Regulation and secretion of Xanthomonas virulence factors

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Abstract

Plant pathogenic bacteria of the genus Xanthomonas cause a variety of diseases in economically important monocotyledonous and dicotyledonous crop plants worldwide. Successful infection and bacterial multiplication in the host tissue often depend on the virulence factors secreted including adhesins, polysaccharides, LPS and degradative enzymes. One of the key pathogenicity factors is the type III secretion system, which injects effector proteins into the host cell cytosol to manipulate plant cellular processes such as basal defense to the benefit of the pathogen. The coordinated expression of bacterial virulence factors is orchestrated by quorum-sensing pathways, multiple two-component systems and transcriptional regulators such as Clp, Zur, FhrR, HrpX and HpaR. Furthermore, virulence gene expression is post-transcriptionally controlled by the RNA-binding protein RsmA. In this review, we summarize the current knowledge on the infection strategies and regulatory networks controlling secreted virulence factors from Xanthomonas species.

Bacteria of the genus Xanthomonas are important plant pathogens

The genus Xanthomonas comprises an important ubiquitous group of Gram-negative plant pathogenic bacteria that belong to the Gamma subdivision of Proteobacteria. Xanthomonas species (spp.) are typically rod shaped with a single polar flagellum, are obligate aerobes and have an optimal growth temperature of 25–30 °C (Bradbury, 1984). Bacterial colonies are usually yellow due to the presence of the membrane-bound pigment xanthomonadin, which might protect the bacteria from photobiological damage (Starr & Stephens, 1964; Jenkins & Starr, 1982; Rajagopal et al., 1997).

Members of the genus Xanthomonas infect approximately 124 monocotyledonous and 268 dicotyledonous plants and are of economical importance in regions with a warm and humid climate (Leyns et al., 1984; Chan & Goodwin, 1999). The bacteria presumably persist as epiphytes on the plant surface before they enter the plant via natural openings such as hydathodes, stomata or wounds. Inside the plant tissue, Xanthomonas spp. multiply either locally in the intercellular space or colonize the xylem vessels and then spread systemically within the plant (Fig. 1). The bacteria are hemibiotic pathogens that initially feed on living host tissue, but in later infection stages, cause the death of plant cells. Plant pathogenic Xanthomonas spp. are evolutionarily related to the opportunistic pathogen Xanthomonas maltophilia, which was renamed Stenotrophomonas maltophilia. Stenotrophomonas maltophilia infects humans and is associated with nosocomial infections; however, some strains are endophytic (Denton & Kerr, 1998). Members of the genus Xanthomonas were originally grouped into separate species on the basis of their host range, but according to the classical nomenclature, most species were later merged into the single species Xanthomonas campestris and subgrouped into different pathovars (Dye & Lelliott, 1974; Starr, 1981). However, due to several taxonomic reclassifications, the nomenclature of the currently approximately 19 species and > 140 pathovars is still under debate (Schaad et al., 2000; Vauterin et al., 2000; Rademaker et al., 2005). In this article, we therefore use the classical nomenclature.

Identification of virulence factors from Xanthomonas spp.

A major goal of past and ongoing research in molecular plant pathology is the identification of bacterial virulence factors that contribute to the host–pathogen interaction. To establish themselves successfully in host plants, plant
pathogenic bacteria should be able to adhere to the plant surface, invade the intercellular space of the host tissue, acquire nutrients and counteract plant defense responses. Successful infection of host plants often depends on bacterial protein secretion systems that secrete proteins into the extracellular milieu or transport proteins and/or DNA directly into the host cell cytosol (Table 1; Fig. 2), a process that is hereafter referred to as translocation. Proteins that are translocated into the host cell are designated effector proteins. Screening of transposon insertion-mutant libraries and targeted mutant approaches showed that mutations affecting bacterial virulence often destroy the function of individual secreted proteins. Several pathogens use a combination of different protein secretion systems to ensure efficient bacterial multiplication and disease progression (Preston et al., 2005). Below, we will summarize the current knowledge on virulence factors from Xanthomonas spp. including bacterial surface structures and secreted proteins. We will also briefly describe the regulatory pathways underlying the control of virulence gene expression. It should be noted that in several cases, strain-specific differences in virulence mechanisms of Xanthomonas spp. have been reported and might reflect bacterial adaptations to specific host plants. We therefore emphasize that the virulence strategies described for individual pathovars and strains of Xanthomonas spp. do not necessarily apply to other bacteria of the genus.

Bacterial surface structures are important virulence factors

Extracellular polysaccharides (EPSs) protect bacteria against environmental stress

Xanthomonas spp. produce a characteristic EPS, xanthan, which leads to the mucoid appearance of the bacterial colonies. Xanthan is a polymer of repeating pentasaccharide units with a cellulose backbone and trisaccharide side chains and is commercially used as a thickening agent in nutritional and pharmaceutical industries (Jansson et al., 1975; Becker et al., 1998). The production of xanthan is directed by several genetic loci including the gum gene cluster, which consists of 12 genes (gumB to gumM) and is highly conserved among Xanthomonas spp. (Katzen et al., 1998; Vojnov et al., 1998). Because of its highly hydrated and anionic consistency, xanthan protects bacteria from environmental stresses (e.g. dehydration, toxic compounds). Furthermore, in vascular pathogens, xanthan might cause wilting of host plants by blocking the water flow in xylem vessels (Denny, 1995; Chan & Goodwin, 1999).

Published genome sequences:

| Xanthomonas spp. | Strain | Reference |
|------------------|--------|-----------|
| Xac              | 306    | Da Silva et al. (2002) |
| Xcc              | 8004   | Qian et al. (2005) |
|                  | ATCC 33913 | Da Silva et al. (2002) |
|                  | B100   | Vorhölt et al. (2008) |
| Xcv              | 85-10  | Thieme et al. (2005) |
| Xoo              | KACC10331 | Lee et al. (2007) |
|                  | MAFF311018 | Ochiai et al. (2005) |
|                  | PXO99A | Salzberg et al. (2008) |

Fig. 1. Model systems of Xanthomonas spp. Examples are given for Xanthomonas spp. with published genome sequences, and the corresponding host plants on which they cause disease. Xanthomonas campestris pv. campestris (Xcc) and Xanthomonas oryzae pv. oryzae (Xoo) spread systemically in the host plant, whereas Xanthomonas axonopodis pv. citri (Xac), X. campestris pv. vesicatoria (Xcv) and X. oryzae pv. oryzicola (Xoc) multiply locally at the infection site.
Kim et al., 2009b). Interestingly, gum genes from X. axonopodis pv. citri are dispensable for bacterial growth and disease symptom formation on Citrus sinensis, but contribute to bacterial virulence in Citrus limon, suggesting that the contribution of xanthan to virulence may depend on the host plant and on the environmental conditions (Dunger et al., 2007; Rigano et al., 2007).

Experimental evidence suggests that xanthan also suppresses basal plant defense responses such as callose deposition in the plant cell wall, presumably by chelation of divalent calcium ions that are present in the plant apoplast and are required for the activation of plant defense responses (Yun et al., 2006; Aslam et al., 2008). Furthermore, in X. campestris pv. campestris and X. axonopodis pv. citri, xanthan has been implicated in the formation of biofilms (Dow et al., 2003; Rigano et al., 2003; Torres et al., 2007). A biofilm is a bacterial population in which bacteria attach to each other or to biotic or abiotic surfaces and are embedded in an extracellular polymeric matrix that mainly consists of EPS, proteins and lipids (Sutherland, 2001; Branda et al., 2005). The formation of a biofilm presumably provides protection against antibiotics and host defense responses and might contribute to bacterial epiphytic survival before colonization of the plant intercellular space or to attachment of vascular bacteria to xylem vessels (Stoodley et al., 2002). However, the role of biofilm formation in the virulence of plant pathogenic bacteria has not yet been studied extensively.

The dual role of lipopolysaccharides in the host–pathogen crosstalk

Another surface-associated virulence factor of Xanthomonas spp. and other plant pathogenic bacteria are lipopolysaccharides (LPS), which are major components of the bacterial outer membrane and protect the cell from hostile environments. LPS which is unique for Gram-negative bacteria, is a tripartite molecule consisting of membrane-anchored lipid A, a core oligosaccharide and polysaccharide side chains (O-antigen) (Raetz & Whitfield, 2002). In X. campestris pv. campestris, the synthesis of LPS is directed by the wxc gene cluster, which comprises 15 genes (Vorhölter et al., 2001). Mutations in LPS gene clusters render the bacteria more susceptible against harsh environmental conditions (e.g. in the plant tissue) and might therefore lead to an attenuation of bacterial virulence as shown for X. campestris pv. campestris and X. campestris pv. citrulli (Kingsley et al., 1993; Dow et al., 1995; Newman et al., 2001).

