Xanthomonas albilineans OmpA1 Appears to be Functionally Modular and Both the OMC and C-like Domains Are Necessary for Leaf Scald Disease of Sugarcane

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Several EZ-Tn5 insertions in gene locus XALc_0557 (OmpA1) of the sugarcane leaf scald pathogen *Xanthomonas albilineans* XaFL07-1 were previously found to strongly affect pathogenicity and endophytic stalk colonization. XALc_0557 has a predicted OmpA N-terminal outer membrane channel (OMC) domain and an OmpA C-like domain. Further analysis of mutant M468, with an EZ-Tn5 insertion in the upstream OMC domain coding region, revealed impaired epiphytic and endophytic leaf survival, impaired resistance to sodium dodecyl sulfate (SDS), structural defects in the outer membrane (OM), and hyperproduction of OM vesicles. Cloned full-length XALc_0557 complemented M468 for all phenotypes tested, including pathogenicity, resistance to SDS, and ability to survive both endophytically and epiphytically. Another construct, pCT47.3, which expressed only the C-like domain of XALc_0557, restored resistance to SDS in M468 but failed to complement any other mutant phenotype, indicating that the C-like domain functioned independently of the OMC domain to help maintain OM integrity. pCT47.3 also complemented pathogenicity, resistance to SDS, and stalk colonization in mutant M1152, which carries an EZ-Tn5 insert in the C-like coding region, indicating that both predicted domains are modular and necessary but neither is sufficient for *X. albilineans* pathogenicity, endophytic survival in, and epiphytic survival on sugarcane.

Xanthomonas albilineans is a gram-negative, xylem-invading phytopathogen that causes sugarcane leaf scald disease (Rott and Davis 2000). *X. albilineans* is close in phylogeny to the systemic xylem-limited pathogen *Xylella fastidiosa* (Pieretti et al. 2009). Like *X. fastidiosa*, *Xanthomonas albilineans* does not have a hypersensitive response and pathogenicity (*hrp*) type III secretion system (T3SS), and is one of only six phytopathogenic bacteria known to have a *Salmonella* pathogenicity island 1 (SPI-1) T3SS, which is associated with animal pathogens and insect endosymbionts (Cornelis 2006; Dale et al. 2001, 2004; Marguerettaz et al. 2010). Furthermore, *X. albilineans* is

the only known xanthomonad that does not produce xanthan gum (Pieretti et al. 2009). The only well-characterized *X. albilineans* pathogenicity factor described to date is albicidin, an antibiotic and DNA gyrase inhibitor that blocks chloroplast differentiation and is responsible for (nearly) all of the foliar symptoms of leaf scald caused by most *X. albilineans* strains (Birch and Patil 1985, 1987a and b; Hashimi et al. 2007). However, there is evidence for additional pathogenicity factors. For example, XaFL07-1 mutant $\Delta albXXI$, which does not produce albicidin, is still able to cause foliar symptoms of leaf scald on sugarcane ‘CP80-1743’, including chlorosis, necrosis, and diagnostic pencil-line stripes (Rott et al. 2011). In addition, albicidin-deficient mutants, including those causing no symptoms, are well able to endophytically colonize sugarcane (Birch 2001; Champoiseau et al. 2006a). Therefore, ability to colonize sugarcane is more fundamental to *X. albilineans* population fitness than ability to cause symptoms of leaf scald; colonization does not depend upon elicitation of pathogenic symptoms.

A search for additional pathogenicity factors in XaFL07-1 using random EZ-Tn5 mutagenesis resulted in 61 mutants with reduced ability to cause disease symptoms or colonize the sugarcane stalk (Rott et al. 2011). Surprisingly, 5 of the 61 (8.2%) were localized to a single gene, the 1,101-bp *X. albilineans* *ompA1* gene (locus tag XALc_0557). XALc_0557 is predicted to encode two distinct domains: an OmpA N-terminal outer membrane channel (OMC) domain (porin superfamily) and an OmpA C-like domain (Fig. 1).

OmpA is the most abundant outer membrane (OM) protein in Enterobacteria (Bosshart et al. 2012); it is present at 100,000 copies per cell in *Escherichia coli* (Koebnik et al. 2000). OmpA of *E. coli* has been found to fold into both open and closed conformations. The predominant state is the closed form, also known as the majority conformer. In this form, the OMC domain folds into an eight-stranded β -barrel and the C-like domain is anchored in the periplasm via noncovalent interactions with the bacterial cell wall (Arora et al. 2000; Nikaido 2003; Smith et al. 2007). The eight-stranded β barrel of the OMC domain of OmpA is thought to allow the passage of ions, although the crystal structure does not show a continuous passage (Pautsch and Schulz 1998). There is evidence of an ion-gating mechanism involving the modification of a salt bridge, allowing the formation of a small pore (Arora et al. 2000; Bond et al. 2002; Smith et al. 2007). The open conformation of OmpA, also known as the minority conformer, was first discovered in unilamellar proteoliposomes in approxi-

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mately 2 to 3% of the OmpA molecules examined (Sugawara and Nikaido 1992). In the open form, OmpA forms a 16-stranded β -barrel, which would produce a channel large enough for the passage of solutes. Existence of both OmpA forms was confirmed in *Salmonella enterica* (Singh et al. 2003).

The OMC domain of OmpA can function without the C-like domain, as in the case of gram-negative porins. Porins form nonspecific channels for small hydrophilic molecules. The major porins, such as OmpC and OmpF of *E. coli* (which form 16-stranded β -barrels and associate in the OM as trimers), have a single OMC domain that extends through most of the protein (Achouak et al. 2001).

In several species of animal pathogens, OmpA has been shown to play a critical role in virulence. In *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*), OmpA[−] mutants were 87% less invasive than the wild type in human cells (Mohan Nair and Venkitanarayanan 2007; Singamsetty et al. 2008). Similarly, OmpA[−] mutants of *Escherichia coli* were less able to invade C6 glioma cells (Wu et al. 2009), less invasive in brain endothelial cells, and less able to penetrate the blood-brain barrier than their wild-type counterparts (Wang and Kim 2002). OmpA[−] mutants of *E. coli* were also shown to be less able to form biofilms on various surfaces (Barrios et al. 2006; Ma and Wood 2009).

Although mutations of *ompA* in a variety of bacteria have been reported, multiple reports also exist documenting difficulty in cloning and expressing *ompA* or homologs from a variety of bacterial strains in *E. coli*: *ompA* of *E. coli* (Bremer et al. 1980), *ompA* of *Shigella dysenteriae* (Braun and Cole 1982), *omp1* of *Fusobacterium nucleatum* (Bolstad and Jensen 1993), *ompU* of *Vibrio cholerae* (Sperandio et al. 1996), *ompH* of *Pasteurella multocida* (Lee et al. 2004), and *mopB* of *Xylella fastidiosa* (Bruening et al. 2005 and 2007). Some reports hypothesized that the difficulty in expression might be due to toxicity of the foreign OmpA hydrophobic domains on the host cell (Laage and Langosch 2001).

