The *exbD2* gene as well as the iron-uptake genes *tonB*, *exbB* and *exbD1* of *Xanthomonas campestris* pv. *campestris* are essential for the induction of a hypersensitive response on pepper (*Capsicum annuum*)

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The *tonB*, *exbB* and *exbD1* genes of *Xanthomonas campestris* pv. *campestris* are essential for ferric iron uptake. In contrast, the *exbD2* gene located in the same gene cluster is not essential. Mutational analysis revealed that the ferric-iron-uptake genes *tonB*, *exbB* and *exbD1* are necessary for the induction of a hypersensitive response (HR) on the nonhost plant pepper (*Capsicum annuum*) and the induction of typical black rot symptoms on the host plant cauliflower (*Brassica oleracea*). Again, the *exbD2* gene behaved differently. It was found to play a role only in the induction of the HR in pepper but not in the induction of black rot symptoms in cauliflower. Due to the low iron concentration in the plant tissue, the titre of viable bacteria of the ferric-iron-uptake mutants *tonB*, *exbB* and *exbD1* decreased after leaf infiltration of pepper. The *exbD2* mutant, however, which is not impaired in ferric iron uptake, multiplied in the pepper leaf tissue and grew even better than the wild-type strain, probably due to its failure to induce the HR. Nevertheless, the *tonB*, *exbB* and *exbD1* mutant strains were able to spread systemically in cauliflower.

Keywords: *Xanthomonas campestris*, *Capsicum annuum*, *tonB*, *exbB*, *exbD*

INTRODUCTION

Xanthomonas campestris pv. *campestris* is the causal agent of black rot disease of crucifers (Williams, 1980; Swings & Civerolo, 1993). After infection of a plant with *X. campestris* pv. *campestris*, two different types of reactions can be observed depending on the susceptibility of the particular plant. If the plant is susceptible, the infection gives rise to blackening of leaf veins, followed by drying and death of the leaf tissue (compatible interaction). In contrast, a hypersensitive response (HR) is induced in a resistant plant or a nonhost plant (incompatible interaction). The HR is a local defence response characterized by rapid tissue necrosis in the inoculated area (Klement, 1982). The *X. campestris* pv. *campestris* genes identified so far are of different importance in compatible and incompatible

interactions. Genes involved in protein secretion, production of protease, endoglucanase and polygalacturonate lyase (reviewed by Dow & Daniels, 1994) and exopolysaccharide (EPS) synthesis (reviewed by Sutherland, 1993) have been identified. Mutants defective in the production of only one of these extracellular enzymes or EPS were still able to cause black rot. Therefore, the exact role of degradative enzymes and of EPS production in pathogenicity is unclear. Regulatory mutants that lack both extracellular enzymes and EPS do not induce disease symptoms on host plants, whereas the induction of the HR in nonhosts is not affected (Daniels *et al.*, 1992).

The *hrp* genes (hypersensitive response and pathogenicity) are essential for virulence on host plants and for induction of a HR on nonhost plants. *hrp* genes have been identified in *Pseudomonas syringae* spp., *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*), *Erwinia amylovora* and *X. campestris* pathovars (Bonas, 1994; Willis *et al.*, 1991). Several *hrp* gene

Abbreviation: HR, hypersensitive response.

products show sequence homology to proteins mediating the type III secretion of virulence factors from animal bacterial pathogens and are therefore predicted to be involved in the secretion of molecules essential for the interaction with the plant (Fenselau *et al.*, 1992; Fenselau & Bonas, 1995; Wengelnik *et al.*, 1996). Hrp-dependent secretion has been demonstrated for the harpin proteins of *E. amylovora*, *Erwinia chrysanthemi* and *P. syringae* pv. *syringae* and the PopA protein of *R. solanacearum* (Arlat *et al.*, 1994; He *et al.*, 1993; Wei *et al.*, 1992).

