Microbial Biotechnology (2009) **2**(4), 465–475 doi:10.1111/j.1751-7915.2009.00095.x

The outer membrane protein TolC is required for phytoalexin resistance and virulence of the fire blight pathogen *Erwinia amylovora*

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Summary

Erwinia amylovora **causes fire blight on several plant species such as apple and pear, which produce diverse phytoalexins as defence mechanisms. An evolutionary successful pathogen thus must develop resistance mechanisms towards these toxic compounds. The *E. amylovora* outer membrane protein, TolC, might mediate phytoalexin resistance through its interaction with the multidrug efflux pump, AcrAB. To prove this, a *tolC* mutant and an *acrB/tolC* double mutant were constructed. The minimal inhibitory concentrations of diverse antimicrobials and phytoalexins were determined for these mutants and compared with that of a previously generated *acrB* mutant. The *tolC* and *acrB/tolC* mutants were considerably more susceptible than the wild type but showed similar levels as the *acrB* mutant. The results clearly indicated that neither TolC nor AcrAB significantly interacted with other transport systems during the efflux of the tested toxic compounds. Survival and virulence assays on inoculated apple plants showed that pathogenicity and the ability of *E. amylovora* to colonize plant tissue were equally impaired by mutations of *tolC* and *acrB/tolC*. Our results allowed the conclusion that TolC plays an important role as a virulence and fitness factor of *E. amylovora* by mediating resistance towards phytoalexins through its exclusive interaction with AcrAB.**

Introduction

Plants produce an array of diverse secondary metabolites that have antimicrobial activities including preformed, so-called phytanticipants and phytoalexins, which are synthesized in response to pathogen attack. For this purpose, flavonoids, isoprenoids and alkaloids comprise three major classes of secondary metabolites synthesized by higher plants. These compounds are essential for diverse plant defence mechanisms and are furthermore implicated in a broad range of physiological processes (Osbourn, 1996; Hammerschmidt, 1999).

Evolutionary successful pathogens are able to circumvent the toxic effect of antimicrobial compounds by enzymatic inactivation, alteration of the cellular target, reduced uptake of the toxic compounds, or active efflux (Walsh, 2000; VanEtten *et al*., 2001). In this context, multidrug efflux (MDE) has been suggested for a broad range of structurally unrelated compounds. So far, most emphasis has been devoted to the investigation of MDE phenomena in human pathogens. However, genome analyses of various microorganisms revealed the ubiquitous occurrence of genes encoding MDE pumps (Paulsen *et al*., 2001). Five well-characterized families of MDE pump exist: the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (Brown *et al*., 1999). Efflux by proteins of the MFS, RND, SMR and MATE families is driven by proton (and sodium) motive force while ATP hydrolysis drives efflux in ABC transporters (Moreira *et al*., 2004; Poole, 2005; Piddock, 2006; Alekshun and Levy, 2007). In Gram-negative bacteria, RND-type transporters reside in the inner membrane and function in concert with two types of proteins: the accessory membrane fusion proteins, which are located in the periplasmic space, and outer membrane protein channels (Piddock, 2006; Alekshun and Levy, 2007). These tripartite transport systems enable drug efflux across both membranes (Zgurskaya and Nikaido, 2000).

For plant pathogens, protection against toxic compounds by MDE transporters has particularly been studied in fungal organisms. Mutation of genes encoding ABC transporters of *Gibberella pulicaris* and *Botrytis cinerea* resulted in decreased tolerance to phytoalexins and thus led to impaired virulence (Del Sorbo *et al*., 2000; Schoonbeek *et al*., 2001; Fleißner *et al*., 2002). In phytopathogenic and other plant-associated bacteria so far only

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few MDE pumps belonging to the RND superfamily have been associated with respective interactions, for example in *Agrobacterium tumefaciens* (Palumbo *et al*., 1998; Peng and Nester, 2001), in the nitrogen-fixing symbiont, *Rhizobium etli* (Gonzáles-Pasayo and Martinez-Romera, 2000), in *Pseudomonas syringae* (Stoitsova *et al*., 2008), in *Erwinia chrysanthemi* (Maggiorani Valecillos *et al*., 2006) and in *Bradyrhizobium japonicum* (Krummenacher and Narberhaus, 2000).

One of the economically most important bacterioses represents fire blight on rosaceous plants, which is particularly destructive to apple and pear trees (Eastgate, 2000). The causal agent of this disease is *Erwinia amylovora*, a member of the *Enterobacteriaceae*. The initial symptom of fire blight is water soaking followed by wilting and rapid necrosis leaving infected tissue with a scorched, blackened appearance. The spread of the pathogen through the host tissue and xylem into all parts of a plant can lead to loss of entire trees (Vanneste and Eden-Green, 2000). The commercial implications of fire blight outbreaks are aggravated by the limited effectiveness of current control strategies (Psallidas and Tsiantos, 2000). Thus, a better understanding of the molecular interactions between plant and pathogen can lead to improved design of antibacterial compounds and genetically engineered plants.