Much less is known about the function of OmpA-like proteins in phytopathogenic bacteria. MopB, an OmpA-like protein in *X. fastidiosa*, was associated with chlorosis-inducing activity on the indicator plant *Chenopodium quinoa* (Bruening et al. 2002) and also with adhesion to xylem-rich balsa wood and cellulose disks (Bruening et al. 2003). Additionally, a knockout mutant of the OmpA-like MopB protein from *Xanthomonas campestris* pv. *campestris* was highly pleiotropic and no longer pathogenic to cabbage (*Brassica* sp.); in vitro, cells aggregated abnormally; were more sensitive to high temperatures, sodium dodecyl sulfate (SDS) and alkaline pH; and were deficient in exopolysaccharide production, adhesion, and motility (Chen et al. 2010). Notably, no distinction was made

between in planta survival or growth and elicitation of pathogenic symptoms in these studies.

We previously reported that the XALc_0557 EZ-Tn5 insertion mutants M468 and M1152, with insertions interrupting the OMC and C-like domains, respectively, of XALc_0557, had lost pathogenicity and ability to colonize sugarcane stalks, and exhibited reduced growth in vitro (Rott et al. 2011). Interestingly, a third reported Tn5 insertion mutant, M768, with an insertion site between the two domains (Fig. 1), inconsistently retained a low level of both pathogenicity and ability to colonize sugarcane stalks. The purposes of this study were to i) utilize complementation and additional in vitro and in planta assays to better characterize the pleiotropic functions of XALc_0557, ii) more closely examine the functions of the predicted OMC and C-like domains of XALc_0557 to determine whether the domains are independently functional, and iii) help define their individual roles in *X. albilineans* epiphytic survival, endophytic colonization, and pathogenesis of sugarcane.

RESULTS

XALc_0557 stabilizes the OM of *X. albilineans*.

Cells of wild-type XaFL07-1 exhibited a regularly shaped, relatively smooth OM (Fig. 2A), whereas the OM proteins of all three mutants were rougher and irregularly shaped (Fig. 2B to D). Additionally, the cells of M468 and M768 appeared thinner (reduced diameter) by transmission electron microscopy (TEM) than those of the wild-type strain and M1152. Both mutants M468 and M768 also seemed to have smaller electron transparent inclusion bodies than the wild type and M1152 by TEM. Although XaFL07-1 produced OM vesicles (OMV), those produced by all three mutants were much more abundant and also larger in size by both TEM (Fig. 2A to D) and scanning electron microscopy (SEM) (Fig. 2E to H). However, M1152 hyperproduced OMV to a level at least 10 $\times$ greater than the other two mutants, with some cells completely covered with OMV (Fig. 2H), and others were also present with very few or no OMV. Additionally, TEM revealed faulty septation and highly elongated cells with M468 that were less evident in M768 and not at all evident in M1152 (Fig. 2A to D).

To further investigate potential defects in the OM, growth in the presence of SDS was examined. Both XaFL07-1 and M768 were able to grow well in Modified Wilbrinks (MW) medium supplemented with SDS at 25 ppm, whereas M468 and M1152 were both strongly inhibited in growth on this medium (Supplementary Fig. S1). The EZ-Tn5 insertion in M768 was confirmed to be stable. These results indicated that the M468 and M1152 mutations in XALc_0557 perturbed the OM barrier function of *X. albilineans*, and that EZ-Tn5 mutations in M468 and M1152 were more severe than the EZ-Tn5 mutation in M768.

Fig. 1. Location of the M468, M768, and M1152 Tn5 insertions in the XALc_0557 gene relative to the regions used to create the complementation constructs. Region A is the region assayed by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) with primer set OmpANtermF + OmpANtermR. Region B is the region assayed by RT-qPCR with primer set OmpACtermF + OmpACtermR.

Fig. 2. Cells of *Xanthomonas albilineans* observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). **A** and **E**, XaFL07-1; **B** and **F**, M468; **C** and **G**, M768; **D** and **H**, M1152. Arrows indicate outer membrane vesicles. TEM bars = 100 nm (**A** to **D**) and SEM bars = 500 nm (**E** to **H**).

pRK-mopB restored SDS resistance to *X. albilineans* M468 but was unstable without selection.

mopB, cloned from *X. campestris* pv. *campestris* into wide host range (RepP) vector pRK415 (Chen et al. 2010), is 83% identical at the nucleotide level to XALc_0557. The predicted MopB protein includes both the OmpA OMC and C-like domains found in the predicted XALc_0557 homolog; therefore, pRK-mopB was expected to complement the XALc_0557 EZ-Tn5 mutations. DNA of pRK-mopB, extracted from *E. coli* DH5 α , proved difficult to directly transform by electroporation into any *X. albilineans* mutant strain. A helper plasmid, pPY0AAB27CH12 (Pieretti et al. 2009), carrying the methylation gene cluster from *X. albilineans* PC-73, was used in *E. coli* DH5 α to methylate pRK-mopB prior to electroporation into *X. albilineans*. Methylated pRK-mopB DNA extracted from *E. coli* carrying pPY0AAB27CH12 was electroporated with high transformation efficiency into M468. Provided that antibiotic selection pressure (i.e., tetracycline in the medium) was maintained, pRK-mopB in M468 restored resistance to SDS at 25 ppm.

However, when selection pressure was dropped and the *X. albilineans* cultures were allowed to grow for 7 days on solid MW medium and subsequently restreaked onto solid MW medium with tetracycline, no colonies grew, indicating that the plasmid was lost and that RepP plasmid pRK-mopB was unstable in *X. albilineans*. Not surprisingly, plants inoculated with M468 carrying pRK-mopB exhibited no complementation of pathogenicity over a 2-month period and no M468/pRK-mopB bacteria were recovered from inoculated plants (data not shown).

XALc_0557 cloned into pUFR047 was stable in *X. albilineans* without selection and restored resistance to SDS.

Full-length (1,101 bp) XALc_0557, including the native Shine-Dalgarno (SD) ribosomal binding site, was cloned into wide-host-range (RepW) vector pUFR047 (DeFeyer et al. 1993), resulting in pLF004.4. This construct was transformed into *E. coli*. DNA of pLF004.4 extracted from *E. coli* also proved difficult to directly transform by electroporation into any *X. albilineans* mutant strain. Again, pPY0AAB27CH12 was used to methylate pLF004.4 prior to electroporation into *X. albilineans* M468, which resulted in high transformation efficiencies.

Complementation using pLF004.4 was first evaluated by plating M468/pLF004.4 on MW medium amended with SDS, both with and without antibiotic selection for the plasmid. Resistance to SDS was restored, regardless of antibiotic supplementation, indicating that RepW vector pUFR047 was stable in *X. albilineans*.

pUFR047 was stable in *X. albilineans* in planta.

Plasmid pUFR047 stability in planta was more closely examined by inoculating sugarcane plants with wild-type strain XaFL07-1 carrying pUFR047, isolating the bacteria from the stalk as described, and comparing the population counts on selective medium with and without gentamycin. In all stalk colonization assays, no differences in cell counts were ever observed after a period of 2 months in planta, demonstrating that pUFR047 was stable in *X. albilineans* without selection.

Full-length XALc_0557 restores pathogenicity in M468.

Complementation of pathogenicity of M468 was examined in planta. Symptoms in sugarcane plants inoculated with M468 containing pLF004.4, although not quite as severe as the wild-type strain, were restored, and exhibited diagnostic pencil lines and necrosis typical of leaf scald disease (Fig. 3B).

XALc_0557 is required for *X. albilineans* endophytic stalk survival and stalk colonization.