Over 30 avirulence (*avr*) genes have been identified as essential for the HR in host and nonhost plants. In a gene-for-gene relationship, avirulence genes confer cultivar-specific interactions on resistant hosts as well as on nonhost plants (Dangl, 1994; Ritter & Dangl, 1995). Members of the avirulence/pathogenicity gene family were shown to encode functional nuclear localization signals (Yang & Gabriel, 1995). These proteins are therefore proposed to be translocated into the plant nucleus. Recently, the AvrBs3 protein from *X. campestris* pv. *vesicatoria* was shown to be recognized inside the plant cell and to require functional nuclear localization signals (Van den Ackerveken *et al.*, 1996).

Moreover, iron is considered to play a crucial role in plant-phytopathogen interactions (Expert *et al.*, 1996; Loper & Buyer, 1991). Iron is one of the factors that limits bacterial growth *in planta* because micromolar concentrations of iron are necessary to support growth and multiplication. The common strategy of bacteria to overcome this problem is the utilization of siderophores with high affinity for ferric iron (Raymond *et al.*, 1989). Ferric iron is complexed by these chelators, which are transported into the cell via an energy-coupled process mediated by the TonB system (reviewed by Braun, 1995). Outer-membrane receptors and the cytoplasmic membrane proteins TonB and ExbBD are involved in this process. In current models, the TonB protein induces a conformational change of the receptor proteins, which causes the release of bound ligands into the periplasmic space. Since the translocation into the periplasm is energy-dependent, TonB is considered to transduce the cytoplasmic membrane proton force to the receptor proteins (Bradbeer, 1993). Two auxiliary proteins, ExbB and ExbD, forming a complex with TonB, are necessary for TonB-dependent uptake. In addition to import processes across the outer membrane, the TonB system mediates signal transduction from the cell surface to the cytoplasm. This was shown for the induction of transcription of ferric citrate and pseudobactin-transport genes of *Escherichia coli* and *Pseudomonas putida*, respectively (Härle *et al.*, 1995; Koster *et al.*, 1994).

Recently, we identified the *tonB*, *exbB* and *exbD1* genes of *X. campestris* pv. *campestris*, which are essential for ferric iron uptake. In contrast to all bacteria investigated for TonB systems so far, a second *exbD* gene was identified in *X. campestris* pv. *campestris* (Wiggerich *et al.*, 1997).

The deduced amino acid sequences of the *exbD1* and *exbD2* genes showed 38.2% identity and 71.3% similarity to each other. Interestingly, we were able to show that *exbD2* mutants are not impaired in ferric iron uptake. Moreover, the ExbD2 protein cannot substitute the ExbD1 protein in ferric-iron-siderophore uptake (Wiggerich *et al.*, 1997). All four genes, *tonB*, *exbB*, *exbD1* and *exbD2*, were found to be clustered in *X. campestris* pv. *campestris*. Due to low coding probability and the wide spread of translational termination codons in all three reading phases, no other open reading frames were identified downstream of *exbD2*. Each gene was predicted to have its individual promoter as could be deduced from the analysis of several strains with insertional mutations (Wiggerich *et al.*, 1997). In the current paper, we report that the *tonB*, *exbB*, *exbD1* and *exbD2* genes are necessary for a HR on the nonhost plant pepper.

METHODS

Bacteria and plasmids. The strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown in Luria broth (LB) medium (Maniatis *et al.*, 1982) at 37 °C. *X. campestris* pv. *campestris* strains were grown in tryptone-yeast (TY) medium (Aguilar *et al.*, 1985) at 30 °C. FeSO₄ was added to a final concentration of 100 µM if needed. Solid media contained agar at 10 g l⁻¹. Swarm agar plates were made with 0.3 g agar l⁻¹. The final concentrations of antibiotics were: ampicillin (Ap), 200 µg ml⁻¹ (*E. coli*); kanamycin (Km), 50 µg ml⁻¹ (*X. campestris* pv. *campestris*); gentamicin (Gm), 10 µg ml⁻¹ (*E. coli*) or 30 µg ml⁻¹ (*X. campestris* pv. *campestris*); and streptomycin (Sm), 800 µg ml⁻¹ (*X. campestris* pv. *campestris*).