In *E. amylovora*, two essential pathogenicity factors have been identified: the *hrp/dsp* genes encoding a type III protein secretion system and disease-specific effector proteins (Steinberger and Beer, 1988; Barny *et al*., 1990; Vanneste *et al*., 1990) and the extracellular polysaccharide amylovoran (Bellemann and Geider, 1992). Further studies revealed a number of additional factors that are not directly involved in the induction of disease symptoms but allow the pathogen to survive *in planta* (Metzger *et al*., 1994; Aldridge *et al*., 1997; Bogs and Geider, 2000).

A resistance mechanism to bypass the antimicrobial effects of secondary metabolites of the host defence has been studied for *E. amylovora* by Burse and colleagues (2004). It was shown that the *E. amylovora* RND-family efflux pump AcrAB was essential for resistance against plant-derived antimicrobial toxins, particularly against the apple phytoalexin, phloretin, and for a successful colonization of plant tissue. Based on that finding, our present study focused on the involvement of the outer membrane protein, TolC, in MDE, its specific interaction with AcrAB, and its involvement in virulence and *in planta* survival of *E. amylovora*. TolC homologues have been shown to be involved in resistance against antimicrobial compounds in mammalian pathogens such as *Escherichia coli* (Fralick and Burns-Keliher, 1994; Sharff *et al*., 2001; Bavro *et al*., 2008), *Salmonella enterica* (Baucheron *et al*., 2004; 2005) and *Borrelia bugdorferi* (Bunikis *et al*., 2008). Furthermore, TolC has been studied to variable extent in the nitrogen-fixing organism, *Sinorhizobium meliloti* (Cosme *et al*., 2008) and in two plant pathogenic bacteria, *Xylella fastidiosa* (Reddy *et al*., 2007) and *Dickeya dadantii* (formerly known as *Erwinia chrysanthemi*) (Barabote *et al*., 2003). Herein, we report on the identification of the gene encoding for TolC in *E. amylovora*, the generation and resistance analyses of single and double mutants for *tolC* and *acrB/tolC*, and the determination of virulence and *in planta* survival of the respective mutants.

Results

In vitro *growth of wild type and mutants of* E. amylovora *Ea1189*

In order to test whether or not individual or combined effects of mutations in *tolC* and *acrB* influenced the overall fitness of respective *E. amylovora* mutants, *in vitro* growth experiments were conducted. Bacteria were cultured in complex Luria–Bertani (LB) and minimal asparagine minimal medium 2 (AMM2) medium at 28°C. The OD_{600} was monitored continuously until cultures had entered the late stationary phase (Fig. 1). Growth of wild-type cultures was generally faster than that of any mutant tested with doubling times of ~60 min for the wild type; ~90 min for the *acrB* mutant; ~105 min for the *tolC* mutant and ~120 min for the *acrB/tolC* double mutant. Growth of the genetically complemented mutants was similar to that of the wild type proving that growth delays were directly due to lack of *tolC* and *acrB/tolC*. Wild type and complemented mutants reached the stationary phase within 7 h with an OD_{600} of ~1.5. In contrast, stationary phases were reached at OD_{600} of ~1, ~0.9 and ~0.8 for the mutants defective in *acrB*, *tolC* and *acrB/tolC* respectively, demonstrating that both, *acrB* and *tolC*, contributed to *in vitro* fitness and that simultaneous mutation of both genes resulted in additive effects. In both media individual growth rates for respective mutants were comparable suggesting that the composition of the growth medium had no effect on particular mutants' phenotypes.

Susceptibility of tolC *mutants towards phytoalexins and other antimicrobial compounds*

Determination of minimal inhibitory concentrations (MICs) of various antibiotics and phytoalexins was used to examine the susceptibility of *tolC* mutants as compared with the wild-type strain and the previously studied *acrB* mutant (Burse *et al*., 2004) in complex and minimal medium (Tables 1 and 2). As potential substrates of TolC and/or AcrAB different isoflavonoids were analysed, which are known phytoalexins in *Rosaceae* plants (Harborne, 1999; Grayer and Kokubun, 2001) and which were previously identified and isolated from apple tissue