Comparative sequence analysis revealed that LPS gene clusters of different Xanthomonas spp. are variable in number and identity of genes and were presumably subject to a strong diversifying selection (generation of multiple different alleles in different species, pathovars and even strains) (Lu et al., 2008). Variations in the composition of LPS might allow the bacteria to evade recognition by the plant’s immune system and presumably also affect bacterial resistance to phage adsorption and/or infection (Ojanen et al., 1993; Hung et al., 2002). Notably, LPS

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**Table 1.** Protein secretion systems from Gram-negative bacteria

| Protein secretion system | Description | Transport across inner membrane | Secreted proteins* | Reference |
|--------------------------|-------------|---------------------------------|-------------------|-----------|
| T1S system               | ABC transporter in IM, periplasm membrane fusion protein, OM channel | Sec-independent | Toxins, degradative enzymes | Gerlach & Hensel (2007) |
| T2S system               | At least 11 components in IM, periplasm and OM; predicted periplasmic pseudopilus | Mediated by Sec or TAT system | Toxins, degradative enzymes | Sandkvist (2001) |
| T3S system               | Evolutionary related to bacterial flagellum; at least 20 components in IM, periplasm and OM; extracellular pilus (plant pathogens) or needle (animal pathogens) | Sec-independent | Extracellular components of T3S system, effector proteins | Ghosh (2004) |
| T4S system†              | Evolutionary related to bacterial conjugation system; spans both bacterial membranes; extracellular pilus | Sec-independent | Extracellular components of T4S system; DNA and/or proteins | Juhas et al. (2008) |
| T5S system               | Protein channel in OM; autotransporters and two-partner secretion systems | Sec-dependent | e.g. adhesins | Gerlach & Hensel (2007) |
| T6S system               | Multicomponent secretion machinery, evolutionary related to phage tail-associated protein complexes | Presumably Sec-independent | Hcp and VgrG, which contains C-terminal actin crosslinking domain | Cascales (2008), Leiman et al. (2009), Wu et al. (2008) |

*T3S, T4S and T6S systems translocate DNA and/or proteins into eukaryotic cells.

†TAT, twin-arginine translocation. The TAT system transports folded proteins with a specific N-terminal secretion signal consisting of two arginine residues (Voulhoux et al., 2001).

Based on their genetic organization and evolutionary relationships, T4S systems were classified into T4AS systems resembling the VirB/VirD4 system and T4BS systems found in intracellular animal pathogenic bacteria. ABC, ATP-binding cassette; IM, inner membrane; OM, outer membrane.
not only act as virulence factors but also induce plant defense responses such as pathogenesis-related gene expression, oxidative burst and thickening of the plant cell wall (Dow et al., 2000; Meyer et al., 2001). Because LPS cannot diffuse through the plant plasma membrane, it is probably perceived by specific plant cell receptors that might be internalized into the plant cell by receptor-mediated endocytosis as was shown for LPS from X. campestris pv. campestris (Rothfield & Pearlman-Kothencz, 1968; Dow et al., 2000; Gross et al., 2005) (Fig. 3). Interestingly, it was reported that pretreatment of pepper leaves with LPS prevents the induction of the hypersensitive response (HR) by an avirulent X. campestris pv. vesicatoria strain, a phenomenon termed localized induced resistance (LIR) (Newman et al., 2000). The HR is a local plant cell death at the infection site and is triggered by individual effector proteins that are translocated by the type III secretion (T3S) system and are recognized in resistant plants that possess cognate resistance (R) genes (Jones & Dangl, 2006). So far, the mechanisms underlying LIR are not yet understood. It is possible that LPS-mediated induction of plant defense restricts bacterial growth or reduces the delivery of type III effectors due to modifications of the plant cell wall (Dow et al., 2000).

**Coming closer – adhesins are bacterial attachment devices**

Adhesion of bacteria to biotic surfaces is key for the invasion of the host tissue. Bacterial attachment depends on specific adhesins that are anchored in the bacterial outer membrane and are classified into fimbrial and nonfimbrial adhesins. Fimbrial adhesins are filamentous proteinaceous structures such as type IV pili, which are structurally related to the predicted periplasmic pilus of T2S systems (Gerlach & Hensel, 2007). Nonfimbrial adhesins include trimeric autotransporters of T5S systems (e.g. YadA from Yersinia spp.) and two-partner secretion substrates (e.g. filamentous hemagglutinin from Bordetella pertussis and YapH from Yersinia spp.) (Gerlach & Hensel, 2007). Bacterial adhesins bind to specific host surface receptors and have been studied intensively in animal pathogenic bacteria. Less is known about the virulence function of adhesins from plant pathogenic bacteria.
Fig. 3. Model of known virulence factors from Xanthomonas spp. Xanthomonas spp. depend on T2S and T3S systems, adhesins, EPS and lipopolysaccharides (LPS) to successfully interact with their host plants. LPS can be released from the bacterial surface and elicit plant defense responses. LPS and other PAMPs are presumably sensed by specific plant receptors that activate plant defense responses [PAMP-triggered immunity (PTI)]. PTI might also be triggered by plant cell wall degradation products that result from the action of degradative enzymes secreted by the T2S system. The T3S system, which translocates effector proteins into the host cell, is essential for bacterial pathogenicity. Effector proteins from Xanthomonas spp. with known localization and/or function in the plant and identified plant interaction partners are shown (see also Table 3). Suppression of PTI was demonstrated for XopN, which interacts with TARK1 and 14-3-3 proteins. Members of the AvrBs3/PthA family modulate host gene expression. The influence of AvrBs4 on host gene expression has not yet been analyzed, but it was shown that AvrBs4 induces catalase crystals in peroxisomes when transiently expressed in the plant. The predicted cysteine proteases XopD and members of the YopJ/AvrRxv family presumably remove SUMO from plant target proteins and/or suppress callose deposition in the plant cell wall as was shown for XopJ. IM, inner membrane; OM, outer membrane; CW, cell wall; PM, plasma membrane.
Comparative genome sequence analysis revealed that plant pathogenic bacteria possess a number of adhesins that presumably mediate bacterial attachment to multiple host cell receptors and might contribute to different stages of the infection process (Das et al., 2009). Adhesins from Xanthomonas spp. include XadA and XadB (both related to YadA from Yersinia spp.), homologs of the autotransporter adhesin YapH from Yersinia spp., filamentous hemagglutinin-like proteins and proteins predicted to be involved in type IV pilus synthesis (Da Silva et al., 2002; Lee et al., 2005; Ochiai et al., 2005; Qian et al., 2005; Thieme et al., 2005; Salzberg et al., 2008; Vorhölter et al., 2008). Type IV pili were proposed to play a role in the attachment of X. campestris pv. hyacinthi to the stomata of its host plant (van Doorn et al., 1994). However, a contribution of type IV pili to the attachment of X. campestris pv. vescicatoria to tomato leaves was not observed (Ojanen-Reuhs et al., 1997). To date, adhesins from X. oryzae pv. oryzae, X. axonopodis pv. citri and Xanthomonas fuscans spp. fuscans were shown to be involved in bacterial virulence and attachment to leaves and/or seeds (Ray et al., 2002; Darsonval et al., 2009; Das et al., 2009; Gottig et al., 2009) (Fig. 3). Mutational analyses of adhesin genes from X. fuscans spp. fuscans revealed that adhesins contribute individually and in a complementary manner to different stages of the infection process (Darsonval et al., 2009). In agreement with this is the finding that XadA and XadB from X. oryzae pv. oryzae are presumably required for bacterial attachment to the leaf surface, whereas the YapH homolog contributes to bacterial colonization of xylem vessels (Das et al., 2009).

**Virulence-associated protein secretion systems and their substrates**

*Xanthomonas* spp. possess at least six types of protein secretion systems, type I to type VI, that differ significantly in their composition and function, and in the recognition of secretion substrates (Gerlach & Hensel, 2007). Only in a few cases is protein secretion into the extracellular milieu mediated by outer membrane vesicles (Mashburn-Warren et al., 2008; Sidhu et al., 2008) (Fig. 2). Type I secretion (T1S) to type VI secretion (T6S) systems from Gram-negative bacteria are briefly summarized in Table 1 (see also Fig. 2), and virulence-associated protein secretion systems are described below. For more details, we refer the reader to excellent recent reviews (Henderson et al., 2004; Preston et al., 2005; Johnson et al., 2006; Gerlach & Hensel, 2007; Filloux et al., 2008; Juhas et al., 2008).

