutant GMI1299 and representation of genomic inserts carried by cosmids pD1D4, pA1C3, pB1E6, and pI1F4. E and B show the recognition sites for *Eco*RI and *Bam*HI, respectively.

The phenotype on tomato plants of our Dsp$^-$ mutants is similar to that of *P. solanacearum* endoglucanase- and polygalacturonase-deficient mutants (Roberts *et al.* 1988; Schell *et al.* 1988). Since strain GMI1000 produces such enzymes, production of both activities by the mutants was assayed on plates as described by Boccara *et al.* (1988) and by Boyer *et al.* (1987). None of the Dsp$^-$ mutants was impaired in the production of these activities (data not shown).

In additional experiments, all of the Dsp$^-$ mutants were shown to be sensitive to low pH since contrary to their wild-type parent and to derivatives carrying insertions outside of the *dsp* region, they failed to grow on MM plates adjusted to pH 4.5 with HCl (results not shown). Furthermore, whereas a shift in pH from 6.5 to 5.5 in MM medium buffered with 50 mM 2-(*N*-morpholino) ethanesulfonic acid resulted in an increase of threefold to fourfold in the generation times of the Dsp$^-$ mutants, it only slightly affected the wild-type strain (1.5-fold increase). It is possible that the *dsp* genes encode products which regulate the growth of *P. solanacearum* under acidic conditions. They could constitute or control some general metabolic pathway(s). This is consistent with the fact that deletion mutants MD34 and MD82 have a reduced growth rate. The *dsp* genes might not directly control pathogenicity, but during the infection process the gene products may be needed for the pathogen to adapt to the new environment in the plant tissues. It is possible that the sensitivity to acidic pH of the Dsp$^-$ mutants reduces their growth rate in some parts of the host plant. This could then affect the efficiency of infection or abolish pathogenicity, as is the case for mutant GMI1299, if the altered gene control is a key function. This hypothesis is in agreement with the fact that mutant GMI1299 is still able to invade the host plant but at a significantly lower level than the wild-type strain (Trigalet and Demery 1986). The *dsp* genes characterized in this work, like the *egl* and *pglA* genes, might modulate the time course of the early events in the infection

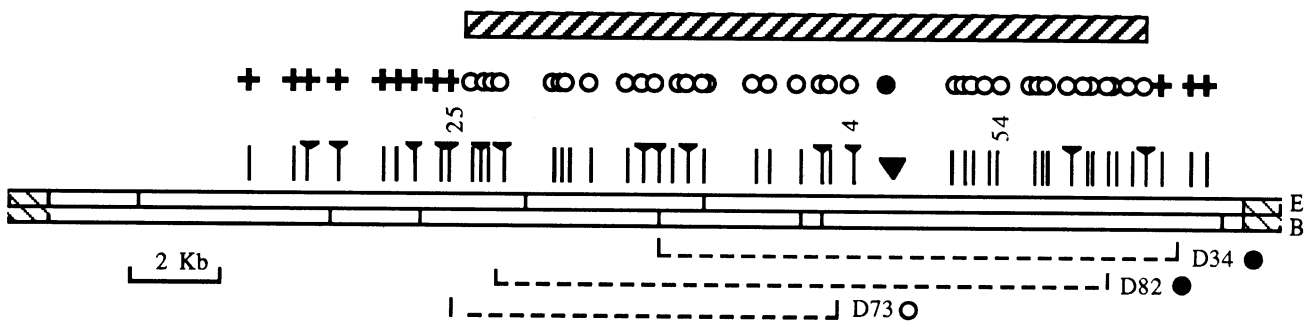

Fig. 2. Localization of Tn*5lac* (|) and MuDII*lac*1734 (⍑) insertions and induced deletions marker-exchanged into the genome of GMI1000 and their corresponding phenotypes on tomato plants. The numbers above some of the insertions refer to mutants for which behavior on tomato is illustrated in Figure 3. The dotted lines below the map show regions that are deleted in mutants MD34, MD82, and MD73. The triangle indicates the site of insertion of Tn*5* in mutant GMI1299. Pathogenicity is indicated by the following symbols: +, wild-type reaction; ●, no visible plant reaction; and ○, hypoaggressive phenotype. The hatched bar shows the cluster of *dsp* genes.

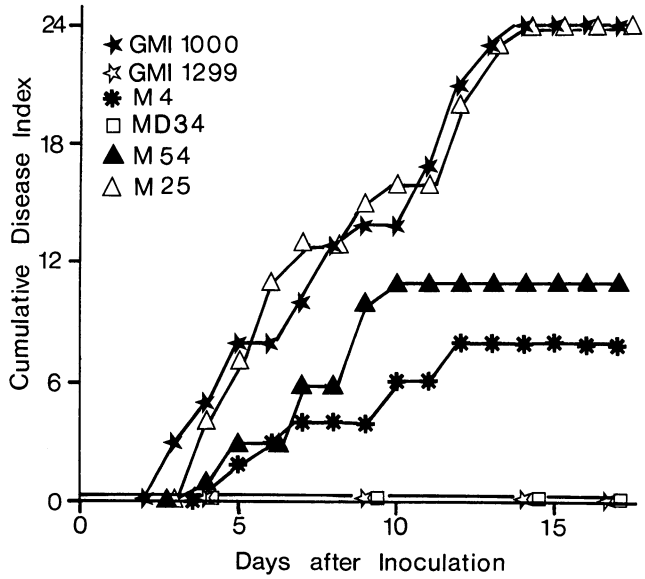

Fig. 3. Cumulative disease index following inoculation of sets of six tomato plants with wild-type strain GMI1000 and with representative mutants.

process and might be important factors in the successful invasion of plants, leading to disease development before the plant has time to build up induced resistance.

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LITERATURE CITED

Boccara, M., Diolez, A., Rouve, M., and Kotoujansky, A. 1988. The role of individual pectate lyases of *Erwinia chrysanthemi* strain 3937 in pathogenicity on saintpaulia plants. Physiol. Mol. Plant Pathol. 33:95-104.

Boucher, C., Barberis, P., Trigalet, A., and Demery, D. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn*5*-induced avirulent mutants. J. Gen. Microbiol. 131:2449-2457.

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischeck, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. 169:5626-5632.

Boyer, M.-H., Cami, B., Chambost, J.-P., Magnan, M., and Cattanèo, J. 1987. Characterization of a new endoglucanase from *Erwinia chrysanthemi*. Eur. J. Biochem. 162:311-316.

Buddenhagen, I. W., and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 2:203-230.

Castilho, B. A., Olfson, P., and Casadaban, M. J. 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposon. J. Bacteriol. 158:488-495.

Kroos, L., and Kaiser, D. 1984. Construction of Tn*5*-*lac*, a transposon that fuses the *lacZ* expression to exogenous promoters, and its introduction in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA 81:5816-5820.

Roberts, D. P., Denny, T. P., and Schell, M. A. 1988. Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in pathogenicity. J. Bacteriol. 170:1445-1451.

Schell, M. A., Roberts, D. P., and Denny, T. P. 1988. Analysis of the *Pseudomonas solanacearum* polygalacturonase encoded by *pglA* and its involvement in pathogenicity. J. Bacteriol. 170:4501-4508.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Trigalet, A., and Demery, D. 1986. Invasiveness in tomato plants of Tn*5*-induced avirulent mutants of *Pseudomonas solanacearum*. Physiol. Mol. Plant Pathol. 28:423-430.