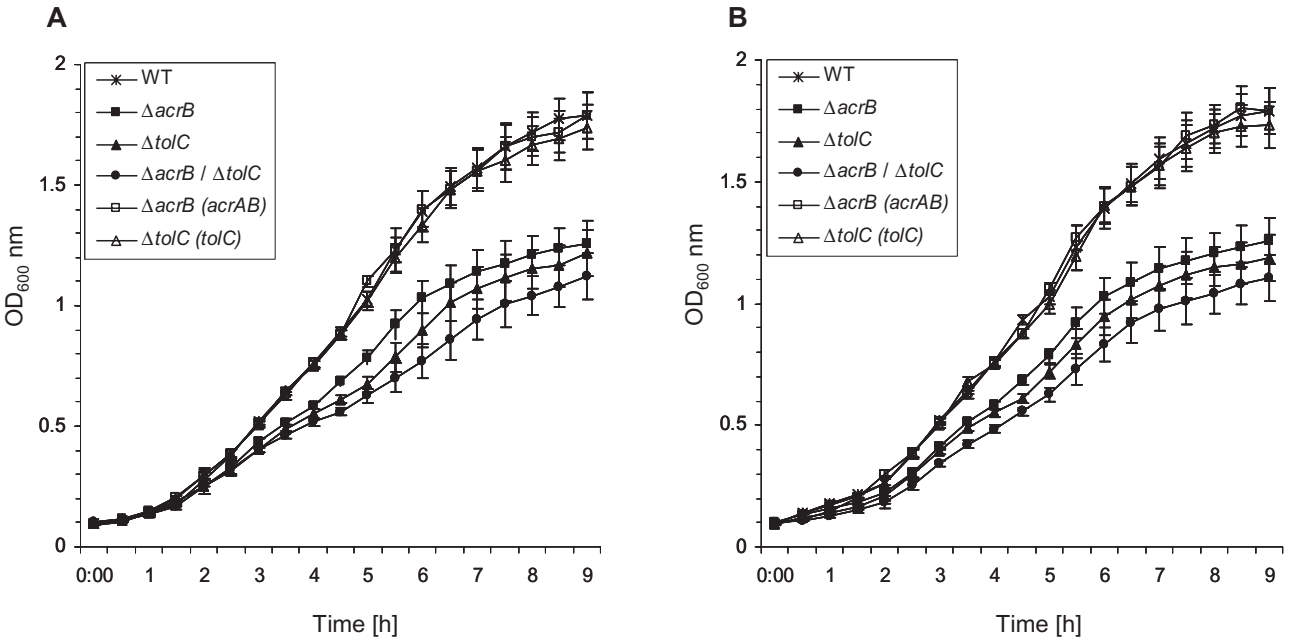

Fig. 1. *In vitro* growth of *E. amylovora* wild type and mutants in LB (A) and AMM2 (B) medium at 28°C as determined by measurement of the OD_{600}. Data represent the means of three independent cultures ± standard deviation.

(Treutter, 2001). Additionally, bile salt as a reported substrate for AcrAB in *E. coli* (Thanassi *et al*., 1997) and several other antimicrobials were tested in order to determine substrate specificity profiles. In general, differences in MIC values were only considered significant if they were at least fourfold. This cut-off is consistent with previous publications by others (Stermitz *et al*., 2000; Andrews, 2001), due to the sensitivity of the used method, and was meant to avoid misinterpretation of data.

In the *tolC* mutant and the *acrB/tolC* double mutant MICs for the dihydrochalcone phloretin, the flavonol quercetin, the flavanone naringenin, the monomeric flavanol

Table 1. Susceptibility of *Erwinia amylovora* strains to different compounds in medium MHB.

	MIC[b] (μg ml^{-1})					
Compounds[a]	Ea1189	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	Δ*tolC* (*tolC*)
Phloretin	1000	125	125	125	> 1000	> 1000
Naringenin	1000	62.5	125	125	1000	1000
(+)-Catechin	> 1000	125	250	250	> 1000	> 1000
Quercetin	1000	62.5	62.5	125	> 1000	1000
Berberine	1000	31.2	31.2	31.2	1000	1000
Bile salt	> 1000	125	125	125	> 1000	> 1000
Acriflavine	15.6	1.56	3.12	3.12	31.2	31.2
Ampicillin	62.5	6.25	12.5	6.25	62.5	62.5
Novobiocin	62.5	1.56	1.56	1.56	62.5	62.5
Cefoperazone	12.5	3.12	3.12	3.12	12.5	12.5
Mitomycin	6.25	0.31	0.62	0.62	12.5	12.5
Tetracycline	6.25	0.62	0.62	0.62	12.5	6.25
Nalidixic acid	1.25	0.12	0.12	0.12	1.25	1.25
Norfloxacin	0.62	0.031	0.062	0.062	0.62	0.62
Ciprofloxacin	0.62	0.062	0.062	0.062	0.62	0.62
SDS	> 1000	100	100	100	> 1000	1000
Ethidium bromide	31.2	3.12	3.12	3.12	62.5	62.5
Crystal violet	3.12	0.62	0.62	0.62	3.12	3.12

a. Reserpine and salicylic acid were also tested (data not shown); however, the mutants and the parent strain showed similar MIC values.
b. Minimal inhibitory concentration determination by the dilution assay was repeated at least three times in each case thereby confirming consistencies of MIC values.

Table 2. Susceptibility of *E. amylovora* strains to different compounds in medium AMM2.

	MIC[b] (μg ml^{-1})					
Compounds[a]	Ea1189	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	Δ*tolC* (*tolC*)
Phloretin	1000	31.2	15.6	15.6	> 1000	> 1000
Naringenin	1000	15.6	31.2	31.2	1000	> 1000
(+)-Catechin	> 1000	31.2	31.2	31.2	> 1000	> 1000
Quercetin	1000	31.2	62.5	62.5	> 1000	> 1000
Berberine	1000	15.6	31.2	31.2	1000	1000
Bile salt	> 1000	31.2	62.5	31.2	> 1000	> 1000
Acriflavine	15.6	3.12	3.12	3.12	31.2	31.2
Ampicillin	31.2	1.56	0.75	0.75	31.2	62.5
Novobiocin	125	1.56	3.12	3.12	250	250
Cefoperazone	6.25	1.56	1.56	1.56	12.5	12.5
Mitomycin	12.5	0.31	0.31	0.31	12.5	12.5
Tetracycline	6.25	0.62	0.31	0.62	12.5	6.25
Nalidixic acid	2.5	0.25	0.25	0.25	2.5	2.5
Norfloxacin	0.31	0.031	0.062	0.062	0.62	0.62
Ciprofloxacin	0.31	0.031	0.015	0.015	0.62	0.62
SDS	250	3.12	3.12	6.25	250	500
Ethidium bromide	62.5	3.12	1.56	1.56	62.5	62.5
Crystal violet	0.75	0.15	0.15	0.31	3.12	1.56

a. Reserpine and salicylic acid were also tested (data not shown); however, the mutants and the parent strain showed similar MIC values.
b. Minimal inhibitory concentration determination by the dilution assay was repeated at least three times in each case thereby confirming consistencies of MIC values.