Complementation of the defect in endophytic stalk colonization of M468 was also examined. M468 is unable to endophytically colonize sugarcane stalk tissue and was never recovered from inoculated stalks (Rott et al. 2011). To extend this work, survival in stalks of M468 and the complementing clone was examined in the present work. As previously observed, M468 was never recovered from inoculated sugarcane stalks. By contrast, M468/pLF004.4 not only survived but colonization was also restored; M468/pLF004.4 colonized sugarcane stalk tissue at population levels and to an extent nearly identical to that achieved by the wild-type strain: on a 0-to-100 scale, mean extent of stalk colonization (ESC) was 0 for M468 with empty vector pUFR047 and 88 for M468/pLF004.4, whereas mean ESC was 91 for wild-type XaFL07-1/pUFR047 (Fig. 4).

XALc_0557 assists *X. albilineans* epiphytic survival on leaves.

Leaves inoculated with XaFL07-1 by simple immersion were pressed onto agar plates 2 weeks after inoculation, resulting in a leaf impression on the agar surface and in growth of numerous colonies—sometimes even confluent growth—on selective medium. Colonies typically appeared in 10 to 100% of the imprint area (Fig. 5A and B). Imprints taken immediately after inoculation (day 0) exhibited confluent growth for all inoculated leaves because inoculum levels were high. Differences between strains (and leaves) only showed up with time; a 2-week assay delay was chosen to see survival differences between strains. Colony counts were taken and the mean extent of epiphytic survival (EES) of the wild-type strain reached values close to 50 (on a 0-to-100 scale). In contrast, imprinting of leaves inoculated with M468 resulted in growth of only a few colonies (Fig. 5C) and a mean EES value of 10 or less. Therefore, M468 appeared to have lost much of its capacity to survive on the sugarcane leaf surface. Epiphytic survival on leaf surfaces was restored by complementation using pLF004.4; M468/pLF004.4 colonized the surface of sugarcane leaves at population levels approaching those of the wild type (Fig. 5D).

XALc_0557 assists endophytic survival of *X. albilineans* in leaves and is required for pathogenic symptoms in leaves.

Levels of endophytic leaf survival and colonization were determined using at least 18 plants per mutant or wild-type strain inoculated using the decapitation method, which allows inoculum to be drawn into the xylem tissue of (usually up to five) inoculated leaves. Inoculations using the wild-type strain XaFL07-1 resulted in relatively consistent leaf colonization, with 28 of 33 leaves (85%) sampled on selective medium showing *X. albilineans* cell counts. Cell counts of the wild-type XaFL07-1 in leaf tissue sampled in this manner were 1.2×10^3 to 7.5×10^9 CFU/g (fresh weight) of macerated leaf tissue examined, for an average of 6.1×10^8 CFU/g of leaf tissue. In these leaves, including those with no cells counted in the sampled leaf area, the disease severity ranged from 4 to 6, and averaged 5.

By contrast with the wild-type strain, no mutant strain exhibited consistent leaf endophytic colonization; M468 was recovered from 6 of 33 leaves (18%) sampled, M768 was recovered from 7 of 25 leaves (28%) sampled, and M1152 was recovered from 2 of 33 leaves (6%) sampled. Surprisingly, cell counts of mutants M468 and M1152, which were never recovered from stalk tissue, were recovered from colonized leaves at relatively high population levels, ranging from 2×10^2 to 7.6×10^6 CFU/g of macerated leaf tissue examined, for an average population count of 9.8×10^5 CFU/g of leaf tissue. Polymerase chain reaction (PCR) was used to verify the mutant identity of

seven randomly chosen colonies of M468, M768, and M1152 recovered from these plants, and no revertants were detected (data not shown). Even in leaves with sampled cell counts of M468 as high as 7.6×10^6 CFU/g, no symptoms were observed up to 2 months postinoculation (i.e., the disease severity was 0 for all leaves inoculated with mutants). These results indicated that the endophytic stalk environment may be harsher for *X. albilineans* than the endophytic leaf environment, or that the mutants are deficient in the ability to efficiently move in leaves. These results also indicated that, although some sugarcane leaves provided an environment that allowed endophytic colonization of M468 and M1152, the bacteria did not cause disease and, therefore, required XALc_0557 not just for stalk colonization but also to condition disease symptoms.

M468 transformed with pLF004.4 was recovered from five of seven leaves (71%) sampled at populations of 3×10^3 to 1.5×10^6 CFU/g of tissue. Disease severity in leaves from these plants was 1 to 5, with an average of 4. Complementation restored ability of M468 to more consistently survive and grow endophytically in leaf tissue, and also restored ability of M468/pLF004.4 to cause disease, even at low population levels.

The OmpA C-like domain of XALc_0557 complements the full range of mutant phenotypes of M1152 but only SDS resistance in M468.

Plasmid pCT47.3 was constructed in an attempt to express only the C-like domain for the purpose of complementation (Fig. 1). The 3' end of XALc_0557 from the wild-type strain

was cloned using primers with an added canonical SD sequence (AGGAG) and methionine start site into pUFR047, forming pCT47.3. This plasmid restored resistance to SDS to both M468 and M1152. Restoration of SDS resistance in M468, with an insertion mutation that eliminates both the OMC and C-like domains, indicated that the C-like domain alone is important for stabilizing at least the barrier function of the OM. pCT47.3 also restored moderate levels of pathogenicity, stalk colonization, and leaf colonization to M1152 but not M468 (Fig. 3E). M1152 transformed with pCT47.3 was recovered from two of eight leaves at populations of 7.9×10^3 to 8.2×10^3 CFU/g of tissue. Disease severity in leaves from these plants was 1 to 4, with an average of 2.7. These results indicated that both the OMC and C-like domains were needed for full pathogenicity and for stalk colonization. These results also indicated that the two domains formed modules capable of independent function.

Regions downstream of the EZ-Tn5 insertions were transcriptionally active and complementation constructs restored transcription to wild-type levels.

Using FindTerm (energy threshold of -10 ; default is -11), a weak Rho-independent terminator was found in Tn5 but no transcriptional terminators were found in EZ-Tn5, using either ARNold or FindTerm. Expression of the XALc_0557 coding sequences corresponding to the OMC and C-like regions from the wild-type strain, M468, M468/pCT47.3, M468/pLF004.4, and M768 were examined to determine expression levels up-

Fig. 3. Complementation of XALc_0557 EZ-Tn5 mutations affecting pathogenicity on sugarcane 'CP80-1743'. A, M468/pUFR047; B, M468/pLF004.4; C, XaFL07-1/pUFR047; D, M1152/pUFR047; E, M1152/pCT47.3; F, H₂O.

stream and downstream of the EZ-Tn5 insertion sites (Fig. 1) by reverse-transcriptase quantitative (RT-q)PCR. The relative abundance of transcripts (ΔC_q) from the C-like region was approximately half that of the N-terminal region in the wild-type strain (data not shown), which is consistent with the 3' to 5' directionality of mRNA decay of *ompA* in *E. coli* (von Gabain et al. 1983). The $\Delta \Delta C_q$ or relative normalized expression method was used to calculate the relative expression of the N-terminal and C-terminal regions; results are presented in Supplementary Fig. S2. The N-terminal region was stably and consistently expressed in all samples. Transcription downstream of EZ-Tn5 was detected in both M468 and M768. The relative normalized expression of the C-like domain in M468 was 15-fold less than in the wild type (P value = 0.003) but, in M768, it was only 3-fold less than in the wild type (P value = 0.016). The expression level of the C-terminal region in M468 carrying either the C-terminal or full-length complementation constructs was restored to the same levels as the wild-type strain (P value = 0.760 and 0.650, respectively).