**The T1S system is required for induction of Xa21-mediated resistance in rice**

The T1S system is a heterotrimetric protein complex that consists of an ATP-binding cassette transporter in the inner membrane, a protein channel in the outer membrane and a membrane fusion protein that links the inner and the outer membrane components (Fig. 2). Substrates of T1S systems are secreted independent of the Sec system, presumably in a one-step process across both bacterial membranes, and include toxins, proteases and lipases (Gerlach & Hensel, 2007). To date, a direct virulence function has not yet been attributed to T1S systems from *Xanthomonas* spp. However, the T1S system from *X. oryzae* pv. *oryzae* was shown to be required for the elicitation of a resistance response in rice plants that carry the disease resistance gene *Xa21* (Shen et al., 2002; da Silva et al., 2004). *Xa21* is a receptor-like kinase with extracellular leucine-rich repeats that presumably perceive the AvrXa21-dependent signal (Song et al., 1995). This is remarkable because most other known avirulence (Avr) proteins from plant pathogenic bacteria are translocated into the plant cell cytosol by the T3S system. Mutagenesis of *X. oryzae* pv. *oryzae* led to the identification of eight genes that are required for AvrXa21 activity and were therefore designated *rax* (required for the AvrXa21 activity of *X. oryzae* pv. *oryzae*) (Shen et al., 2002; da Silva et al., 2004). Rax A, B and C are predicted components of a T1S system, RaxH and RaxR are part of a two-component regulatory system and RaxP, Q, S and T are involved in sulfur metabolism, suggesting that *Xa21* recognizes a sulfated molecule (Shen et al., 2002; Burdman et al., 2004; da Silva et al., 2004). It remains to be investigated whether the T1S system from *X. oryzae* pv. *oryzae* and other *Xanthomonas* spp. secretes additional factors that contribute to the plant–pathogen interaction.

**T2S systems and extracellular degradative enzymes**

The major protein secretion system that mediates protein transport from the bacterial periplasm to the extracellular milieu is the T2S system. It secretes toxins and extracellular enzymes such as proteases, lipases and cell wall-degrading enzymes that might contribute to the host–pathogen interaction. The T2S apparatus consists of 12–15 components, most of which are associated with the bacterial inner membrane (Sandkvist, 2001). A member of the secretin protein family forms a multimeric transmembrane channel in the outer membrane. It is assumed that secretion across the outer membrane depends on a predicted periplasmic pilus that is continuously assembled and disassembled and thus pushes T2S substrates through the secretin channel (Johnson et al., 2006).

The T2S system was initially discovered in the animal pathogenic bacterium *Yersinia pseudotuberculosis* and was identified in many other bacteria (d’Enfert et al., 1987; Gianciotto, 2005). Notably, however, it is absent in several pathogens including the human pathogens *Salmonella enterica* and...
**Table 2. Known virulence factors from Xanthomonas spp.**

| Species* | Protein secretion systems and secreted virulence factors† | Others† | References |
|----------|----------------------------------------------------------|---------|------------|
| Xac      | T3S system                                               |         |            |
|          | TSS system (filamentous hemagglutinin-like protein XacFhaB) |         |            |
|          | ABC transporters (XAC2072, XAC3600)                      |         | Laia et al. (2009) |
|          | TonB-dependent receptor (XAC0144)                        |         | Gottig et al. (2009) |
|          | Protease (XAC3980)                                      |         | Laia et al. (2009) |
|          | Amylase (XAC0798)                                       |         | Laia et al. (2009) |
| Xag      | T3S system                                               |         |            |
|          | Sucrose hydrolase SUH                                   |         | Kim et al. (2003) |
| Xcc      | Xps-T2S system (polygalacturonases PghAxc and PghBxc; amylases) |         | Chen et al. (2005), Gough et al. (1988), Qian et al. (2005), Wang et al. (2008b) |
|          | T3S system (effector XopXccN)                            |         | Jiang et al. (2008) |
|          | T4AS system†                                            | Genes involved in EPS/LPS synthesis; rpf genes; TonB-dependent receptor SuxA | He et al. (2007a), Qian et al. (2005), Blanvillain et al. (2007), Qian et al. (2005), Tang et al. (1991) |
| Xcv      | T3S system (lytic transglycosylase HpaH; effectors AvrBs2, XopD, XopN, XopX) |         |            |
| Xoo      | Xps-T2S system (lipase/esterase, cellobiosidase, cellulase, endoglucanase EgIXoB)† |         |            |
|          | T3S system (effectors AvrXa7, AvrXa10, PthXo6, PthXa7)  |         | Hu et al. (2007), Jha et al. (2007), Rajeshwari et al. (2005), Ray et al. (2000), Sun et al. (2005), Wang et al. (2008a) |
|          | TSS system (adhesins XadA, XadB, YapH)                   |         | Sugio et al. (2007), Yang & White (2004), Yang et al. (2000) |
| Xoc      | T3S system                                               |         |            |
|          | rpf genes; genes involved in EPS and LPS synthesis; type IV pilus component PilQ |         | Lim et al. (2008), Tang et al. (1996), Wang et al. (2008a) |

*Xac, X. axonopodis pv. citri; Xag, X. axonopodis pv. glycines; Xcc, X. campestris pv. campestris; Xcv, X. campestris pv. vesicatoria; Xoo, X. oryzae pv. oryzae; Xoc, X. oryzae pv. oryzicola.
†Protein secretion systems and cognate substrates (given in brackets) that were experimentally shown to contribute to virulence of Xanthomonas spp.
‡Genes or proteins that were shown to contribute to virulence.
†The T4AS system from Xcc strain 8004 was reported to be required for bacterial virulence on cabbage but not on radish.
*Secretion of EgIXoB by the Xps-T2S system has not been shown.

**Shigella flexneri** and the plant pathogen *Agrobacterium tumefaciens* (Goodner et al., 2001; Wood et al., 2001; Cianciotto, 2005). The precise contribution of individual T2S substrates to bacterial virulence and the identity of their cognate plant targets are not yet understood. It is conceivable that type II-secreted enzymes contribute to the degradation of the plant cell wall, which is a major obstacle for plant pathogenic bacteria (McNeill et al., 1984). Thus, T2S substrates might facilitate the assembly of extracellular appendages of virulence-associated protein secretion systems such as T3S, T4S and T6S systems that are dedicated to effector protein translocation (Fig. 2). In agreement with this model is the finding that the synthesis of type II-secreted enzymes is coregulated with the expression of T3S genes, suggesting a functional interplay between both secretion systems (Furutani et al., 2004; Wang et al., 2008b; Yamazaki et al., 2008).

T2S systems were identified as virulence factors in the plant pathogenic bacteria *Ralstonia solanacearum* and *Erwinia* spp. and *Xanthomonas* spp. (Cianciotto, 2005; Jha et al., 2005). Genome sequence analysis revealed that *Xanthomonas* spp. are equipped with one (*X. oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola*) or two (*X. campestris* pv. *vesicatoria, X. campestris* pv. *campestris, X. axonopodis* pv. *citri*) predicted T2S systems, which are encoded by *xcs* and *xps* gene clusters. So far, mutations that affect bacterial virulence have only been mapped to the *xps* gene cluster (Dow et al., 1987; Ray et al., 2000; Qian et al., 2005; Rajeshwari et al., 2005; Sun et al., 2005; Lu et al., 2008). Proteins secreted by the Xps-T2S systems from *X. campestris* pv. *campestris, X. axonopodis* pv.
citric and X. oryzae pv. oryzae include degradative enzymes such as cellulases, cellobiosidases, lipases, xylanases, endoglucanases, polygalacturonases and proteases (Dow et al., 1987; Ray et al., 2000; Schröter et al., 2001; Furutani et al., 2004; Rajeshwari et al., 2005; Sun et al., 2005; Jha et al., 2007; Wang et al., 2008b; Yamazaki et al., 2008). A direct influence on the plant–pathogen interaction was shown for an endoglucanase and polygalacturonases from X. campestris pv. campestris as well as for a lipase/esterase, a cellulase, an endoglucanase and a xylanase from X. oryzae pv. oryzae (Gough et al., 1988; Rajeshwari et al., 2005; Hu et al., 2007; Jha et al., 2007; Wang et al., 2008a, b) (Table 2). The reduction in virulence is more pronounced upon mutation of multiple T2S substrate-encoding genes, suggesting functional redundancies among type II-secreted proteins (Rajeshwari et al., 2005; Jha et al., 2007).

Interestingly, T2S substrates are not only associated with bacterial virulence but can also induce plant defense responses such as the deposition of callose in the cell wall as was shown for T2S substrates from X. oryzae pv. oryzae (Jha et al., 2007). T2S-dependent induction of basal plant defense is suppressed by X. oryzae pv. oryzae wild-type strains that contain a functional T3S system (Jha et al., 2007) (Fig. 3). This suggests that type III effector proteins counteract basal plant defense that is elicited by T2S substrates.