DNA methods, hybridization, transformation and electroporation. Plasmid DNA was isolated using a rapid boiling lysis technique (Arnold & Pühler, 1988). Total DNA was isolated by lysozyme/sarcosyl/proteinase K lysis, followed by phenol/chloroform extraction and 2-propanol precipitation (Meade *et al.*, 1982). Restriction enzymes and other commercially available enzymes were obtained from Pharmacia or BRL and used in accordance with the manufacturer's recommendations. Restriction-endonuclease-generated fragments of total DNA were separated by gel electrophoresis and subsequently vacuum blotted on nylon filters as described by Simon *et al.* (1991). Labelling of the probe (vector and DNA region) and hybridization were done as described elsewhere (Simon *et al.*, 1991). Transformation of *E. coli* cells and electroporation of *X. campestris* pv. *campestris* with plasmid DNA were performed as described by Morrison (1977) and Wiggerich *et al.* (1997), respectively. Agarose gel electrophoresis was carried out as described by Maniatis *et al.* (1982).

Construction of plasmids. Plasmids pHGW100 and pHGW31 were described previously (Wiggerich *et al.*, 1997). Plasmids pHGW241, pHGW242 and pHGW243 harboured the *tonB*, *exbB* and *exbD1* gene of *X. campestris* pv. *campestris*, respectively (Wiggerich *et al.*, 1997). A 1.3 kb *Bam*HI-*Xba*I

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>E. coli</i>		
294	<i>thi pro hsdR⁻</i> , Pro	Bachmann <i>et al.</i> (1976)
<i>Xanthomonas campestris</i>		
B100	DSM 1526, Sm ^r	Hötte <i>et al.</i> (1990)
B100-5.05	<i>tonB</i> , omegon-Km(<i>cat</i>)-induced mutant	Wiggerich <i>et al.</i> (1997)
B100-6.01	omegon-Km(<i>cat</i>)-induced mutant	Wiggerich <i>et al.</i> (1997)
B100-7.03	<i>exbB</i> , omegon-Km(<i>cat</i>)-induced mutant	Wiggerich <i>et al.</i> (1997)
B100-9.01	<i>exbD1</i> , omegon-Km(<i>cat</i>)-induced mutant	Wiggerich <i>et al.</i> (1997)
B100-9.21	<i>exbD1</i> , <i>aacC1</i> -induced mutant	Wiggerich <i>et al.</i> (1997)
B100-11.03	<i>exbD2</i> , omegon-Km(<i>cat</i>)-induced mutant	Wiggerich <i>et al.</i> (1997)
Plasmids		
pHGW31	pHIP1 <i>aacC1</i> Δ BglIII carrying the multiple cloning site of pSVB30	Wiggerich <i>et al.</i> (1997)
pHGW100	pSVB30, Ap ^r , 3.6 kb <i>HindIII</i> – <i>SmaI</i> wild-type fragment	Wiggerich <i>et al.</i> (1997)
pHGW241	pHGW31, Gm ^r , <i>tonB</i> _{Xcc}	Wiggerich <i>et al.</i> (1997)
pHGW242	pHGW31, Gm ^r , <i>exbB</i> _{Xcc}	Wiggerich <i>et al.</i> (1997)
pHGW243	pHGW31, Gm ^r , <i>exbD1</i> _{Xcc}	Wiggerich <i>et al.</i> (1997)
pHGW244	pHGW31, Gm ^r , <i>exbD2</i> _{Xcc}	This work

Fig. 1. (a) Genetic and physical map of the 3.6 kb *HindIII*–*SmaI* *X. campestris* pv. *campestris* DNA fragment involved in the uptake of ferric iron. A detailed restriction map is given in Wiggerich *et al.* (1997). The locations of *tonB*, *exbB*, *exbD1* and *exbD2* are indicated below the restriction map. (b) Subfragment cloned for complementation of the *X. campestris* *exbD2* mutant. Open flags indicate the orientation of the *cat* gene of the omegon-Km(*cat*) insertions; a filled flag indicates the orientation of the *aacC1* gene insertion. (i) +, Typical HR; –, no HR; i, intermediate HR. (ii) +, Typical black rot symptoms; –, single spots of dried leaf tissue. (iii) +, Growth; –, no growth (Wiggerich *et al.*, 1997). (iv) +, Siderophore production; ++ and +++, enhanced siderophore production (Wiggerich *et al.*, 1997).

subfragment of pHGW100 carrying the *exbD2* gene of *X. campestris* pv. *campestris* resulted from nested deletions with exonuclease III and S1 nuclease (nested deletion kit from

Pharmacia). This 1.3 kb *BamHI*–*XbaI* DNA fragment was cloned into pHGW31 and the resulting plasmid was designated pHGW244 (Fig. 1).