M768 likely formed a translational fusion that functionally separated the two OmpA1 domains.

Examination of the EZ-Tn5 transposon insertion site in M768 revealed a potential SD ribosomal binding site [AGG(G)] beginning at the 1,196th base of the EZ-Tn5 sequence and an ATG start site 6 bp immediately downstream of the potential SD, which would form an in-frame translational fusion with the XALc_0557 open reading frame (ORF) at the site of the M768 insertion (Supplementary Fig. S3). Because no transcriptional terminator was predicted to be downstream from the *kan2* gene on the transposon, the Kan-2 promoter of EZ-Tn5 could drive a translational gene fusion. This predicted gene fusion included the entire C-like domain of XALc_0557, adding six amino acids from the transposon to the N-terminus of the XALc_0557 native sequence, and functionally separating the OMC domain from the C-like domain. Levels of expression of

the C-like domain of the predicted translational fusion were threefold lower than that of the wild type.

DISCUSSION

OmpA of *E. coli*, the archetypical integral OM protein, has been called a molecular Swiss army knife (Smith et al. 2007). Consistent with that characterization of multifunctionality, the present study shows that XALc_0557 plays a central role in *X. albilineans* OM integrity, epiphytic survival, endophytic survival, and pathogenicity. Mutations affecting the OM proteins of several gram-negative bacterial plant pathogens have been shown to compromise the critically important barrier function of OM proteins and allow host defense compounds, including phytoalexins or reactive oxygen species, to be much more effective against bacteria suffering these mutations (Balsanelli et al. 2010; Kingsley et al. 1993). XALc_0557 knockout mutants M468 and M1152 were never recovered from inoculated sugarcane stalks, and both mutants exhibited greater sensitivity to SDS, which strongly indicated that the barrier function of the OM was severely compromised, at least in these mutants; which, in turn, likely affected both epiphytic and endophytic survival in planta.

The phenotypes of mutant M768, particularly the inconsistent low level of pathogenicity and stalk colonization as compared with four other EZ-Tn5 insertions in the same locus (Rott et al. 2011), were at first puzzling. Transposon insertions are generally considered to be polar, although there have been exceptions reported with Tn5 (de Bruijn and Lupski 1984). In terms of a gene encoding two predicted domains, a Tn5 insertion should eliminate transcriptional read-through to the downstream portion of the affected gene. However, M768 exhibited transcriptional read-through and the downstream region was clearly expressed, although expression was diminished. The phenotypes of M768 were similar to those of M1152/pCT47.3, except that pathogenicity and stalk colonization of M768 were much weaker, indicating that the translationally fused C-like domain in M768 was expressed at a much lower level than the same domain in pCT47.3, expressed in M1152. Together, the

Fig. 4. Stalk colonization of *Xanthomonas albilineans* wild-type, mutant, and complemented strains. Strains were recovered from a minimum of eight sugarcane stalks, all inoculated as described (arrow indicates the region of attachment of the inoculated leaves) and at similar initial cell densities (5×10^8 CFU/ml). Bracket thickness (width) corresponds to cell density recovered 2 months postinoculation from the indicated stalk region; * indicates strain carries empty vector pUFR047.

Fig. 5. Growth of *Xanthomonas albilineans* on selective medium shown imprinting of sugarcane leaves (upper and lower surfaces). Growth shown 2 weeks after immersion of the leaves in a suspension of *X. albilineans* (1×10^7 CFU/ml). **A**, XaFL07-1 (wild-type); **B**, XaFL07-1/pUFR047; **C**, M468; **D**, M468/pLF004.4.

phenotypes of M768 and M1152/pCT47.3 (SDS resistance, stalk colonization, and ability to cause symptoms of leaf scald) support the idea that both the OMC and OmpA C-like domains of XALc_0557 are needed for pathogenicity and stalk colonization by *X. albilineans* and that both domains can function independently of each other.

The intact OmpA C-like domain appeared to be required for robust growth on SDS media. Both pLF004.4 and pCT47.3 restored SDS resistance to M468. M468 carries a Tn5 insertion in the upstream OMC domain that virtually abolished expression of the OmpA C-like domain. Because pCT47.3 restored SDS resistance to M468 and encoded only the OmpA C-like domain of XALc_0557, it appears that the OmpA C-like domain of XALc_0557 alone was responsible for restoring SDS resistance, likely by stabilizing the OM. Despite the overall appearance by TEM of the M1152 cells being more similar to the wild type (compare Fig. 2A with D) than to the other two mutants, the disrupted portion of the C-like domain in M1152 may destabilize the predicted periplasmic anchoring of the OM to the bacterial cell wall. In addition to a defensive role, XALc_0557 also appeared to play a role in pathogenic offense. Surprisingly, and despite the fact that knockout mutants M468 and M1152 were never recovered from stalk tissues, endophytic leaf populations of M468 and M1152 mutants were sometimes recovered at levels similar to those observed with the wild-type strain. Nevertheless, the inoculated leaves that retained endophytic *X. albilineans* mutant bacteria remained completely asymptomatic. The mutants were unable to cause the characteristic pencil-line streaks and chlorosis in leaf tissue caused by the wild type, despite their ability to produce albidin when grown on agar plates (Rott et al. 2011).

Mutants M468 and M768 produced more and much bigger OMV than the wild type, and M1152 hyperproduced OMV (at least 10× more) when compared with the other two mutants. M1152 may be hypervesiculating due to the greater abundance of misfolded OmpA1 in the OM. When misfolded or toxic levels of proteins accumulate in the OM proteins of bacteria, the result can be production of OMV that package these proteins so that they are removed from the cell (McBroom and Kuehn 2007). M768 produced less of the C-terminal region than the wild type because its expression was driven off of the Tn5 transposon and translation was dependent on a predicted weaker SD sequence (AGG) than that of XALc_0557 (AAGGAG). In addition, the two expressed (OMC and C-like) domains would be physically unlinked. By contrast, M1152, with a predicted truncated C-like region, would likely have levels of expression of the C-like region equivalent to that of the wild type but the mutant C-like domain would be physically linked to the OMC region. Consequently, M1152 should produce abundant misfolded OmpA1, while M768 produced both domains intact but physically separated.

XALc_0557 proved difficult to clone in high copy vectors allowing expression in *E. coli*, in line with multiple reports of such difficulties (see above). More than 30 PCR amplicons from this locus, most created using *Taq* polymerase with proof-reading activity, were TA-cloned in pCR2.1-TOPO (high copy in *E. coli*) and sequenced; all of them had one or more mutations affecting the predicted coding sequence (data not shown). In order to achieve a functional full-length clone, domains containing errors were swapped with error-free fragments and cloned directionally in order to avoid expression of XALc_0557. The final clone was directionally recloned to allow expression in a vector (pUFR047, repW) that replicates at low-moderate copy in *E. coli* (DeFeyter et al. 1990). The resulting *E. coli* strains carrying XALc_0557 appeared to grow normally. Notably, a low-copy vector was also used in the work reported by Chen and associates (2010).