The T3S system is essential for bacterial pathogenicity

One of the key pathogenicity factors of most Gram-negative plant and animal pathogenic bacteria is the T3S system (Ghosh, 2004). In plant pathogenic bacteria, the T3S system is encoded by the chromosomal hrp (HR and pathogenicity) gene cluster, which contains more than 20 genes that are organized in several transcriptional units (Büttner & Bonas, 2002a). hrp genes were first discovered by the analysis of transposon insertion mutants in the plant pathogen Pseudomonas syringae pv. phaseolicola and were shown to be essential for bacterial pathogenicity and HR induction in host and nonhost plants, respectively (Lindgren et al., 1986). Since their initial discovery, hrp genes were identified in most Gram-negative plant pathogenic bacteria, with the exception of A. tumefaciens and of Xylella fastidiosa, which is closely related to Xanthomonas spp. (Willis et al., 1991; Bonas, 1994; Lindgren, 1997; Simpson et al., 2000; Goodner et al., 2001; Wood et al., 2001).

Clues about the function of hrp genes emerged from the finding that several hrp genes are homologous to components of T3S systems from animal pathogenic bacteria that inject bacterial effector proteins directly into eukaryotic host cells (Fenselau et al., 1992; Gough et al., 1992; Rosqvist et al., 1994; Sory & Cornelis, 1994). The core secretion apparatus, which spans both bacterial membranes, is presumably conserved among plant and animal pathogenic bacteria and is associated with an extracellular Hrp pilus (plant pathogens) or needle (animal pathogens) that serve as transport channels for secreted proteins to the host–pathogen interface (Ghosh, 2004). It is assumed that the Hrp pilus from plant pathogens spans the plant cell wall and is connected to the T3S translocon, a predicted proteinaceous transmembrane channel that inserts into the eukaryotic plasma membrane and mediates the translocation of effector proteins (Büttner & Bonas, 2002b). Because mutations of individual translocon components often lead to a complete loss or a drastic reduction of pathogenicity, translocation of effector proteins is presumably crucial for bacterial proliferation and elicitation of disease symptoms (Büttner et al., 2002; Kim et al., 2003; Sugio et al., 2005).

Identification of type III effector proteins from Xanthomonas spp.

The T3S system from individual Xanthomonas strains localizes a cocktail of different effector proteins into the plant cell (Rodent et al., 2004b; Thieme et al., 2005; Furutani et al., 2009) (Table 3). Based on experimental and bioinformatic analyses, 24 effectors or effector candidates have been identified in X. axonopodis pv. citri strain 306, 30 in X. campestris pv. vesicatoria strain 85-10, 23 in X. campestris pv. campestris strains ATCC 33913 and 8004, respectively, 32 in X. oryzae pv. oryzae strain KACC10331 and 37 in X. oryzae pv. oryzae strains MAFF 311018 and PXO99A, respectively (http://www.xanthomonas.org). Inactivation of individual effector genes often does not significantly affect bacterial virulence, presumably due to functional redundancies among effector proteins (Vivian & Arnold, 2000; Noël et al., 2003; Roden et al., 2004b). The lack of mutant phenotypes has significantly hampered the identification and functional characterization of type III effectors. In fact, the first known effector proteins were not identified because of a virulence function, but due to their ability to induce specific defense responses in resistant plants that carry corresponding R genes (White et al., 2000). Plant R proteins activate defense responses upon direct recognition of an effector protein, detection of effector-triggered modifications of plant target molecules or effector-mediated activation of R gene expression (Van der Hoorn & Kamoun, 2008). Effector-triggered immunity is often associated with the HR, and effector proteins that elicit the HR in corresponding resistant plants were designated Avr proteins (Jones & Dangl, 2006). However, this nomenclature is misleading because effector proteins presumably act as virulence factors in susceptible plants to the benefit of the pathogen (Mudgett, 2005; Grant et al., 2006).

Since 2002, the identification of effector proteins was significantly fostered by the availability of the genome sequences of several plant pathogenic bacteria, including
| Effector *          | Homology/conserved motifs                                                                 | Induction of plant defense reactions¹ | (Possible) virulence function                                                                 | Subcellular localization | Plant interaction partners                                                                 | References                                                                 |
|--------------------|-------------------------------------------------------------------------------------------|--------------------------------------|-----------------------------------------------------------------------------------------------|--------------------------|-----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| AvrBs1 (Xcv)       | Present in Xcc, Xcv; homolog of AvrA from P. syringae pv. glycinea                         | Recognition in pepper ECW-10R (Bsl1) | Decreases starch content in chloroplasts²; increases number of vesicles; induces chlorotic symptoms in N. benthamiana; contributes to disease under field conditions | CP                       | NA                                                                                           | Escolar et al. (2001), Gürlebeck et al. (2009), Minsavage et al. (1990), O’Garro et al. (1997), Wichmann & Bergelson (2004) |
| AvrBs2 (Xcv)       | Widespread in Xanthomonas spp.; predicted glycerophosphoryl diester phosphodiesterase     | Recognition in pepper ECW-20R (Bsl2) | Major virulence factor; phosphodiesterase activity could not be shown                           | NA                       | NA                                                                                           | Gassmann et al. (2000), Kearney & Staskavicz (1990), Minsavage et al. (1990), Swords et al. (1996), Wichmann & Bergelson (2004) |
| AvrXccC/AvrXccFM [XopAH] (Xcc) | Present in Xcc; N-myristoylation motif; AvrB family                                          | Recognition in mustard (Brassica napiformis) | Required for virulence on cabbage                                                               | PM                       | NA                                                                                           | Castaneda et al. (2005), Wang et al. (2007b) |
| AvrXv3 [XopAF] (Xcv) | Present in Xcv, Xoc; homolog of HopAF1 from P. syringae                                   | Recognition in Solanum lycopersicum and pepper | Modulates gene expression in tomato                                                            | NA                       | NA                                                                                           | Astua-Monge et al. (2000a), Balaji et al. (2007), Gibly et al. (2004) |
| AvrRxo1 [XopAI] (Xoc) | Present in Xcv, Xoc                                                                        | Recognition in maize lines (Rxa1)     | NA                                                                                                | PM                       | NA                                                                                           | Zhao et al. (2004) |
| AvrAC [XopAC] (Xcc) | Present in Xcc, LRR                                                                         | Recognition in vascular tissue of Arabidopsis Col-0 | No virulence function detected                                                                | NA                       | NA                                                                                           | Xu et al. (2008) |
| Ecf [XopAA] (Xcv)  | Present in Xcv, Xoo, Xoc; homolog of HopAE1 from P. syringae                             | Unknown                              | Induces chlorosis in bean                                                                     | NA                       | NA                                                                                           | Morales et al. (2005) |
| XopC (Xcv)         | Present in Xcv, Xap, Xff                                                                   | NA                                   | No virulence function detected                                                                | NA                       | NA                                                                                           | Alavi et al. (2008), Noël et al. (2003) |
| XopD (Xcv)         | Present in Xcc, Xcv; predicted SUMO cysteine protease (C48 family); HLH domain; EAR motifs | NA                                   | SUMO peptidase and SUMO isopeptidase; modulates defense gene expression; delays tissue collapse and promotes bacterial growth | NF                       | NA                                                                                           | Kim et al. (2008), Hotson et al. (2003) |
| XopF1 (Xcv)        | Present in Xcv, Xoo, Xoc                                                                  | NA                                   | No virulence function detected                                                                | NA                       | NA                                                                                           | Büttner et al. (2007), Roden et al. (2004b) |
| XopF2 (Xcv)        | Present in Xcv                                                                            | NA                                   | No virulence function detected                                                                | NA                       | NA                                                                                           | Büttner et al. (2007), Roden et al. (2004b) |
| XopN (Xcv)         | Present in Xac, Xcc, Xcv, Xoo, Xoc; homolog of HopA1 from P. syringae; α-helical ARM/HEAT repeats | NA                                   | Suppresses PTI; promotes bacterial growth and disease symptom formation (virulence role was also shown for XopNcc) | CP                       | Tomato atypical receptor-like kinase 1 (TARK1), tomato 14-3-3 isoforms (TFT1, 3, 5, 6) | Jiang et al. (2008), Kim et al. (2009a), Roden et al. (2004b) |
Table 3. Continued.