(+)-catechin, the isoquinoline alkaloid berberine, and bile salt were similar to that of the *acrB* mutant in both media and were decreased 8-fold, 16-fold, 16-fold, 8-fold, 32-fold and 8-fold respectively, as compared with the MICs in the wild type. There were no significant (i.e. more than fourfold) differences between the mutants with single *tolC* knockout or with simultaneous mutations in both, *tolC* and *acrB*, suggesting that both gene products were essential for the efflux of the tested compounds and that neither protein significantly interacted with additional third components to accomplish the efflux.

Complementation of the two single mutants with plasmids pNK7 (for the *tolC* mutant) and pNK8 (for the *acrB* mutant), respectively, fully restored tolerance towards the tested isoflavonoids thereby confirming that these hydrophobic compounds were substrates of the AcrAB–TolC efflux system in *E. amylovora*. The results furthermore suggested that lack of TolC resulted in increased susceptibility possibly due to accumulation of antimicrobials in the bacterial cells.

The three tested mutants were susceptible to the polyketides, tetracycline with a fivefold reduction of MIC values, and to hydrophobic quinolon derivatives such as nalidixic acid, norfloxacin and ciprofloxacin, with a 10-fold reduction of the MICs. In contrast to the wild type, the mutants were sensitive to the beta-lactam antibiotics, ampicillin and cefoperazone. In addition, the mutants showed increased susceptibility with about 10-fold reduction of the MIC to the aminocoumarin novobiocin, the aziridine mitomycin, the antiseptic acriflavine, the detergent SDS, and the dyes, crystal violet and ethidium bromide. Again complementation of either single mutant with the respective genetic locus restored wild-type resistance levels (Tables 1 and 2). Only the MICs of reserpine and salicylic acid did not differ from those determined for the wild type (data not shown) suggesting that neither compound acted as a substrate for AcrAB–TolC in *E. amylovora*. Just as observed for the *in vitro* growth, there was no significant (i.e. at least fourfold difference in MIC values) influence of the used growth medium indicating that activity of AcrAB–TolC was not significantly triggered by the physiological state of the cells.

Contribution of TolC to virulence and in planta *survival of* E. amylovora

First, any potential role for TolC on virulence of the fire blight pathogen was tested by inoculation of apple rootstock MM106 plants with wild type, the *tolC* mutant, and the *acrB/tolC* double mutant. The previously reported *acrB* mutant (Burse *et al*., 2004) was used as a control. Shoot tips of the plants were inoculated by the so-called prick technique (May *et al*., 1997), which mimics the natural infection process and allows for defined numbers of bacterial cells to be inoculated. Observable fire blight symptoms were the typical shepherd's crook-like bending of the shoot tip after 1 week post inoculation as well as ooze formation and necrosis after 3 weeks post inoculation. Such typical symptoms were induced by the wild type of *E. amylovora* Ea1189 (Fig. 2). In contrast to this finding and comparable to the previously published

Fig. 2. Pathogenicity assay on apple plant. Shoot tips were inoculated with 5 μl of 1×10^7 cfu ml^{-1} bacterial suspensions. Disease symptoms such as the typical 'shepherd's crook' formation and wilting developed on plants inoculated with *E. amylovora* wild type after 1 week of inoculation.

results for the *acrB* mutant of Ea1189, neither the *tolC* mutant nor the *acrB/tolC* double mutant exhibited any disease symptoms (Fig. 2). As expected, genetic complementation rendered the mutants virulent with typical symptoms produced. These result suggested that TolC plays an important role in virulence of the fire blight pathogen. As no significant differences in the lack of symptom developments were observed between the *tolC*, *acrB* and the *acrB/tolC* mutants it could be concluded that both proteins, i.e. TolC and AcrB, were fully required for symptom formation.

Next, bacterial colonization of the host plant 24 h post inoculation was studied postulating that TolC and AcrB might be likewise important for this initial step of the disease development. *Erwinia amylovora* Ea1189 and its mutants were inoculated at different cell numbers (10^3–10^7 cfu/inoculation sites) and bacterial multiplication was monitored by re-isolating bacterial cells from infected plant tissue after 24 h (Table 3). In contrast to the wild type, inoculation of all three mutants in low cell numbers (10^3 and 10^4 cfu/shoot) yielded in no re-isolatable colony forming units suggesting that the mutants did not survive. Inoculation of the mutants with cell numbers $> 10^5$ cfu/shoot were necessary for re-isolation of mutant cells. However, the populations decreased approximately 1000-fold for both single mutants and for the *tolC–acrB* double mutant indicating that mutants with knockout in *tolC* were clearly impaired in multiplication in apple tissue. Noteworthy, mutation of *tolC* in the genomic background of the *acrB* mutant did not lead to any additive effects in terms of this phenotype. In contrast, the wild type reached an approximately 10-fold increase in population density at 24 h post inoculation regardless of the initially cell numbers used for inoculation (10^3–10^7 cfu/shoot). As observed above, complementation of the mutants with plasmids, pNK7 (for the *tolC* mutant) and pNK8 (for the *acrB* mutant), restored the wild-type phenotype.