HR test. The HR-inducing ability of *X. campestris* pv. *campestris* strains was tested on *Capsicum annuum* cultivars California Wonder, Yolo Wonder and Pusztagold (pepper) as described elsewhere (Arlat *et al.*, 1991; Conrads-Strauch *et al.*, 1990). Seedlings were individually planted into flowerpots filled with sterile vermiculite. Once a week the plants were watered with MS medium (Sigma-Aldrich). After 6 weeks growth in a growth room at 25 °C and with illumination for 16 h d^{–1} the plants were inoculated by infiltrating approximately 10 µl bacterial culture (1 × 10⁸ cells ml^{–1}) suspended in sterile water into the leaf tissue with a plastic syringe. For the first 2 d after inoculation the plants were set under high humidity by using closed, transparent chambers. For the rest of the incubation time the plants were maintained under growth room conditions. The growth of bacteria in pepper leaf tissue was studied by infiltrating groups of plants with approximately 100 µl of each bacterial suspension containing 10⁷ cells ml^{–1}. For a period of 10 d, at 1 d intervals, 0.95 cm² discs of infiltrated plant tissue were taken and homogenized in 0.5 ml sterile water. At each time interval discs of five different plants were studied. Suitable dilutions of the homogenates were plated on streptomycin- and kanamycin-supplemented agar. Plates were incubated for 2 d at 30 °C. Bacterial titres determined were related to 1 cm² tissue.

Pathogenicity test. *Brassica oleracea* cultivar Braunschweiger (cauliflower) was used for inoculation of mature host plants. The plants were maintained under growth room conditions described for the HR test. The plants were inoculated by injecting approximately 10 µl bacterial culture (1 × 10⁹ cells ml^{–1}) suspended in sterile water into the leaf margin. Symptom development was scored for 14 d after infection. Reisolation of bacteria from cauliflower leaf tissue was carried out as described for pepper.

B100-WT B100-7.03 *exbB*
 B100-5.05 *tonB* B100-9.01 *exbD1*
 B100-6.01 B100-11.03 *exbD2*

Fig. 2. Symptoms induced in pepper leaves (*Capsicum annuum* cv. California Wonder) by the wild-type strain *X. campestris* pv. *campestris* B100 and its *tonB*, *exbB*, *exbD1* and *exbD2* mutants. The *tonB*-*exbB* intergenic mutant B100-6.01 is described in Fig. 1. Approximately 1×10^6 cells suspended in sterile water were infiltrated into the leaf mesophyll tissue with a plastic syringe. Picture of the pepper leaf was taken 4 d after infiltration.

Fig. 3. Viable bacterial titre *in planta* determined by reisolation of the wild-type strain *X. campestris* pv. *campestris* B100 and its *tonB*, *exbB*, *exbD1* and *exbD2* mutants from infiltrated *Capsicum annuum* cv. California Wonder (pepper) leaves. □, B100-11.03 (*exbD2*); ◇, B100-6.01; ◆, B100 (wt); ○, B100-7.03 (*exbB*); ■, B100-9.01 (*exbD1*); △, B100-5.05 (*tonB*). Mutant strain B100-6.01 is described in Fig. 1. Approximately 1×10^6 cells suspended in sterile water were infiltrated into the leaf mesophyll tissue with a plastic syringe. The first value (0 d) is represented by viable bacterial titre 0.5 h after infiltration of pepper leaf tissue. The following values were obtained by daily reisolation of bacteria of infected tissue. Each value represents the mean of five samples taken. Data shown represent the results of one out of three independent experiments. Within these experiments, values only differed in a range of 10%.