| Effector* | Homology/conserved motifs | Induction of plant defense reactions | (Possible) virulence function | Subcellular localization | Plant interaction partners | References |
|-----------|---------------------------|-------------------------------------|-----------------------------|------------------------|--------------------------|------------|
| XopO (Xcv) | Present in Xcv, Xoc; homolog of AvrRps4 and HopK1 from *P. syringae* | NA | No virulence function detected | NA | NA | Roden et al. (2004b) |
| XopP (Xcv) | Present in Xac, Xcc, Xcv, Xoo, Xac | NA | No virulence function detected | NA | NA | Roden et al. (2004b) |
| XopQ (Xcv) | Present in Xac, Xcc, Xcv, Xoo, Xoc; homolog of HopQ1 from *P. syringae*; structural homology to inosine-uridine nucleoside N-ribohydrolase | NA | No virulence function detected | NA | NA | Roden et al. (2004b) |
| XopX (Xcv) | Present in Xac, Xcc, Xcv, Xoo, Xoc; homolog of HopAE1 from *P. syringae*; Recognition in *N. benthamiana* (depends on a cofactor delivered by the T3S system) | Promotes bacterial growth; contributes to bacterial virulence | Major virulence factor; virulence function is due to contribution of HpaA to T3S in the bacterial cytosol | NA | NA | Metz et al. (2005) |
| HpaA (Xcv) | Present in Xac, Xcc, Xcv, Xoo, Xoc | NA | No virulence function detected | PM | NA | Lorenz et al. (2008) |
| **HopX (AvrPphE) family** | | | | | | |
| XopE1 (Xcv) | Present in Xac, Xcc, Xcv; N-myristoylation motif; predicted transglutaminase | Recognition in *N. benthamiana* and *N. clevelandii* | No virulence function detected | PM | NA | Thieme et al. (2007) |
| XopE2 (Xcv) | Present in Xac, Xcc, Xcv; N-myristoylation motif; predicted transglutaminase | Recognition in *Solanum pseudocapsicum*; weak recognition of AvrXccE (XopE2 homolog from Xcc) in bean (R2) | No virulence function detected | PM | NA | Castaneda et al. (2005), Nimchuk et al. (2007), Thieme et al. (2007) |
| **YopJ/AvrRxv family (members present in Xcv)** | | | | | | |
| XopJ [XopJ1] (Xcv) | N-myristoylation motif; predicted cysteine protease (C55 family) | Recognition in *N. benthamiana* and *N. clevelandii* | Suppresses callose deposition in the plant cell wall | PM | NA | Bartetzko et al. (2009), Thieme et al. (2007) |
| AvrBsT [XopJ2] (Xcv) | Present in Xcv strain 75-3, but not in strain 85-10; predicted cysteine protease (C55 family) | Recognition in *Arabidopsis* Pi-0, *Capsicum annuum*, *Capsicum pubescens* and *N. benthamiana* | NA | NA | Ciesiolka et al. (1999), Escolar et al. (2001), Minsavage et al. (1990), Orth et al. (2000) |
| Effector * | Homology/conserved motifs | Induction of plant defense reactions | (Possible) virulence function | Subcellular localization | Plant interaction partners | References |
|------------|--------------------------|-------------------------------------|------------------------------|------------------------|----------------------------|------------|
| AvrRxv [XopJ3] (Xcv) | Predicted cysteine protease (C55 family) | Recognition in tomato, bean (Rxv) and in several nonhost plants | Modulates host gene expression | Mainly CP | 14-3-3 protein (tomato) | Bonshtien et al. (2005), Ciesiolk et al. (1999), Whalen et al. (1993, 1988, 2008) |
| AvrXv4 [XopJ4] (Xcv) | Predicted cysteine protease (C55 family) | Recognition in tomato (XV4) and N. benthamiana | SUMO isopeptidase¹ | CP | NA | Astua-Monge et al. (2000b), Roden et al. (2004a) |
| AvrBs3 (Xcv) | 17.5 × 34 aa repeats | Recognition in pepper ECW-30R (Bs3) | Induces hypertrophy in Solanaceae; binds to conserved UPA box in promoter of UPA genes; activates UPA gene expression | N | Importin α from pepper | Gürlebeck et al. (2009), Kay et al. (2007), Marois et al. (2002), Minsavage et al. (1990), Römer et al. (2007), Szurek et al. (2001, 2002) |
| AvrBs4 (Xcv) | 17.5 × 34 aa repeats | Recognition in potato¹, tomato (Bs4) and C. pubescens | Induces catalase crystals in peroxisomes¹ | N | NA | Gürlebeck et al. (2009), Minsavage et al. (1999), Schornack et al. (2004) |
| Hax2 (Xcr) | 21.5 × 35 aa repeats | Unknown | Hax2, 3 and 4 contribute additively to disease symptoms on radish | NA | NA | Kay et al. (2005) |
| Hax3 (Xcr) | 11.5 × 34 aa repeats | Recognition in tomato (Bs4) | Hax2, 3 and 4 contribute additively to disease symptoms on radish | NA | NA | Kay et al. (2005) |
| Hax4 (Xcr) | 14.5 × 34 aa repeats | Recognition in tomato (Bs4) | Hax2, 3 and 4 contribute additively to disease symptoms on radish | NA | NA | Kay et al. (2005) |
| AvrXa7 (Xoo) | 25.5 × 34 aa repeats | Recognition in rice (Xa7) | Contributes to bacterial aggressiveness under field conditions; contributes to symptom development; HR suppressor activity; binds to DNA | N | NA | Bai et al. (2000), Fujikawa et al. (2006), Hopkins et al. (1992), Vera Cruz et al. (2000), Yang et al. (2000, 2006), Yang & White (2004) |
| AvrXa10 (Xoo) | 15.5 × 34 aa repeats | Recognition in rice (Xa10) | HR suppressor activity | NA | NA | Fujikawa et al. (2006), Hopkins et al. (1992) |
| AvrXa27 (Xoo) | 16.5 × 34 aa repeats | Recognition in rice (Xa27) | Induces expression of Xa27 | NA | NA | Gu et al. (2005) |
| PthXo1 (Xoo) | 22.5 × 34 aa repeats | Unknown | Induces expression of rice susceptibility gene Os8N3 | NA | NA | Yang et al. (2006) |
| PthXo6 (Xoo) | 22.5 × 34 aa repeats | Unknown | Contributes to virulence; induces expression of transcription factor gene Os7FX1 | NA | NA | Sugio et al. (2007) |
| Effector * | Homology/conserved motifs | Induction of plant defense reactions | (Possible) virulence function | Subcellular localization | Plant interaction partners | References |
|------------|---------------------------|----------------------------------|-------------------------------|------------------------|---------------------------|------------|
| PthXo7 (Xoo) | 21.5 × 34 aa repeats | Unknown | Contributes to lesion length and bacterial growth when expressed in Xoo strains with reduced virulence; induces expression of the gene that encodes the small subunit of the transcription factor OsTRIAgamma1 | NA | NA | Sugio et al. (2007) |
| PthA (Xac) | 17.5 × 34 aa repeats | Recognition in cotton and bean | Causes cell division and enlargement in citrus; contributes to disease symptom formation | NA | NA | Duan et al. (1999), Swanup et al. (1991, 1992), Yang et al. (1994) |
| AvrTaw (Xac) | 18.5 × 33/34 aa repeats | Recognition in tomato | NA | NA | Rybak et al. (2009) |
| AvrHah1 (Xg) | 13.5 × 34 aa repeats | Recognition in pepper | Contributes to disease in pepper; induces hypertrophy in tomato and N. benthamiana | N | NA | Schornack et al. (2008) |

*Listed are effectors with known virulence or avirulence function and effectors that were experimentally shown to be translocated by the T3S system. Abbreviations indicate Xanthomonas spp., in which effectors were analyzed: Xaa, X. axonopodis pv. aurantifolii; Xac, X. axonopodis pv. citri; Xap, X. axonopodis pv. phaseoli; Xca, X. citri pv. aurantifolia; Xci, X. campestris pv. armoraciae; Xcc, X. campestris pv. campestris; Xcm, X. campestris pv. maltacearum; Xcma, X. campestris pv. manihottis; Xcv, X. campestris pv. vesicatoria; Xff, X. fuscans ssp. fuscans; Xg, Xanthomonas gardneri; Xoo, X. oryzae pv. oryzae; Xoc, X. oryzae pv. oryzicola. Effector designations following an alternative nomenclature (http://www.xanthomonas.org) are given in squared brackets.

†For these observations, effectors were transiently expressed in planta after Agrobacterium tumefaciens-mediated gene delivery.

aa, amino acids; LRR, leucine-rich repeat; HLH, helix–loop–helix; EAR, ERF-associated amphiphilic repression; CP, cytoplasm; PM, plasma membrane; N, nucleus; NF, nuclear foci.
eight Xanthomonas strains (Da Silva et al., 2002; Lee et al., 2005; Ochiai et al., 2005; Qian et al., 2005; Thieme et al., 2005; Salzberg et al., 2008; Vorhölter et al., 2008) (Fig. 1). Effector gene candidates were uncovered by comparative genomic sequence analyses based on homologies to known type III effectors from plant and animal pathogens or the presence of typical eukaryotic motifs in corresponding gene products, indicating a protein function inside the host cell (Böttner et al., 2003). Furthermore, effector candidates were identified by bioinformatic approaches that include the presence of conserved promoter elements and specific N-terminal amino acid compositions as search criteria (Fouts et al., 2002; Guttmann et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002; Arnold et al., 2009; Furutani et al., 2009; Samudrala et al., 2009). Despite the fact that a virulence function was shown only for a few effector proteins from plant pathogenic bacteria, accumulating experimental evidence suggests that individual effector proteins counteract the plant innate immune response that is triggered upon recognition of conserved pathogen-associated molecular patterns (PAMPs) such as flagellin, cell wall degradation products or LPS (Espinosa & Alfano, 2004; Keshavarzi et al., 2004; Grant et al., 2006; Jones & Dangl, 2006; Jha et al., 2007; Block et al., 2008). Suppression of PAMP-triggered immunity by type III effectors might therefore be a major requirement for the successful establishment and multiplication of bacteria in the plant tissue.