Table 3. Survival of *E. amylovora* strains in apple rootstock MM106.

Inoculums[a] cfu/shoot	Re-isolated bacteria cells[b] (cfu/shoot)					
	Ea1189	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	Δ*tolC* (*tolC*)
1.2×10^3	$4.4 \times 10^3 \pm 2 \times 10^2$	0	0	0	$4.1 \times 10^3 \pm 2 \times 10^2$	$4.3 \times 10^3 \pm 2 \times 10^2$
1.1×10^4	$1.6 \times 10^5 \pm 2 \times 10^3$	0	0	0	$1.5 \times 10^5 \pm 2 \times 10^3$	$1.5 \times 10^5 \pm 2 \times 10^3$
1.0×10^5	$5.4 \times 10^5 \pm 1 \times 10^3$	$1.2 \times 10^2 \pm 9 \times 10^1$	$1.5 \times 10^2 \pm 6 \times 10^1$	$1.9 \times 10^2 \pm 6 \times 10^1$	$3.4 \times 10^5 \pm 1 \times 10^3$	$3.4 \times 10^5 \pm 1 \times 10^3$
1.2×10^6	$7.1 \times 10^7 \pm 1 \times 10^4$	$6.4 \times 10^3 \pm 2 \times 10^2$	$8.1 \times 10^3 \pm 2 \times 10^2$	$9.9 \times 10^3 \pm 2 \times 10^2$	$7.6 \times 10^7 \pm 1 \times 10^4$	$7.5 \times 10^7 \pm 1 \times 10^4$
1.0×10^7	$8.8 \times 10^8 \pm 2 \times 10^5$	$1.7 \times 10^4 \pm 3 \times 10^3$	$1.7 \times 10^4 \pm 3 \times 10^3$	$1.8 \times 10^4 \pm 3 \times 10^3$	$8.4 \times 10^8 \pm 1 \times 10^5$	$8.5 \times 10^8 \pm 1 \times 10^5$

a. Bacteria were inoculated by prick technique in the shoot tip of plants.
b. Establishment of a population of *E. amylovora* strains was determined 24 h after inoculation. Data represent the average cfu numbers from 15 individual plants.

Discussion

Herein the role of the outer membrane protein, TolC, for antibiotics resistance, virulence, and *in planta* survival of the fire blight pathogen, *E. amylovora*, was investigated demonstrating its significance for each of these bacterial processes. Burse and colleagues (2004) had previously shown that mutation of *acrB* caused a clear reduction in resistance towards different antibiotics as well as towards plant isoflavonoids, isoprenoids and alkaloids. Furthermore, mutation of *acrB* led to an avirulent phenotype and caused a significant reduction in bacterial *in planta* survival (Burse *et al*., 2004).

The AcrAB–TolC tripartite system of *E. coli* mediates resistance towards a wide variety of lipophilic and amphiphilic compounds including bile salt, detergents, dyes and antimicrobial agents and is one of the best-characterized bacterial RND-type MDE pumps (Zgurskaya and Nikaido, 2000). AcrAB–TolC complexes were also described for *Salmonella enterica* (Baucheron *et al.*, 2004; 2005) and *Enterobacter cloacae* (Perez *et al*., 2007). However, in *E. coli*, TolC can also interact with several other transport systems besides AcrAB (Sharff *et al*., 2001; Ramos *et al*., 2002; Koronakis *et al*., 2004) prompting the question whether this is also the case for the enterobacterial fire blight pathogen. In Gram-negative bacteria other than *E. coli*, individual TolC-like outer membrane channels are often unique for a given RND-type transporter and their genes are consequently co-expressed in the same gene cluster as the RND-type pumps, such as in the case of the *oprJMN* cluster in *Pseudomonas aeruginosa* (Li *et al*., 1995; Koehler *et al*., 1997) or for the outer membrane channel, MtrE, in *Neisseria gonorrhoeae* (Hagmann *et al*., 1995). In contrast, in *Klebsiella pneumonia* AcrAB homologues specifically interact with the outer membrane protein, KocC, but their respective genes are not co-transcribed (Li *et al*., 2008). At least in terms of genomic localization of *acrAB* and *tolC*, *E. amylovora* seems to group along with *E. coli* and some of the other enterobacterial representatives mentioned above. The bicistronic *acrAB* operon (nucleotide numbers 1120951–1125307) is not co-transcribed with *tolC*, which is located at a very distant position (nucleotide numbers 3269783–3271233) within the *E. amylovora* genome (http://www.sanger.ac.uk/projects/E.amylovora).