Fig. 4. Symptoms evoked by *X. campestris* pv. *campestris* B100 (a) and *X. campestris* pv. *campestris* mutants B100-11.03 (b) and B100-5.05 (c) on the host plant cauliflower (*Brassica oleracea*) 14 d after infection. Suspensions of the bacteria were injected into the petioles at the leaf margins. Whereas *X. campestris* pv. *campestris* mutant B100-11.03 (b) induced the same symptoms of black rot as the wild-type B100 (a), the *X. campestris* pv. *campestris* mutant B100-5.05 (c) causes only small spots of dried leaf tissue.

RESULTS AND DISCUSSION

X. campestris pv. *campestris* *tonB*, *exbB*, *exbD1* and *exbD2* mutants do not elicit a HR on pepper

Previously, we identified the *tonB*, *exbB*, *exbD1* and *exbD2* genes encoding the central structure of the TonB system on a 3.6 kb *HindIII*–*SmaI* DNA fragment of the *X. campestris* pv. *campestris* wild-type strain B100 (Fig. 1). Mutations in the *tonB*, *exbB* and *exbD1* genes, but not in the *exbD2* gene, affected ferric iron uptake *ex planta* (Wiggerich *et al.*, 1997). Since the acquisition of iron is considered to play a crucial role in plant–phytopathogen interactions (Expert *et al.*, 1996; Loper & Buyer, 1991), we analysed whether the *tonB/exb* gene region also contributes to the induction of a HR on nonhost plants. Therefore, the *X. campestris* pv. *campestris* mutants B100-5.05 (*tonB*), B100-7.03 (*exbB*), B100-9.01 (*exbD1*), B100-11.03 (*exbD2*) and B100-6.01 with an omegon-Km(*cat*) insertion in the intergenic region between *tonB* and *exbB* (Fig. 1) were infiltrated into the leaves of *C. annuum* cv. California Wonder (pepper). The results are presented in Fig. 2. Within 48 h the HR induced by the wild-type strain *X. campestris* pv. *campestris* B100 resulted in the formation of typical dark, parchment-like necrotic lesions. The affected dark mesophyll tissue was strictly separated from the unaffected green tissue. In contrast to the *X. campestris* pv. *campestris* wild-type strain B100, none of the mutant strains induced these typical HR symptoms. Only slight chlorosis occurred in the infected leaf tissue infiltrated with the *tonB*, *exbB*, *exbD1* or *exbD2* mutants. An HR with strongly reduced symptoms was induced by the intergenic mutant B100-6.01. In this case, the induced HR was characterized by the formation of yellow to brown lesions of the infiltrated mesophyll tissue. The symptoms induced by all mutants mentioned did not develop to wild-type symptoms during longer infection periods (data not shown).

Although typical characteristics of an HR induced by the wild-type strain B100 occurred within 48 h, the photograph presented in Fig. 2 was taken after 96 h to reach a better differentiation of the symptoms induced by the mutant strains.

The HR-inducing abilities of the mutant strains were also tested on the pepper cultivars Yolo Wonder and Pusztagold. The types of reaction were identical to those with cultivar California Wonder (data not shown).

To confirm that all effects observed were due to the insertions located in the genes investigated, we tested the *tonB*, *exbB*, *exbD1* and *exbD2* mutants complemented with a copy of the particular wild-type gene for their ability to induce an HR. To complement the *exbD2* mutant B100-11.03, the suicide vector pHGW244 (*exbD2*), carrying the corresponding subfragment of the 3.6 kb *HindIII*–*SmaI* wild-type DNA, was constructed and integrated into the genome by a single-crossover event. The correct integration was confirmed by Southern hybridization (data not shown). Mutants B100-5.05::pHG241 (*tonB*), B100-7.03::pHG242

(*exbB*) and B100-9.01::pHG243 (*exbD1*) were described previously (Wiggerich *et al.*, 1997). Each mero-diploid mutant regained the ability to induce wild-type HR symptoms when infiltrated into pepper leaves. However, symptom formation was delayed by 2 d.