Type III effector proteins from Xanthomonas spp. interfere with host cellular processes

Comparative sequence analysis of type III effectors from Xanthomonas spp. revealed that several effectors belong to conserved protein families, members of which are present in different plant and animal pathogens. Furthermore, some effectors are homologous to proteins with known enzymatic activities such as cysteine proteases, phosphatases or transglutaminases (Mudgett, 2005; Grant et al., 2006). As examples, we will briefly describe members of the YopJ/AvrRxv family of predicted cysteine proteases, the putative cysteine protease XopD and members of the AvrBs3/PthA family of transcription factors from Xanthomonas spp. For more details on known type III effectors from Xanthomonas spp., we refer to Table 3 and excellent recent reviews (Mudgett, 2005; Gürlebeck et al., 2006; Schornack et al., 2006; Kay & Bonas, 2009).

Members of the YopJ/AvrRxv family are present in both plant and animal pathogenic bacteria and belong to the C55 family of the CE clan of cysteine proteases (Orth, 2002; Mudgett, 2005; Angot et al., 2007). The most prominent member of this protein family, YopJ from Yersinia spp. (designated YopP in Yersinia enterocolitica), interferes with mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signaling pathways (Angot et al., 2007). Experimental evidence suggests that YopJ acts on small ubiquitin-like modifier (SUMO)-conjugated proteins; however, specific targets are unknown (Orth et al., 2000). Furthermore, it was shown that YopJ removes K63-linked polyubiquitin chains from tumor necrosis factor receptor-associated factor 6 (TRAF6) and K48-linked polyubiquitin chains from the inhibitor of NF-κB, IκBz, suggesting that YopJ is a protease (Zhou et al., 2005). K63-linked polyubiquitin activates TRAF6 and thus MAPK-mediated signaling pathways, whereas K48-linked polyubiquitin targets proteins for proteasome-dependent degradation (Pickart & Fushman, 2004). YopJ-mediated removal of K48-linked polyubiquitin therefore presumably stabilizes IκBz, thus preventing import of NF-κB into the nucleus (Angot et al., 2007). Interestingly, however, YopJ also acts as an acetyltransferase on a MAPK kinase, suggesting that it has a dual enzymatic activity (Mittal et al., 2006; Mukherjee et al., 2006).

Members of the YopJ/AvrRxv family of effector proteins have mainly been identified in X. campestris pv. vesicatoria, which contains four YopJ homologs including XopJ and the Avr proteins AvrXv4, AvrRxv and AvrBsT, which trigger the HR in corresponding resistant plants (Table 3). Mutant analyses revealed that the conserved residues in the catalytic triad (histidine, glutamate and cysteine) of AvrRxv, AvrBsT and AvrXv4 are required for the HR induction, suggesting that the enzymatic activity of these proteins is important for recognition in resistant plants (Orth et al., 2000; Roden et al., 2004a; Bonshtien et al., 2005). Notably, it was shown that a chimeric protein containing the N-terminal domain of AvrRxv and the C-terminal catalytic domain of YopP still elicits the HR on tomato cultivar Hawaii 7998 (Whalen et al., 2008). It is therefore possible that the catalytic mechanisms underlying the activity of YopJ homologs are conserved. Because in planta expression of AvrXv4 from X. campestris pv. vesicatoria leads to a reduction of SUMO-conjugated plant proteins, AvrXv4 presumably acts as a SUMO protease on yet unknown plant target proteins (Roden et al., 2004a). However, the enzymatic activity of other YopJ homologs from X. campestris pv. vesicatoria is still elusive.

Another predicted cysteine protease from Xanthomonas spp. is the effector protein XopD, which belongs to the C48 family and is structurally similar to the yeast ubiquitin-like protease ULP1 (Noël et al., 2002; Hotson et al., 2003). Experimental evidence suggests that XopD cleaves SUMO precursors and removes SUMO from plant proteins (Hotson et al., 2003). Because XopD localizes to subnuclear foci and binds unspecifically to DNA via a putative helix–loop–helix domain, it might target components of the plant transcription machinery. Sequence analysis revealed that XopD possesses two conserved ethylene responsive factor-associated amphiphilic repression motifs, which are
characteristic for plant transcription factors that repress gene expression (Kim et al., 2008). In line with this observation is the finding that in planta expression of XopD leads to reduced promoter activity of salicylic acid- and jasmonate-induced genes (Kim et al., 2008).

Nuclear targeting and effects on the host transcription machinery were also shown for members of the conserved AvrBs3/PthA effector family (also designated TAL, for transcription activator-like) from Xanthomonas spp. (Schornack et al., 2006; Kay & Bonas, 2009) (Fig. 3). AvrBs3 and homologous proteins share features of eukaryotic transcription factors, i.e. nuclear localization signals and an acidic activation domain, and modulate host gene expression presumably by direct interaction with the host transcription machinery (Schornack et al., 2006; Kay & Bonas, 2009). For AvrBs3-like effectors from X. oryzae pv. oryzae and X. campestris pv. vesicatoria, plant target genes were identified including, for example the susceptibility gene Os8N3 from rice, the pepper Bs3 resistance gene and the pepper UPA20 (UPA, upregulated by AvrBs3) gene (Marois et al., 2002; Yang et al., 2006; Kay et al., 2007; Römer et al., 2007; Sugio et al., 2007). UPA20 encodes a plant transcription factor that induces plant cell hypertrophy, a phenotype that is also induced by AvrBs3 (Table 3) (Marois et al., 2002; Kay et al., 2007). Sequence analysis of the promoter regions of UPA20 and other UPA genes revealed a conserved motif (UPA box) that is bound by AvrBs3 (Kay et al., 2007, 2009; Römer et al., 2007, 2009). DNA binding of AvrBs3 is determined by two hypervariable amino acids in the central repeat region that confer binding specificity to defined base pairs of the UPA box (Boch et al., 2009; Moscou & Bogdanove, 2009). Taken together, the data suggest that AvrBs3 and its homologs specifically modulate plant gene expression by binding to target gene promoters (Fig. 3).

**Regulatory networks underlying the virulence of Xanthomonas spp.**

Foliar plant pathogens such as Xanthomonas spp. undergo different life stages and often colonize leaf surfaces as epiphytes before they invade the intercellular space. Like other pathogens, Xanthomonas spp. have evolved regulatory systems to adapt the expression of virulence factors to different extracellular stimuli such as population density, availability of nutrients, oxygen levels and the presence of plant-derived molecules. Perception and transduction of external signals is often mediated by two-component signal transduction systems that usually consist of a membrane-bound histidine kinase sensor and a cytoplasmic response regulator. Upon perception of the external stimulus, the histidine kinase sensor is autophosphorylated and transfers a phosphoryl group to the receiver domain of the cognate response regulator, which in turn activates the expression of target genes (Qian et al., 2008a). Comparative sequence analysis revealed that X. campestris pv. campestris contains > 50 predicted sensor kinases and response regulators (Qian et al., 2008b). To date, a contribution to virulence has only been studied for a few of them including RpfC/RpfG [involved in quorum sensing (QS) and regulation of virulence factors], RavS/RavR [regulation of adaptation and virulence (Rav)] (involved in the regulation of virulence factors), RavX/H/RaxR (required for AvrXa21 activity), CoIR/ColS (contribute to hrp gene expression) and the response regulator HrpG (essential for hrp gene expression), which will be briefly described below (Qian et al., 2008b; Zhang et al., 2008; He et al., 2009). For more details on bacterial regulatory networks that control virulence, we refer the reader to recent reviews (Dow et al., 2006; Tang et al., 2006; He & Zhang, 2008; Qian et al., 2008a).