The initial finding that the *in vitro* growth of the *acrB/tolC* double mutant was more impaired than that of the single mutants seemed to support the idea that AcrAB and TolC of *E. amylovora* may also interact with other proteins. However, simultaneous mutation of *acrB* and *tolC* did not yield in any significant additive or synergistic effects in terms of resistance towards phytoalexins or any other antimicrobial compound suggesting that at least for these functions AcrAB and TolC, respectively, might not recruit other transport partners. For proper *in vitro* growth in both complex and minimal medium, there might be a particular yet-to-be identified additional function, which does not require an exclusive AcrAB–TolC interaction.

In comparison with the wild type, the single *tolC* mutant and the *acrB/tolC* double mutant showed significant but similar reductions in resistance towards hydrophobic and amphiphilic antibiotics, dyes and detergents. A wide range of constitutively synthesized isoflavonoids has been described for members of the *Rosaceae* plant family (Harborne, 1999; Grayer and Kokubun, 2001). Notably, Mayr and colleagues (1995) found that the glycosides, phloretin, phloridzin and quercetin represented the quantitatively most abundant compounds in apple leaves of the cultivar 'Golden Delicious'. In the current study, the impact of various antibacterial compounds previously described for apple plants (Treutter, 2001) on *E. amylovora* wild type and its mutants were examined. Phloretin, quercetin, (+)-catechin and naringenin inhibited growth of the *tolC* mutant to very similar extent as the growth of the *acrB/tolC* double mutant and the *acrB* mutant. Consequently, it can be assumed that these compounds are likely to be exported by the AcrAB–TolC complex but not by AcrAB and TolC respectively, recruiting other components in *E. amylovora*. A total of six different MDE systems are present in the genome of *E. amylovora*. A BLAST search with the genome sequence of *E. amylovora* Ea237 using the amino acid sequence of AcrB from *E. coli* K12 strain DH10B (accession number YP-001729367) as query identified six homologous sequences in the genome of Ea237. At the amino acid sequence level, the respective predicted *E. amylovora* proteins showed the following identities (similarities given in brackets): AcrB with 83% (92%), AcrD with 78% (89%), MdtB with 81% (90%), MdtC with 73% (86%), and two MdtB- and MdtC-like proteins with 63% (79%) and 56% (73%) respectively (http://www.sanger.ac.uk/projects/E.amylovora).

The increased sensitivity towards diverse antibiotics was comparable to that of the previously described *acrB* mutant of *E. amylovora* and was corresponding to the previously reported broad substrate spectrum of AcrAB–TolC from *E. coli* and other homologous MDE pumps (Poole, 2000; Nishino and Yamaguchi, 2001; Sulavik *et al*., 2001). However, neither of the tested *E. amylovora* mutants showed increased sensitivity towards the indole alkaloid, reserpine, or the plant hormone, salicylic acid, which consequently might not belong to the substrate spectrum of AcrAB–TolC of *E. amylovora*.

In plant-associated bacteria, TolC was predicted to function in protection against phytoalexins, whose syntheses represent important plant defence mechanisms. For the phytopathogen, *Xylella fastidiosa*, it was shown that a *tolC* knockout impaired virulence and increased sensitivity to the grapevine phytoalexin, resveratrol

(Reddy *et al*., 2007). Likewise, in the phytopathogen, *Dickeya dadantii* (*E. chrysanthemi*), the importance of *tolC* for virulence and resistance towards plant-derived chemicals was shown (Barabote *et al*., 2003). Cosme and colleagues (2008) demonstrated that mutation of *tolC* in the nitrogen-fixing plant symbiont, *Sinorhizobium meliloti*, strongly affected the resistance against antimicrobial agents and was important for symbiotic functions. Herein, we demonstrated that individual or simultaneous disruption of single components of a MDE pump in *E. amylovora* resulted in similarly reduced symptom formation and in significantly reduced *in planta* survival shortly after infection. These findings indicated that the detoxifying activity might be linked to AcrAB–TolC as a complex. Thus, the infected plants were obviously able to block the infection and kill bacterial cells if those lacked the gene products of *acrB* or *tolC*. The results were substantiated by genetic complementation of the mutants to wild-type levels with recombinant *acrAB* and *tolC*. It is tempting to speculate that the AcrAB–TolC complex might enable bacterial cells to survive at and outgrow from the local infection site, in which antimicrobial substances of plant defence accumulate during early stages of infection.

Erwinia amylovora wild type and its mutants were additionally inoculated on tobacco leaves to monitor the development of the hypersensitive response (data not shown). Regardless of their reduced virulence, the *acrB* and *tolC* mutants elicited typical hypersensitive responses during the incompatible reaction with tobacco plants demonstrating that the *hrp* type III secretion system (Nissinen *et al*., 2007) was not influenced by the MDE system. For *E. chrysanthemi*, the TolC analogue, PrtF, was shown to be required for the secretion of a protease important for virulence (Létoffé *et al*., 1990). Our future studies will therefore focus on the in-depth analysis of potential function(s) of TolC in protein secretion as well as on the genetic exchange of individual AcrAB–TolC components between *E. amylovora* and *E. coli* in order to determine ecological niche-mediated specializations of either system. Additionally, future work will be directed towards the transcriptional regulation of the *tolC* gene of *E. amylovora* in order to determine whether its expression is substrate-dependent and whether expression of *acrAB* (Burse *et al*., 2004) and that of *tolC* is co-regulated by any potential substrate(s).