To rule out the possibility that the inability of the *exbD1* mutant B100-9.01 to induce an HR was due to the lack of both ExbD1 and ExbD2 proteins, we tested strain B100-9.21 carrying an *aacC1* gene in the *exbD1* gene bringing the *exbD2* gene under the control of the constitutively active P_{out} promoter of the *aacC1* gene (Wiggerich *et al.*, 1997). Like strain B100-9.01 mutant strain B100-9.21 induced only slight chlorosis in the infected leaf tissue (data not shown).

The *X. campestris* pv. *campestris* *tonB*, *exbB* and *exbD1* mutants died rapidly, but the *exbD2* mutant grew even better than the wild-type strain in pepper leaf tissue

As has been shown previously, the TonB system of *X. campestris* pv. *campestris* is involved in ferric iron uptake *ex planta* (Wiggerich *et al.*, 1997). Therefore, the investigated mutant strains could have failed to acquire iron *in planta*, resulting in a lack of growth. To analyse if this was the reason why the *tonB/exbBD1D2* mutant strains failed to induce an HR on pepper we scored the viable bacteria by reisolation from infiltrated pepper leaf tissue for the first 2 d after infection (Fig. 3). Surprisingly, the mutants behaved differently. During the first 4 d after infiltration the bacterial titre of the wild-type strain B100 increased from 1×10^6 bacteria initially infiltrated to 7×10^7 bacteria per cm² tissue. As expected, within the next 4 d, the bacterial titre decreased rapidly to a value of 5×10^6 cells per cm². In contrast, the viable bacterial titre of the mutant strains B100-5.05 (*tonB*), B100-7.03 (*exbB*) and B100-9.01 (*exbD1*) decreased immediately after infiltration from a value of 1×10^6 bacteria per cm² tissue to values two to three orders of magnitude lower. Not only were these mutants unable to grow in the pepper leaf tissue, they even died rapidly. Since these mutant strains were also found to grow only on medium supplemented with ferrous iron (Wiggerich *et al.*, 1997), they were considered to be unable to acquire iron *in planta*, thus explaining the absence of growth. The decreasing number of bacteria seems to be the reason that pepper did not establish an HR. An unexpected result was obtained with the *exbD2* mutant B100-11.03. The bacterial titre of this mutant strain increased during the first 4 d after infiltration, as did the titre of the wild-type strain. However, in contrast to the viable bacterial titre of the wild-type strain B100, the bacterial titre of the *exbD2* mutant B100-11.03 did not decrease at all. Instead, the bacteria multiplied to a titre of 9×10^7 cells per cm² during the following 6 d. Similarly to the *ex planta* state (Wiggerich *et al.*, 1997), this mutant was probably able to acquire iron *in planta*. Interestingly, despite its higher titre, the *exbD2* mutant strain did not induce HR symptoms on pepper, indicating the dis-

turbance of the interaction between this mutant and the nonhost plant pepper. The bacterial titre of the intergenic mutant B100-6.01 increased during the first 5 d to 5×10^7 bacteria per cm² tissue, then decreased to a value of 2×10^7 bacteria per cm² tissue. The intergenic mutant B100-6.01, which is not affected in iron import (Wiggerich *et al.*, 1997), induced an HR with strongly reduced symptom formation. In accordance we found that this strain could multiply for a longer period of time in infected pepper leaf tissue than the wild-type strain. However, even this reduced HR led to a decreasing number of viable bacteria. Although the insertional mutation in strain B100-6.01 is intergenic, this mutation affected the interaction between this mutant strain and pepper. A reason for this effect can be the disturbance of a common regulation of the *tonB/exbBD1D2* genes. Since the complemented mutant strains were in a merodiploid state, this regulation may also be affected in the complemented strains, resulting in a delay of symptom formation of 2 d. We conclude that the recognition between *X. campestris* pv. *campestris* and its nonhost plant pepper is disturbed in mutant strains with an ineffective TonB system independently of their capacity to import iron.

It appeared that the wild-type strain B100 and all mutant strains were restricted to the infiltrated tissue.