The mechanism by which XALc_0557 in *X. albilineans* plays an offensive role in pathogenicity remains speculative. The fact that wild-type *X. albilineans* cells produce vesicles (Fig. 2A and E) indicates a potential secretion mechanism that has not previously been reported for *X. albilineans* but has for *X. campestris* pv. *campestris* (Sidhu et al. 2008). Indeed, OMV appear to be produced by all gram-negative bacteria examined to date and comprise budded, released spheres of OM surrounding periplasmic content that can include signaling and virulence compounds, including toxins (Schwechheimer et al. 2013). It is possible that XALc_0557 may indirectly affect the efficiency of albidin export and secretion from *X. albilineans* cells in planta, because loss of OM integrity may, in turn, cause misassembly or loss of function of the albidin efflux pump (Pieretti et al. 2009). Alternatively or in addition, XALc_0557 may play a role in attachment of *X. albilineans* to sugarcane leaf surfaces as a first step in colonization or invasion, as suggested by Chen and associates (2010) for *X. campestris* pv. *campestris* and Bruening and associates (2003) for *Xylella fastidiosa*. *Xanthomonas albilineans* is aeri ally transmitted (Daugrois et al. 2003), which presumably involves the need for attachment, and OmpA1 mutant M468 proved to be deficient in survival on sugarcane leaves. The hypervesiculation observed with all XALc_0557 mutants examined indicates that OmpA1 of *X. albilineans* plays a key role in vesicle production or stability that deserves closer examination in terms of conditioning pathogenicity.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

All strains and constructs used are summarized in Table 1. *X. albilineans* XaFL07-1, isolated in 2007 from sugarcane sampled in Canal Point, FL, was used in all experiments. XALc_0557 mutants 468, 768, and 1152 were created by electroporation of XaFL07-1 with 20 ng of the transposase-EZ-Tn5 DNA synaptic complex (Epicentre Biotechnologies, Madison, WI, U.S.A.), and previously reported by Rott and associates (2011). Bacteria were routinely cultured on MW medium (10 g of sucrose, 5 g of peptone, 0.50 g of K₂HPO₄ · 3H₂O, 0.25 g of MgSO₄ · 7H₂O, 0.05 g of Na₂SO₃, 15 g of agar, and 1 liter of deionized water, pH 6.8 to 7.0) at 28 to 30°C. EZ-Tn5 insertion mutants were grown on MW agar supplemented with kanamycin at 20 mg/liter. All strains were stored at –80°C as turbid cell suspensions in sterile distilled water.

E. coli strains Mach1, TOP10, and DH5α were grown in Luria Broth (LB) (Sambrook et al. 1989) or PYGM (5 g of peptone, 3 g of yeast extract, 40 ml of 50% glycerol, and 15 g of agar in 900 ml of deionized water, pH 7.4; after autoclaving, 100 ml of morpholinepropanesulfonic acid buffer was added) (Defeyter et al. 1990) at 37°C. Antibiotics were used at the following concentrations: ampicillin, 40 µg/ml; kanamycin, 50 µg/ml; tetracycline, 10 µg/ml; chloramphenicol, 25 µg/ml; and gentamycin, 3 µg/ml. Chemically competent *E. coli* was transformed using 10 to 150 ng of DNA in 50 µl of cells. After adding DNA, cells were incubated for 10 min on ice and were heat shocked at 42°C for 30 s, and 250 µl of room-temperature SOC medium (Sambrook et al. 1989) was immediately added. The mixture was then incubated at 37°C for 1 h and plated on appropriate selective media.

Electrocompetent *X. albilineans*.

Liquid cultures of XALc_0557 mutants or the wild type in a total volume of 25 ml MW medium were grown at 28°C for 2 to 3 days (to an optical density at 600 nm [OD₆₀₀] = 0.4 to 0.7) with shaking at 125 rpm in a rotary water bath shaker. Cultures were chilled in ice water for 15 to 30 min and centrifuged at 4°C

at 3,200 × g for 15 min. Cells were gently washed first in 50 and then in 25 ml of ice-cold distilled water and centrifuged at 4°C at 3,200 × g for 15 min. Pellets were resuspended in 1 ml of sterile distilled water and stored as 50-µl aliquots at –80°C.

Electroporation of *X. albilineans*.

Electrocompetent *X. albilineans* cells were thawed on ice. Plasmid DNA or ligation mixes (1 to 5 µl or approximately 50 to 150 ng) were added to prechilled cuvettes with 1-mm gaps and put on ice. Competent cells (40 µl) were pipetted into the cuvettes and incubated on ice for 30 s. Cells were electroporated at 1,800 kV using an Eppendorf 2510 electroporator (Westbury, NY, U.S.A.). Typically, time constants were between 5.6 and 6.2. Immediately after electroporation, 900 µl of MW medium was added and cells were transferred to 14-ml Falcon tubes and allowed to recover at 28°C for 3 to 4 h. Transformed cells were plated on MW medium with appropriate antibiotics.

Verification of EZ-Tn5 Stability in M768.

Colony touch PCR of M768 was performed directly from cells growing on media containing SDS to verify that this strain was not a revertant and the transposon was still intact. Three different primer sets were used: i) Kan2FP (5' ACC TAC AAC AAA GCT CTC ATC AAC C 3') + ompAR1 (5' AAA GCT TCA CTT GTT CTC GAC GTT CAG 3'), ii) Kan2RP (5' GCA ATG TAA CAT CAG AGA TTT TGA G 3') + ompAF2 (5' AGG ATC CAA CCG ATG CCA CCA CGG AC 3'), and iii) ompAF2 + ompAR1. Using M768 as PCR substrate, primer set 1 should amplify a 437-bp band, set 2 a 1,379-bp band, and set 3 a 2,884-bp band. The wild-type strain should not be amplified by primer sets 1 or 2, and should display a 1,663-bp amplicon with primer set 3.

Construction of pLF004.4.

A 1,156-bp fragment comprising the full-length XALc_0557 predicted ORF, annotated as *X. albilineans ompA1* and including the native SD region, was amplified by PCR from strain XaFL07-1. Accuprime *Taq* High Fidelity polymerase (Invitrogen Corp., Carlsbad, CA, U.S.A.), 2× Failsafe Buffer D (Epicentre), primer ompAF1 (5' CGG ATC CTG CCT CTA TCG TTT TAT CTC 3') with an added *Bam*HI site, and ompA R1 (5' AAA GCT TCA CTT GTT CTC GAC GTT CAG 3') with an added *Hind*III site were used. PCR products were cloned

into pCR2.1-TOPO (Invitrogen) and transformed into *E. coli* Mach 1 cells. Plasmid DNA was extracted and sequenced, resulting in pLF003. The plasmid was digested sequentially with *Bam*HI and *Hind*III and the resulting fragment was gel purified and ligated into broad-host-range, repW shuttle vector pUFR047 (DeFeyer et al. 1993), resulting in plasmid pLF004.4.

Construction of pCT47.3.

A 351-bp fragment corresponding to the C-Terminal OmpA C-like domain of XALc_0557 was amplified by PCR from XaFL07-1 using Accuprime *Taq* High Fidelity polymerase (Invitrogen), 2× Failsafe Buffer D (Epicentre), primer CTermF (5' TG AAT TCA GGA GTC TAG ATG CTG AAG GGT GTC AAC TTT GAC TTC 3') with an added *Eco*RI site and SD sequence, and primer CTermR (5' TTA AGC TTA TCA CTT GTT CTC GAC GTT CAG CT 3') with an added *Hind*III site. The PCR product was digested with *Eco*RI and *Hind*III and the resulting fragment was gel purified and ligated into pUFR047 and directly transformed into electrocompetent XALc_0557 M1152. Ten colonies were obtained. All colonies were screened using several primer sets to verify the presence of the gene within the vector. In all, 7 of the 10 colonies were PCR positive. Four of the seven colonies were inoculated in 10-ml liquid cultures in MW medium and grown for 3 days, and plasmids were extracted and sequenced. Only one plasmid extracted from colony 3 had a sequence that was 100% identical to the expected XALc_0557 sequence, and this construct was given the name pCT47.3.