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study were listed in Tables 4 and 5. *Erwinia amylovora* strains were cultured at 28°C on LB medium or AMM2. Asparagine minimal medium 2 has the following composition (per litre of deionized water): fructose, 10 g; L-asparagine, 4 g; $Na_2HPO_4 \times 7\ H_2O$, 12.8 g; K_2HPO_4, 3 g; NaCl, 3 g; $MgSO_4 \times 7\ H_2O$, 0.2 g; nicotinic acid, 0.25 g; thiamine, 200 μg. *Escherichia coli* DH5α was used as cloning host. *Escherichia coli* cells were routinely maintained at 37°C in LB medium. Cultures were supplemented with 50 μg ml^{-1} ampicillin (Ap), 25 μg ml^{-1} chloramphenicol (Cm), gentamicin (Gm) at 2 μg ml^{-1} and 25 μg ml^{-1} kanamycin (Km) when necessary. Bacterial growth was monitored using a spectrophotometer (OD at 600 nm).

Standard genetic procedures

Restriction digestions, agarose gel electrophoresis, electroporation, PCR, ligation of DNA fragments, plasmid DNA isolation, and other routine molecular methods were performed by standard techniques (Sambrook and Russel, 2001). Purification of PCR products and DNA fragments from agarose gels were performed using Nucleospin columns (Macherey-Nagel, Dueren, Germany).

Generation of tolC *in* E. amylovora

Erwinia amylovora tolC-deficient mutants were generated by marker exchange mutagenesis as follows. Two fragments flanking the *tolC* gene were PCR-amplified from strain Ea1189 using the oligonucleotide primer pairs Ea-tolC-FwdI, Ea-tolC-RevI and Ea-tolC-FwdII, Ea-tolC-RevII respectively (Table 6). PCR products were cloned into pGEM-T Easy

Table 4. *E. coli* and *E. amylovora* strains used in this study.

Strain	Relevant characteristics	Reverence or source
E. coli		
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook and Russel (2001)
S17-1 λ-*pir*	λ-*pir* lysogen of S17-1	Wilson *et al.* (1995)
E. amylovora		
1189	Wild type	GSPB[a]
1189-3	Km^r, *acrB* mutant carrying Km^r cassette in the *acrB* gene	Burse *et al.* (2004)
1189-25	Gm^r, *tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene	This study
1189-3-3	Km^r, Gm^r, *acrB/tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene and Km^r cassette in *acrB* gene	This study
1189-25-1	Gm^r, Cm^r, complemented *tolC* mutant carrying pNK7	This study
1189-3-1	Km^r, Cm^r, complemented *acrB* mutant carrying pNK8	This study

a. GSPB, Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany.

Table 5. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference or source
pGEM-T Easy	Ap^r, ColE1 origin	Promega
pNK1	Ap^r, contains a 984 bp XhoI PCR fragment that located upstream of *tolC*.	This study
pNK2	Ap^r, contains a 912 bp XhoI/BamHI PCR fragment located downstream of *tolC*	This study
pPS858	Source of the Gm-GFP cassette	Hoang *et al.* (1998)
pNK3	Ap^r, Gm^r, 1843 bp Gm-GFP cassette from pPS858 cloned into BamHI-linearized pNK2	This study
pNK4	Ap^r, Gm^r, 2755 bp SpeI-XhoI GFPGm-downstream fragment from pNK3 cloned into SpeI-XhoI-linearized pNK1	This study
pCAM-MCS	Ap^r, pCAM140-derivative without mini-Tn5, contains the MCS of pBluescript II SK (+)	Burse *et al.* (2004)
pNK5	Ap^r, Gm^r, 3739 bp ScaI-EcoRI upstream-GFPGm-downstream fragment cloned into EcoRI-linearized pCAM-MCS	This study
pNK6	Ap^r, contains a 2630 bp PCR fragment carrying the *tolC* gene, upstream and downstream of the gene	This study
pNK7	Cm^r, contains a 2.6 kb fragment carrying *tolC* of *E. amylovora* cloned in pBBR1MCS by SpeI and SacII in opposite direction of *lacZ*	This study
pNK8	Cm^r, contains a 4.9 kb fragment carrying *acrAB* of *E. amylovora* cloned in pBBR1MCS by SpeI and SacI in opposite direction of *lacZ*	This study

Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Cm, chloramphenicol; GFP, green fluorescent protein.

(Promega, Mannheim, Germany) yielding plasmids pNK1 (3999 bp) and pNK2 (3927 bp). A 1843 bp BamHI fragment containing a green fluorescent protein–gentamicin-resistance cassette (GFP-Gm^r) was derived from pPS858 (Hoang *et al.*, 1998) and ligated into BamHI-digested pNK2, yielding the 5767 bp plasmid pNK3. A 984 bp XhoI-SpeI fragment was derived from pNK1 and ligated into XhoI-SpeI-digested pNK3, yielding the 6751 bp plasmid pNK4. A 3739 bp EcoRI fragment was obtained from pNK4 and ligated into EcoRI-digested suicide plasmid, pCAM-MCS (Burse *et al.*, 2004), yielding the final *tolC* replacement plasmid pNK5 (7439 bp). *Escherichia coli* S17-1 *λ-pir* was used as host for the suicide plasmid. The plasmid was transferred via electroporation to electro-competent *E. amylovora* wild type and its *acrB* mutant, which subsequently were grown at 28°C for 1 h in SOC broth and plated on LB containing gentamicin. Putative mutants were screened for homologous recombination events by checking for antibiotics resistance and GFP fluorescence. To confirm the altered genotypes, PCR amplification of *tolC* using primers Ea-tolC-FwdI and Ea-tolC-RevII (Table 6) was carried out, yielding a 3739 bp fragment for the two mutants, as opposed to the presence of wild-type *tolC*, forming a 2630 bp PCR fragment in samples of wild type and *acrB* mutant.