The production of ferric-siderophores has been suggested as an elicitor of plant defence reactions (Expert *et al.*, 1996). Chrysobactin, a siderophore produced by *E. chrysanthemi*, has been identified as a virulence factor in plant disease (Enard *et al.*, 1988). Since the *X. campestris* pv. *campestris* *tonB*, *exbB* and *exbD1* mutant strains exhibited an increased siderophore production (Wiggerich *et al.*, 1997), the siderophore of *X. campestris* pv. *campestris* cannot be the elicitor of HR induction in pepper. Additional work is necessary to determine whether signal molecules are recognized or imported via the TonB system in *X. campestris* pv. *campestris*.

The *X. campestris* pv. *campestris* *exbD2* mutant, but not the *tonB*, *exbB* and *exbD1* mutants, induced typical black rot symptoms on the host plant cauliflower

Since the *exbD2* mutant strain B100-11.03 was able to grow in pepper, we wanted to know whether this strain was also affected in the interaction with host plants. Therefore, the *tonB*, *exbB*, *exbD1* and *exbD2* mutant strains and the wild-type strain B100 were injected into leaf margins of *B. oleracea* (cauliflower). Symptoms caused by the wild-type strain were blackening of the leaf veins followed by sectorial drying and death of the leaf tissue (Fig. 4a). The iron-uptake genes *tonB*, *exbB* and *exbD1* are therefore not only necessary for the interaction with nonhost plants but also with the host plant cauliflower, because strains carrying a mutation in one of these genes no longer induced the typical black rot symptoms. Since the sectorial drying of the leaf tissue is missing, the blockage of veins, which is commonly

considered to be the reason for death of leaf tissue, did not occur. The symptoms caused by the *exbD2* mutant strain were identical to those induced by the wild-type strain (Fig. 4b). Only a slight delay of symptom formation during the first 2 d after injection could be detected (data not shown).

Interestingly, the *tonB*, *exbB* and *exbD1* mutants caused symptoms which were completely different from those observed for the wild-type strain. In all leaves infected with the mutant strains blackening of the veins did not occur, even after 14 d following infection. Also sectorial drying of the leaf tissue was missing. Instead, small single spots of dried leaf tissue occurred on parts of the leaf, as shown in Fig. 4(c) for the *tonB* mutant strain B100-5.05. Reisolation experiments revealed that the *tonB*, *exbB*, *exbD1* and *exbD2* mutants and the wild-type strain had spread systemically over the whole leaves (data not shown). This means that they can multiply in *B. oleracea*. Therefore, these strains had to be able to utilize ferrous iron provided by the host plant.

The *exbD2* gene of *X. campestris* pv. *campestris* increases the known scope of the TonB system function

The TonB system of Gram-negative bacteria is not only involved in ferric-iron-siderophore uptake but also serves as the central structure of the import pathways of several macromolecules (Postle, 1990; Braun, 1995). Moreover, the TonB system and related components normally used for iron uptake were shown to mediate the induction of transcription of ferric citrate and pseudobactin transport genes from the cell surface in *E. coli* and *P. putida*, respectively (Härle *et al.*, 1995; Koster *et al.*, 1994). In *X. campestris* pv. *campestris*, a second ExbD protein (ExbD2) is present, which cannot substitute for the ExbD1 protein in ferric iron uptake (Wiggerich *et al.*, 1997). We have demonstrated here that this ExbD2 protein was found to be involved in the induction of an HR in pepper. To our knowledge, the existence of a second ExbD protein has not been reported for other Gram-negative bacteria so far. With respect to the results published here it would be interesting to know whether a second *exbD2* gene is present in other HR-inducing bacteria. Mutational analysis revealed that although the *tonB*, *exbB*, *exbD1* and *exbD2* genes are likely to form one operon, the *exbD1* and *exbD2* genes can be independently expressed from the *tonB* and *exbB* genes (Wiggerich *et al.*, 1997). Since *tonB*, *exbB* and *exbD1* mutant strains, like the *exbD2* mutant strain, were unable to induce an HR on pepper, one can speculate that the ExbD2 protein interacts with the central structure of the TonB system, consisting of the TonB, ExbB and ExbD1 proteins, to increase the scope of the TonB system. Further investigations of the TonB system might give new insights into the signal exchange between phytopathogenic bacteria and plants.

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