**Control of virulence gene expression by QS**

Bacteria synthesize small diffusible signal molecules (DSFs) that accumulate with increasing population size and regulate gene expression via corresponding receptor proteins, a phenomenon termed QS (Miller & Bassler, 2001; Von Bodman et al., 2003). In X. campestris pv. campestris, a QS signal (DSF) was identified as cis-11-methyl-2-dodecanoic acid and shown to regulate the expression of at least 165 genes including putative virulence genes (Barber et al., 1997; Wang et al., 2004; He et al., 2006; Ryan et al., 2007; He & Zhang, 2008). Synthesis of DSF is driven by the putative enoyl-CoA hydratase RpfF and the fatty acyl-CoA ligase RpfB, which are both encoded by the regulation of pathogenicity factor (rpf) gene cluster (Tang et al., 1991). It is assumed that DSF can diffuse across the bacterial membranes due to its lipophilic nature and accumulates in the early stationary growth phase in the external milieu (Crossman & Dow, 2004). DSF is presumably sensed by a two-component signal transduction system consisting of the sensor kinase RpfC and the response regulator RpfG (Slater et al., 2000) (Fig. 4). Mutations in rpfF, rpfG or rpfC lead to decreased production of EPS, extracellular enzymes and altered biofilm formation in certain media, suggesting that DSF signaling is involved in the regulation of virulence factors (Slater et al., 2000; Dow et al., 2003; Ryan et al., 2007; Torres et al., 2007; Jeong et al., 2008; Thowthampitak et al., 2008). Furthermore, in X. campestris pv. campestris, rpf-dependent signaling is required for the bacterial ability to revert stomatal closure that is activated as part of the plant defense response upon pathogen attack (Gudesblat et al., 2008).

The response regulator RpfG contains an HD-GYP domain (letters refer to conserved amino acids) that is conserved in Gram-positive and Gram-negative bacteria and is presumably involved in the hydrolysis of cyclic di-GMP (Galperin et al., 2001; Dow et al., 2006; Ryan et al.,...
In agreement with this hypothesis is the finding that RpfG is an active cyclic di-GMP phosphodiesterase. The enzymatic activity is required for RpfG-dependent control of gene expression, suggesting that cyclic di-GMP is an important messenger molecule (Ryan et al., 2006b) (Fig. 4). Cyclic di-GMP was first described as an allosteric inhibitor of the cellulose synthase from Gluconacetobacter xylinus (Römling & Amikam, 2006). Intracellular cyclic di-GMP acts as a second messenger that presumably regulates a variety of cellular functions such as biofilm formation and expression of virulence genes. It was shown that high levels of cyclic di-GMP promote biofilm formation, whereas lower levels promote motility and expression of virulence factors (Simm et al., 2004; Tischler & Camilli, 2004; Römling et al., 2005) (Fig. 4). Cyclic di-GMP signaling presumably involves proteins with a conserved PilZ domain that might provide a binding site for cyclic di-GMP (Römling & Amikam, 2006). A contribution to bacterial virulence was recently shown for two PilZ proteins from X. campestris pv. campestris (McCarthy et al., 2008). Besides PilZ proteins, additional targets of cyclic di-GMP including transcription factors, a protein with the GGDEF domain and riboswitches have been identified in other Gram-negative bacteria, suggesting that cyclic di-GMP is involved in the regulation of a variety of cellular functions (Pesavento & Hengge, 2009).

**Cyclic di-GMP signaling activates the transcriptional activators Clp and Zur**

Experimental evidence suggests that DSF and RpfC/RpfG activate the transcriptional regulator Clp (CAP [catabolite...
activator protein, also called cAMP receptor protein (CRP)-like protein), which contains nucleotide- and DNA-binding domains and binds to promoters of target genes as was demonstrated, for example for a pectate lyase and an endoglucanase from *X. campestris pv. campestris* (Hsiao et al., 2005, 2009; He et al., 2007b; Ge & He, 2008).

DNA binding of Clp from *A. xanopopodis pv. citri* is inhibited in vitro by cyclic di-GMP (Leduc & Roberts, 2009). In *X. campestris pv. campestris*, it was shown that Clp induces the expression of genes belonging to the DSF regulon, for example genes that encode extracellular enzymes, components of T2S and T3S systems, and genes involved in EPS synthesis. By contrast, Clp is not involved in the DSF-dependent regulation of biofilm formation (He et al., 2007b) (Fig. 4). The analysis of Clp-regulated genes from *X. campestris pv. campestris* led to the identification of two transcription factors, i.e. FhrR, which contains a TetR family transcription factor domain, and the zinc uptake regulator Zur that belongs to the Fur family of transcription factors (He et al., 2007b). While FhrR regulates the expression of genes that encode flagellar, Hrp and ribosomal proteins, Zur is involved in the regulation of iron uptake, multidrug resistance and detoxification (He et al., 2007b). Zur was initially identified as a repressor of Zn$^{2+}$ uptake systems in *E. coli* and *Bacillus subtilis*, but was also shown to contribute to the virulence of *X. campestris pv. campestris* (Hantke, 2005; Tang et al., 2005). A recent study revealed that Zur from *X. campestris pv. campestris* strain 8004 positively regulates *hrp* gene expression presumably via the transcriptional activator HrpX (Huang et al., 2009). By contrast, *hrp* genes in *X. campestris pv. campestris* strain XC1 appear to be repressed by Clp and FhrR (He et al., 2007b) (Fig. 4). It therefore cannot be excluded that multiple signaling cascades are involved in the regulation of virulence factors or that the mechanisms underlying DSF-mediated control of gene expression vary among different strains of the same pathovar of *Xanthomonas* spp.

To date, DSF-dependent signaling has been studied most extensively in *X. campestris pv. campestris*. Furthermore, *rpf* genes from *O. eryzae pv. eryzae* and *A. xanopopodis pv. glycines* were shown to contribute to virulence, expression of genes encoding extracellular enzymes and EPS production (Tang et al., 1996; Jeong et al., 2008; Thowthampitak et al., 2008). Interestingly, the analysis of DSF signaling in *S. malophila* revealed that RpfF might also be involved in LPS production (Fouhy et al., 2007).

### The two-component system RavS/RavR modulates cyclic di-GMP levels

The genomes of *Xanthomonas* spp. encode a number of proteins with conserved HD-GYP, GGDEF and EAL domains that are involved in the synthesis (GGDEF domains) or the degradation (EAL and HD-GYP domains) of cyclic di-GMP (Dow et al., 2006). Genome-wide mutational analyses of proteins with HD-GYP, GGDEF or EAL domains in *X. campestris pv. campestris* led to the identification of additional virulence factors that presumably regulate cyclic di-GMP levels (Ryan et al., 2007). Among these is the two-component system response regulator RavR that contains GGDEF-EAL domains and contributes to EPS production and extracellular protease and cellulose activities (He et al., 2009). RavR promotes the virulence of *X. campestris pv. campestris* strain XC1 on Chinese cabbage. However, deletion of the homologous gene X2228 in *X. campestris pv. campestris* strain 8004 does not affect lesion length in Chinese radish, suggesting that the virulence functions of regulatory proteins depend on the genetic background or the host plant (Ryan et al., 2007; He et al., 2009).

Biochemical approaches revealed that RavR is a cyclic di-GMP phosphodiesterase, which is in agreement with the presence of the EAL domain (He et al., 2009). RavR is activated by the cognate histidine kinase RavS, which contains two PAS domains that are also present in signaling proteins of several other Gram-negative bacteria and were shown to act as oxygen sensors. The second PAS domain of RavS is required for protein function, suggesting that RavS/RavR regulate virulence factor production in response to low oxygen levels (He et al., 2009). In agreement with this hypothesis is the finding that a predicted cyclic di-GMP phosphodiesterase (XC2324) with a PAS domain is required for virulence factor production under low-oxygen conditions in *X. campestris pv. campestris* strain 8004 (Ryan et al., 2007). Microarray analyses in *X. campestris pv. campestris* strain XC1 revealed that RavR activates the expression of at least 206 genes including *clp, hrp* genes and genes involved in the synthesis of extracellular enzymes, EPS and LPS (He et al., 2009). Thus, RavR presumably regulates virulence gene expression via Clp, suggesting that the synthesis of virulence factors is controlled in response to both QS and low oxygen (He et al., 2009) (Fig. 4).

### Regulation of *hrp* gene expression

*hrp* genes are not constitutively expressed, but are activated when the bacteria enter the plant or are cultivated in certain minimal media (Tang et al., 2006). Although *hrp* gene expression in *X. campestris pv. campestris* is regulated by DSF and cyclic di-GMP levels, QS is not sufficient to induce *hrp* gene expression. Mutant analyses revealed that *hrp* gene expression depends on the key regulator HrpG, which is an OmpR-type response regulator and presumably perceives an environmental signal via a yet unknown sensor kinase (Wengelnik et al., 1996a, b; Wengelnik et al., 1999) (Fig. 4). *hrpG* expression is slightly induced in certain minimal media and on the plant surface; however, a significant...
induction of \textit{hrpG} expression is observed when the bacteria enter the plant apoplast (Wengelnik \textit{et al.}, 1996b; Zhang \textit{et al.}, 2009). Experimental evidence reported for \textit{X. oryzae pv. oryzae} suggests that \textit{hrpG} expression is controlled by multiple regulatory pathways including the two-component system PhoPQ, the H-NS protein XrVA and Trh, a member of the GntR regulator family (Furutani \textit{et al.}, 2004; Metz \textit{et al.}, 2005; Wei \textit{et al.}, 2008). Notably, however, HrpX-dependent gene expression was also shown for genes without the PIP box, suggesting that the presence of a PIP box is not required for HrpX inducibility (Noël \textit{et al.}, 2001; Tsuge \textit{et al.}, 2005).