Methylation of pLF004.4 and pRK-mopB.

E. coli DH5α containing pPY0AAB27CH12, a plasmid containing the methyltransferase gene XALc_2634 from *X. albilineans* PC73 (Champoiseau et al. 2006b; Pieretti et al; 2009), was inoculated into 1 ml of LB broth supplemented with chloramphenicol. Cells were cultured at 37°C for 16 h. A 50-µl aliquot was inoculated in 10 ml of LB supplemented with chloramphenicol and incubated at 37°C for 2 h. Cells were transferred to a 40-ml Oakridge tube and pelleted at room temperature. The cell pellet was resuspended in 700 µl of cold 0.1 M CaCl₂ solution, and 200-µl aliquots of cells were transferred to four sterile 16-by-100-mm glass tubes and placed on ice. Approximately 250 ng of plasmid DNA (pLF004.4 or pRK-mopB) was added to the cells and incubated on ice for 20 min. Tubes were trans-

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics ^a	Reference or source ^b
<i>Escherichia coli</i>		
<i>E. coli</i> DH5α	F [–] , <i>endA1</i> , <i>hsdR17</i> (rk [–] ,mk ⁺), <i>supE44</i> , <i>λ-thi-1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>Φ80lacZΔM15</i> , <i>Δ(lacZYA-argF)U169</i> , <i>phoA</i>	Invitrogen Corp.
<i>E. coli</i> Mach1-T1 ^R	F [–] , <i>Φ80lacZΔM15</i> , <i>ΔlacX74</i> , <i>hsdR</i> (rk [–] ,mk ⁺), <i>ΔrecA1398</i> , <i>endA1</i> , <i>tonA</i> (confers resistance to phage T1)	Invitrogen Corp.
<i>E. coli</i> TOP10	F [–] , <i>mcrA</i> , <i>Δ(mrr-hsdRMS-mcrBC)</i> , <i>Φ80lacZΔM15</i> , <i>ΔlacX74</i> , <i>recA1</i> , <i>araD139</i> , <i>Δ(ara leu) 7697</i> , <i>galU</i> , <i>galK</i> , <i>rpsL</i> , <i>StrR</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen Corp.
XaFL07-1	<i>Xanthomonas albilineans</i> , wild-type strain isolated in Florida	Rott et al. 2011
M468	Nonpathogenic Tn5 mutant derivative of XaFL07-1; XALc_0557::EZ-Tn5, Kn ^r	Rott et al. 2011
M768	Nonpathogenic Tn5 mutant derivative of XaFL07-1; XALc_0557::EZ-Tn5, Kn ^r	Rott et al. 2011
M1152	Nonpathogenic Tn5 mutant derivative of XaFL07-1; XALc_0557::EZ-Tn5, Kn ^r	Rott et al. 2011
Plasmids		
pPY0AAB27CH12	10.6-kb region from <i>X. albilineans</i> strain PC73 containing XALc_2634 (methyltransferase) cloned into pCNS, Cm ^r	Pieretti et al. 2009
pCR2.1-TOPO	3.9 kb; PCR cloning vector, Amp ^r , Kn ^r	Invitrogen Corporation
pUFR047	8.6 kb; IncW, Mob ⁺ , <i>lacZα</i> ⁺ , Par ⁺ , Gm ^r , Amp ^r	DeFeyer et al. 1993
pLF003	5.0 kb; 1.1-kb region containing XALc_557 of XaFL07-1 cloned into pCR2.1-TOPO, Amp ^r , Kn ^r	This work
pLF004.4	9.7 kb; 1.1-kb <i>Bam</i> HI/ <i>Hind</i> III fragment containing XALc_0557 from pLF003 cloned into pUFR047, Gm ^r , Amp ^r	This work
pCT47.3	8.9 kb; 351bp <i>Eco</i> RI/ <i>Hind</i> III fragment containing the C-terminal OmpA domain of XALc_0557 of XaFL07-1 cloned into pUFR047, Gm ^r , Amp ^r	This work
pRK-mopB	11.7 kb; 1.1-kb region containing mopB of <i>X. campestris</i> pv. <i>campestris</i> cloned into pRK415, Tet ^r	Chen et al. 2011

^a Amp = ampicillin, Gm = gentamicin, Kn = kanamycin, St = streptomycin, and Tet = tetracycline. PCR = polymerase chain reaction.

^b Invitrogen Corp., Carlsbad, CA, U.S.A.

ferred to 42°C for 1 min and incubated on ice for an additional 10 min. A volume of 1.8 ml of LB medium was added to each tube and cells were incubated without shaking at 37°C for 2 h. Aliquots of 100 µl were plated on PYGM agar supplemented with gentamycin and chloramphenicol. Methylation was verified by comparing the *HincII* restriction profile of methylated and unmethylated pLF004.4 or pRK-mopB extracts.

SDS tolerance assay.

MW agar medium was prepared as described above and autoclaved, and 10% filtered SDS in water was added to a final concentration of 25 ppm. *X. albilineans* wild-type and mutant cultures (2 to 3 days old) were streaked onto these plates and incubated at 30°C for 4 days, and relative growth was compared.

Epiphytic survival bioassay.

Tissue-cultured plantlets of CP68-1026 sugarcane exhibiting two to three fully expanded leaves were immersed for 5 s in a suspension of *X. albilineans* at 10^7 CFU/ml of distilled sterile water. After immersion, plantlets were placed in 200-by-20-mm test tubes containing nutritive plant growth medium (Rott and Chagvardieff 1984), and incubated in a growth chamber at 28°C with 12 h of light. Two weeks after inoculation, the upper and lower surfaces of leaves were imprinted on WSD selective medium (Davis et al. 1994). The plates were examined for the presence or absence of bacterial colonies of *X. albilineans* after 5 days of incubation at 28°C.

Epiphytic survival was estimated by using a scale of 0 to 6, where 0 = no colonies in the leaf imprint, 1 = 1 to 50 colonies in the leaf imprint, 2 = more than 50 colonies and no confluent growth of bacteria in the leaf imprint, 3 = confluent growth of bacteria in less than 10% of the leaf imprint, 4 = confluent growth of bacteria in 10 to 40% of the leaf imprint, 5 = confluent growth of bacteria in 41 to 80% of the leaf imprint, and 6 = confluent growth of bacteria in 81 to 100% of the leaf imprint. EES was expressed as $EES = 100 [(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6)/6 \times N_T]$, where N_i = number of the leaf surfaces with score i and N_T = total number of leaf surfaces per plantlet.

Plantlets were inoculated with wild-type strain XaFL07-1, XALc_0557 mutant M468, M468/pLF004.4, and sterile distilled water as control. Six plantlets were inoculated per strain and randomly distributed in the growth chamber. The experiment was repeated independently once.

Inoculation of sugarcane by decapitation.