Complementation of mutants

Genetic complementation of *tolC* mutants was performed as follows: a 2630 bp fragment including the *tolC* gene and its upstream and downstream regions was PCR-amplified from strain Ea1189 using the oligonucleotide primers, Ea-tolC-FwdI and Ea-tolC-RevII (Table 6), cloned into pGEM-T Easy yielding the 5645 bp plasmid pNK6. A 2630 bp SpeI-SacII fragment was derived from pNK6 and ligated into SpeI-SacII-digested pBBR1MCS (Kovach *et al.*, 1995) yielding the final *tolC* complementation plasmid pNK7 (7337 bp). Complementation of the *acrB* mutant was performed as follows: a 4917 bp fragment including *acrAB* and its upstream and downstream region was PCR-amplified from Ea1189 using the oligonucleotide primers, Ea-acr-Com-Fwd and Ea-acr-Com-Rev (Table 6), cloned into SpeI-SacI-digested pBBR1MCS (Kovach *et al.*, 1995) yielding the final *acrAB* complementation plasmid pNK8 (9624 bp). Plasmids pNK7 and pNK8 were transferred to electro-competent cells of the *E. amylovora tolC* and *acrB* mutants respectively, which subsequently were grown at 28°C for 1 h in SOC broth and plated on LB containing appropriate antibiotics. To confirm complementation of the genotypes, plasmids were re-isolated from the complemented cells and the target gene(s) were PCR-amplified using appropriate oligonucleotides.

Table 6. Oligonucleotide primers used in this study.

Primer	Nucleotide sequence (5′-3′)[a]
Ea-tolC-FwdI	GCTCACCACATGCACAAG
Ea-tolC-RevI	CTCGAGAGTGTTGCTGTTAGAGCCAC
Ea-tolC-FwdII	GCCAAATTCAGCCACGCA
Ea-tolC-RevII	CTCGAGGATCCGCAAGTGAACAGCTCGAAG
Ea-acr-Com-Fwd	CGAGAGCTCCGCCAGTGACGTATTAGC
Ea-acr-Com-Rev	GATACTAGTCGGTATAGTAAACGTGCG

a. Restriction sites incorporated into primers are underlined: CTCGAG, XhoI; GGATCC, BamHI; GAGCTC, SacI; ACTAGT, SpeI.

Drug susceptibility tests

The MICs of diverse antibiotics were determined by a dilution assay in Mueller–Hinton broth (MHB) (Becton Dickinson, Heidelberg, Germany) and AMM2 respectively. All tests were done in triplicate following the National Committee for Clinical Laboratory Standards (2000) recommendations. Briefly, testing of MIC was carried out in micro-titer plates with 96 flat-bottomed wells. With the exception of those wells used as control, each well received 100 μl of a twofold dilution series of an antibiotic solution in the appropriate medium. Than each well except those used as sterility controls received 100 μl of bacterial suspension at 1×10^6 cfu ml^{-1}. The MIC was defined as the lowest concentration of an antibiotic that completely stopped visible cell growth. Growth of *E. amylovora* at 28°C was examined by visual and photometric inspection after 24 h of incubation.

Plant materials and pathogenicity assay on apple plants

Apple plants (rootstock *Malus* MM106) were grown in a light chamber at 20–25°C, 60% humidity, with a 12 h photoperiod (15 000 lux). *Erwinia amylovora* strains were grown on LB agar plates for 48 h, resuspended in sterile 0.9% NaCl solution and diluted to a cell density of 1×10^7 cfu ml^{-1} for inoculation. Apple plants were inoculated by the prick technique as described by May and colleagues (1997). Each bacterial strain was inoculated into shoots of five individual plants by placing 5 μl of bacterial suspensions onto each wound on the shoot tip. Plants were monitored for symptom development daily.

To study bacterial survival in plant tissue, 5 μl of bacterial suspensions (10^2–10^6 cfu ml^{-1}) was placed onto each wound on the shoot tips of nine individual plants. Sterile 0.9% NaCl solution was used as negative controls. Survival of bacteria in the plant tissue was examined by re-isolation of bacterial cells 24 h post inoculation. One centimetre of the shoot tip around the inoculation site was cut off. Samples from apple plants were pooled, homogenized in 0.9% NaCl solution, serially diluted, and appropriate dilutions were spread on LB agar plates.

All greenhouse experiments were repeated at least three times to confirm reproducibility and to calculate standard deviations.

Nucleotide sequence accession number

The nucleotide sequence of the *tolC* gene of *E. amylovora* 1189 was deposited in GenBank under Accession No. FJ462442.

Acknowledgment

This project was partially funded by a stipend from the Deutscher Akademischer Austauschdienst (DAAD).

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