Mutant studies revealed that HrpG and HrpX from \textit{X. campestris pv. vesicatoria} are essential for pathogenicity and contribute to the epiphytic survival of the bacteria (Wengelnik & Bonas, 1996; Wengelnik \textit{et al.}, 1996a, b; Moss, 2000). Interestingly, epiphytic survival is more compromised upon mutation of \textit{hrpG/hrpX} than upon mutation of the Hrp pilus gene \textit{hrpE}, which is essential for T3S (Moss, 2000). This implies that HrpG and HrpX activate factors independent of the T3S system that contribute to the epiphytic phase. In agreement with this hypothesis, cDNA amplified fragment length polymorphism analysis in \textit{X. campestris pv. vesicatoria} and expression studies in other \textit{Xanthomonas} spp. revealed that HrpG controls the expression of a genome-wide regulon including predicted virulence genes that encode, for example type III effectors and T2S substrates (Noël \textit{et al.}, 2001, 2003; Furutani \textit{et al.}, 2004; Metz \textit{et al.}, 2005; Wang \textit{et al.}, 2008b; Yamazaki \textit{et al.}, 2008). Similar findings were reported for the HrpG homolog from the plant pathogenic bacterium \textit{R. solanacearum} (Valls \textit{et al.}, 2006). Interestingly, HrpG-regulated genes from \textit{X. campestris pv. campestris} include two proteins with a PilZ domain, which are required for bacterial virulence and presumably are involved in cyclic di-GMP sensing (McCarthy \textit{et al.}, 2008). Furthermore, HrpG and HrpX from \textit{X. campestris pv. campestris} activate expression of the regulatory gene \textit{hpaR}, which encodes a transcriptional regulator of the MarR family (Wei \textit{et al.}, 2007). Deletion of \textit{hpaR} abolishes the virulence of \textit{X. campestris pv. campestris} strain 8004 on the host plant cabbage and leads to a reduction of HR induction in the nonhost plant pepper. Furthermore, \textit{hpaR} mutants exhibit increased extracellular protease activity, suggesting that HpaR inhibits protease production (Qian \textit{et al.}, 2005; Wei \textit{et al.}, 2007) (Fig. 4). In agreement with this finding, a HrpG/HrpX-dependent inhibition of extracellular protease activity was also shown for \textit{X. campestris pv. vesicatoria} (Noël \textit{et al.}, 2001).

In addition to HrpG and HrpX, expression of the \textit{hrpC} and \textit{hrpE} operons from the \textit{hrp} gene cluster of \textit{X. campestris pv. campestris} is controlled by the two-component regulatory system ColR/ColS, suggesting that various signal transduction pathways are involved in the regulation of \textit{hrp} gene expression and that individual \textit{hrp} operons might be targeted by alternative signaling pathways (Zhang \textit{et al.}, 2008) (Fig. 4). In agreement with this hypothesis is the finding that several \textit{hrp} genes from \textit{X. axonopodis pv. citri} are induced in the minimal medium XVM2, whereas \textit{hrpB1} is repressed (Astu-Monge \textit{et al.}, 2005). However, there might also be pathovar-specific differences in \textit{hrp} gene expression because microarray experiments revealed that \textit{hrp} genes from \textit{X. oryzae pv. oryzae} and \textit{X. oryzae pv. oryzicola} are differentially regulated (Seo \textit{et al.}, 2008). Taken together, the analysis of HrpG-dependent gene expression profiles clearly demonstrates the complexity of signaling pathways underlying the control of bacterial virulence and the interplay between various regulation cascades.

\textbf{Post-transcriptional control of virulence gene expression by repressor of secondary metabolism (RsmA)}

Interestingly, virulence gene expression is also controlled at the post-transcriptional level by the RNA-binding protein RsmA as reported recently for \textit{X. campestris pv. campestris} (Chao \textit{et al.}, 2008). RsmA belongs to a conserved family of RNA-binding proteins that were initially identified as repressors of carbon metabolism [carbon storage regulator (CsrA)] (Lapouge \textit{et al.}, 2008). Members of the RsmA/CsrA family bind to specific binding sites near the Shine–Dalgarno sequence of target mRNAs and thus block ribosome binding. Translational repression by RsmA/CsrA can be relieved by small RNAs that bind to RsmA/CsrA proteins (Lapouge \textit{et al.}, 2008). Notably, RsmA/CsrA proteins are involved in the regulation of virulence gene expression in animal pathogens and \textit{Erwinia} spp. (Lapouge \textit{et al.}, 2008).

In \textit{X. campestris pv. campestris}, a mutation of \textit{rsmA} results in complete loss of bacterial motility, loss of virulence in Chinese radish and of HR induction in nonhost plants and enhanced biofilm formation (Chao \textit{et al.}, 2008). RsmA from \textit{X. campestris pv. campestris} appears to contribute to the synthesis of EPS, extracellular endoglucanases and amylases and to the transcript levels of \textit{hrp} and effector genes. By contrast, extracellular protease activity and expression of HrpG and HrpX are not affected (Chao \textit{et al.}, 2008) (Fig. 4). Because RsmA often acts as a negative translational repressor, positive regulation of gene expression by RsmA is presumably indirectly achieved by RsmA-mediated post-transcriptional control of additional regulatory factors. The identity of potential small RNAs that bind to RsmA in \textit{Xanthomonas} spp. remains to be elucidated.
Concluding remarks
In the last decade, a large number of publications revealed the complexity of virulence factors used by Xanthomonas spp. to conquer their respective host plants that include important crop species worldwide. Successful infections often depend on the bacterial ability to adhere to and to communicate with host cells. Most known virulence factors therefore include bacterial surface structures and secreted proteins that presumably promote nutrient acquisition by the bacterium and suppress plant defense responses. For a better manipulation of plant cells, bacteria also translocate effector proteins into the plant cell cytosol. Effector protein translocation by the T3S system is one of the key events during the host–pathogen interaction and has therefore been studied intensively. However, the functional characterization of effector proteins and other virulence factors is often complicated by the fact that individual deletion mutants are not impaired in virulence, presumably due to functional redundancies among secreted proteins. The comprehensive analysis of molecular mechanisms underlying the activity of secreted virulence factors is therefore one main focus of future research. The detailed characterization of effector proteins will not only shed light on bacterial virulence strategies but also provide clues about plant developmental processes.

Besides the T3S system, Xanthomonas spp. presumably use other protein secretion systems to conquer their host plants including the recently discovered T6S systems, which are dedicated to the translocation of bacterial proteins into eukaryotic cells. We now need to focus on the characterization of these protein secretion systems and their corresponding secretomes to deepen our understanding of plant–pathogen interactions. Furthermore, the comparative analysis of virulence-associated protein secretion machineries might help to unravel a functional interplay between different secretion systems as was shown for T2S and T3S systems from X. oryzae pv. oryzae. The concept of a functional crosstalk between different protein secretion systems is supported by the finding that expression of several virulence factors such as type III effectors and degradative enzymes is orchestrated by common regulators. The elucidation of virulence-associated regulatory networks, which do not only include transcriptional activators but also RNA-binding proteins and small RNAs, is one of the major challenges of ongoing research. In conclusion, the aim of this article was not only to provide a summary of our current knowledge on virulence factors from Xanthomonas spp. but also to show that we are just beginning to understand bacterial virulence strategies.

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I. Introduction

Infection strategies of *Xanthomonas*

II. Gene for gene-mediated recognition of nuclear-targeted AvrBs3-like bacterial effector proteins. *J Plant Physiol* 163: 256–272.

III. Characterization of AvrHa1, a novel AvrBs3-like effector from *Xanthomonas gardneri* with virulence and avirulence activity. *New Phytol* 179: 546–556.

IV. Infection strategies of *Starr MP (1981) The genus Xanthomonas*.

V. Characterization of the hrpF pathogenicity peninsula of *Xanthomonas oryzae* pv. oryzae. *Mol Plant Microbe In* 18: 546–554.

VI. Analysis of outer membrane vesicle associated proteins isolated from the pathovar *Xanthomonas campestris* pv. citri. *Appl Microbiol Biot* 55: 727–233.

VII. A putative homolog from *Xanthomonas axonopodis* pv. citri responsible for host-specific suppression of virulence. *J Bacteriol* 189: 3271–3279.

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X. 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.

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XII. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol Microbiol* 14: 583–594.

XIII. The genus *Xanthomonas*. *The Prokaryotes* (Starr MP, Stolp H, Trüper HG, Balows A & Schlegel HG, eds), pp. 742–763. Springer Verlag, Berlin.

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