Suspensions of *X. albilineans* strains to be tested for pathogenicity were standardized to $OD_{600} = 0.30 \pm 0.02$ in sterile distilled water. Sugarcane CP80-1743 with at least three developed stalk internodes was inoculated with approximately 300 to 600 µl of *X. albilineans* cell suspension by the decapitation method as described (Rott et al. 1997), in greenhouse conditions. Briefly, leaves that had developed sufficiently to expose the dewlaps were numbered from the top of the stalk downward and the tightly whorled leaf region between the third and fourth dewlaps, which usually included leaves attached to newly formed stalk nodes, was cut through with sterile pruning shears. The *X. albilineans* cell suspension was then applied with a pipette to the cut leaf surface, and the suspension was naturally drawn into the xylem. In each inoculation experiment, at least eight plants were inoculated with each strain or water (as a control).

Symptom assessments.

One month postinoculation, visual observations of qualitatively assessed leaf symptoms were recorded for at least three emerging leaves per plant. The symptoms, expressed as dis-

ease severity on a scale of 0 to 6, were scored as follows: 0 = no symptoms, 1 = one to five pencil lines, 2 = six to ten pencil lines, 3 = more than 10 pencil lines, 4 = leaf chlorosis or less than 10% necrosis, 5 = 10 to 50% leaf necrosis, and 6 = more than 50% necrosis.

Assessment of leaf colonization.

One month postinoculation, leaves were sampled using scissors sterilized with 95% ethanol. Leaves were cut with a scalpel into approximately 2-in. sections and weighed. Leaf tissue was sterilized by brief submersion in 95% ethanol and flaming. Sterilized leaf tissue was then chopped into small pieces using a sterile scalpel and forceps in plastic petri dishes and 1 ml of Tris-buffered saline buffer was pipetted onto the chopped leaf fragments. After 2 h of incubation at room temperature, homogenates were serially diluted and plated in triplicate on MW medium supplemented with cephalixin at 25 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WCNCB medium) (Rott et al. 2011) and MW medium supplemented with kanamycin at 20 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WKNCB medium) (Rott et al. 2011). Plates were incubated for 3 to 5 days at 30°C.

Assessment of stalk colonization.

Two months postinoculation, leaves were removed and stalks were cut at the soil level by sterile pruning shears. The rind of the stalk was cleaned with 95% ethanol and paper towels. It was sprayed again with 95% ethanol and flame sterilized. Using sterilized pruning shears, the stalk was cut in between nodes and the cut section was pressed onto WCNCB and WKNCB for Tn5 mutants or MW medium supplemented with gentamycin at 3 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WGNCB) for plasmid constructs with gentamycin resistance or MW medium supplemented with tetracycline at 10 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WTNCB) for plasmid constructs with tetracycline resistance. Stalk colonization was assessed in 10 locations: I – 4 (representing four internodes below the first stunted internode) through I + 5 (representing five internodes above the first stunted internode, called I0). Stalk colonization was quantified with the following scoring system: 0 = no bacterial colony in the stalk imprint, 1 = 1 to 10 colonies in the stalk imprint, 2 = more than 10 colonies or confluent growth of bacteria in less than 25% of the stalk imprint, 3 = confluent growth of bacteria in 25 to 75% of the stalk imprint, and 4 = confluent growth of bacteria in more than 75% of the stalk imprint. ESC was expressed as $ESC = 100 [(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)/4 \times N_T]$, where N_i = number of internodes with score i and N_T = total number of internodes.

TEM.

Bacteria that grew for 2 to 3 days on Wilbrink's medium were fixed in a 4% glutaraldehyde and 0.1 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide, dehydrated using a series of acetone washes, and embedded in TAAB 812 epon resin. Ultrathin sections (60 nm) were mounted on collodion carbon-coated copper grids, contrasted using uranyl acetate and lead citrate, and examined at 80 kV with a transmission electron microscope (Jeol 100CX II).

SEM.

Bacteria that grew for 2 to 3 days on Wilbrink's medium were deposited upon a polylysine coated cover glass and fixed with 2% glutaraldehyde in cacodylate buffer, pH 7.2, for 2 h at room temperature. Bacteria were then washed in cacodylate

buffer and fixed samples were dehydrated using a graded ethanol series (30 to 100%), followed by 2 min in hexamethyldisilazane. Subsequently, the samples were coated with an approximately 10-nm-thick gold film and examined with a scanning electron microscope (Hitachi S4000).

DNA sequence analysis.

ORF were predicted using pDRAW32 by AcaClone Software. Conserved OmpA domains were identified using BLASTp, and homologues of *X. albilineans ompA1* were identified using the BLASTn algorithms from the National Center for Biotechnology Information.

Transcription terminator analysis of EZ-Tn5 and Tn5.

The DNA sequences of EZ-Tn5 and Tn5 were obtained from Epicentre and GenBank, respectively. Web-based transcriptional terminator analysis sites ARNold and FindTerm, available from Softberry (Mount Kisco, NY, U.S.A.) were used with default settings. In the case of FindTerm, the default energy threshold setting was adjusted higher to allow detection of weaker terminators.

Extraction of RNA, DNase treatment, and cDNA synthesis.

X. albilineans strains were inoculated into liquid MW media supplemented with appropriate antibiotics and grown until early log stage ($OD_{600} = 0.15 \pm 0.02$). Starter cultures were transferred to fresh media and grown until reaching $OD_{600} = 0.30 \pm 0.10$. RNA was immediately stabilized by the addition of 2 ml of RNeasy Protect Bacteria Reagent (Qiagen, Valencia, CA, U.S.A.) to 1 ml of culture. The mixture was vortexed for 5 s, incubated at room temperature for 5 min, and centrifuged at $3,700 \times g$ for 15 min. The supernatant was poured off and RNA was extracted using the RNeasy Protect Bacteria Mini Kit (Qiagen) Protocol 4 (Enzymatic Lysis and Proteinase K Digestion of Bacteria) followed by Protocol 7 (Purification of Total RNA from Bacterial Lysate Using the RNeasy Mini Kit). All extracts were analyzed for yield and purity using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). The A_{260}/A_{280} (absorbance) values of samples were 2.06 to 2.12 and the A_{260}/A_{230} values were greater than 0.17, indicating that extracts were relatively free of protein contaminants. No RT controls in initial experiments revealed significant DNA contamination of RNA extracts. RNA extracts were diluted in nuclease-free water to 120 ng/ μ l, and contaminating DNA in RNA extracts was removed by treatment with TURBO DNA-free Kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's protocol for "rigorous DNase treatment", and RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen).

Real-Time PCR.

The expression of the mRNA corresponding to the N-terminal OM-channels domain was assayed using the primer set Omp-ANtermF (5' CTG GTT TCA ATT TCC AGG ACG 3') + Omp-ANtermR (5' GTT CGG GTT CTG ATA GTT CAG 3'), which amplified a 125-bp fragment upstream of all EZ-Tn5 insertion sites. The expression of the mRNA corresponding to the C-terminal OmpA C-like domain was assayed using primer set Omp-ACtermF (5' CAA CTT TGA CTT CAA CAA GTC G 3') + Omp-ACtermR (5' TTG TCG CTA CCG ATG GAG TC 3'), which amplified a 135-bp fragment between the insertion sites of M768 and M1152. The specificity of the two primer sets was initially assessed by conventional PCR. RT-qPCR reactions were carried out using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.). The reaction mixture contained 10 μ l of 2 $\times$ QuantiTect SYBR Green PCR Master Mix (Qiagen), 25 ng of cDNA, and 300 nM each primer in a

total volume of 20 μ l. Reactions were incubated at 95°C for 15 min, and cycled 40 times at 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Standard curves were prepared to calculate the amplification efficiencies for the two primer sets across a linear dynamic range of six $\log_{10}$ concentrations, with $r^2 = 0.990$ and 0.993 for the N-terminal and C-terminal primer sets, respectively. Melting curve analyses were performed to assess the specificity of the products. Three technical replicates were performed for each of two biological replicates, along with no-RT controls (NRTC) and no-template controls (NTC) for each strain and primer set. Of 74 NRTC runs, only 3 amplified, with Cq values of 38.93, 34.76, and 37.29. The mean Cq values of the corresponding samples were 14.50, 14.56, and 14.97, respectively, indicating that the levels of DNA contamination were negligible. All NTC runs failed to amplify. Relative normalized expression levels were calculated with the Bio-Rad CFX Manager Software Package 3.0. A correction factor to account for the difference in amplification efficiencies between the N-terminal and C-terminal primer sets was applied and the wild-type strain was set as a control, with relative expression level set to 1. Student's *t* tests ($\alpha = 0.05$) were performed using the Bio-Rad CFX Manager Software Package 3.0.

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LITERATURE CITED

- Achouak, W., Heulin, T., and Pagès, J.-M. 2001. Multiple facets of bacterial porins. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 199:1-7.
- Arora, A., Rinehart, D., Szabo, G., and Tamm, L. K. 2000. Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. J. Biol. Chem. 275:1594-1600.
- Balsanelli, E., Serrato, R. V., de Baura, V. A., Sasaki, G., Yates, M. G., Rigo, L. U., Pedrosa, F. O., de Souza, E. M., and Monteiro, R. 2010. *Herbaspirillum seropedicae rfbB* and *rfbC* genes are required for maize colonization. Environ. Microbiol. 12:2233-2244.
- Barrios, A. F. G., Zuo, R., Ren, D., and Wood, T. K. 2006. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. Biotechnol. Bioeng. 93:188-200.
- Birch, R. G. 2001. *Xanthomonas albilineans* and the antipathogenesis approach to disease control. Mol. Plant Pathol. 2:1-11.
- Birch, R. G., and Patil, S. S. 1985. Preliminary characterization of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. J. Gen. Microbiol. 131:1069-1075.
- Birch, R. G., and Patil, S. S. 1987a. Correlation between albicidin production and chlorosis induction by *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Physiol. Mol. Plant Pathol. 30:199-206.
- Birch, R. G., and Patil, S. S. 1987b. Evidence that an albicidin-like phytoxin induces chlorosis in sugarcane leaf scald disease by blocking plastid DNA replication. Physiol. Mol. Plant Pathol. 30:207-214.
- Bolstad, A. I., and Jensen, H. B. 1993. Complete sequence of *ompI*, the structural gene encoding the 40-kDa outer membrane protein of *Fusobacterium nucleatum* strain Fev1. Gene 132:107-112.
- Bond, P. J., Faraldo-Gómez, J. D., and Sansom, M. S. P. 2002. OmpA: A pore or not a pore? Simulation and modelling studies. Biophys. J. 83:763-775.
- Bosshart, P. D., Iordanov, I., Garzon-Coral, C., Demange, P., Engel, A., Milon, A., and Müller, D. J. 2012. The transmembrane protein KpOmpA anchoring the outer membrane of *Klebsiella pneumoniae* unfolds and refolds in response to tensile load. Structure 20:121-127.
- Braun, G., and Cole, S. T. 1982. The nucleotide sequence coding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Res. 10:2367-2378.
- Bremer, E., Beck, E., Hindennach, I., Sonntag, I., and Henning, U. 1980.

- Cloned structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12: Localization on hybrid plasmid pTU100 and expression of a fragment of the gene. *Mol. Gen. Genet.* 179:13-20.
- Bruening, G., Civerolo, E., Kirkpatrick, B., and Gilchrist, D. 2002. Virulence analysis of the Pierce's disease agent *Xylella fastidiosa*. Pages 286-289 in: Proceedings of the 2002 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA. U.S.A.
- Bruening, G., Civerolo, E., Dandekar, A. M., and Gupta, G. 2003. Roles of *Xylella fastidiosa* proteins in virulence. Pages 135-137 in: Proceedings 2003 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA. U.S.A.
- Bruening, G., Civerolo, E., Feldstein, P., Francis, M., Dandekar, A. M., and Gupta, G. 2005. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control. Pages 221-224 in: Proceedings 2005 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA. U.S.A.
- Bruening, G., Civerolo, E., Feldstein, P., Dandekar, A. M., and Gupta, G. 2007. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control. Pages 173-176 in: Proceedings 2007 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA. U.S.A.
- Champoiseau, P., Daugrois, J.-H., Girard, J.-C., Royer, M., and Rott, P. 2006a. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. *Phytopathology* 96:33-45.
- Champoiseau, P., Daugrois, J.-H., Pieretti, I., Cociancich, S., Royer, M., and Rott, P. 2006b. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. *Phytopathology* 96:1081-1091.
- Chen, Y.-Y., Wu, C.-H., Lin, J.-W., Weng, S.-F., and Tseng, Y.-H. 2010. Mutation of the gene encoding a major outer-membrane protein in *Xanthomonas campestris* pv. *campestris* causes pleiotropic effects, including loss of pathogenicity. *Microbiology* 156:2842-2854.
- Cornelis, G. R. 2006. The type III secretion injectisome. *Nat. Rev. Microbiol.* 4:811-825.
- Dale, C., Young, S. A., Haydon, D. T., and Welburn, S. C. 2001. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U.S.A.* 98:1883-1888.
- Dale, C., Jones, T., and Pontes, M. 2004. Degenerative evolution and functional diversification of type-III secretion systems in the insect endosymbiont *Sodalis glossinidius*. *Mol. Biol. Evol.* 22:758-766.
- Daugrois, J.H., Dumont, V., Champoiseau, P., Costet, L., Boisne-Noc, R., and Rott, P. 2003. Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. *Eur. J. Plant Pathol.* 109:445-458.
- Davis, M. J., Rott, P., Baudin, P., and Dean, J. L. 1994. Evaluation of selective media and immunoassays for detection of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. *Plant Dis.* 78:78-82.
- De Bruijn, F. J., and Lupski, J. R. 1984. The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—A review. *Gene* 27:131-149.
- DeFeyer, R., Kado, C. I., and Gabriel, D. W. 1990. Small, stable shuttle vectors for use in *Xanthomonas*. *Gene* 88:65-72.
- DeFeyer, R., Yang, Y., and Gabriel, D. W. 1993. Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum* *avr* genes. *Mol. Plant-Microbe Interact.* 6:225-237.
- Hashimi, S. M., Wall, M. K., Smith, A. B., Maxwell, A., and Birch, R. G. 2007. The phytotoxin albicidin is a novel inhibitor of DNA gyrase. *Antimicrob. Agents Chemother.* 51:181-187.
- Kingsley, M. T., Gabriel, D. W., Marlow, G. C., and Roberts, P. D. 1993. The *opsX* locus of *Xanthomonas campestris* affects host range and biosynthesis of lipopolysaccharide and extracellular polysaccharide. *J. Bacteriol.* 175:5839-5850.
- Koebnik, R., Locher, K. P., and Van Gelder, P. 2000. Structure and function of bacterial outer membrane proteins: Barrels in a nutshell. *Mol. Microbiol.* 37:239-253.
- Laage, R., and Langosch, D. 2001. Strategies for prokaryotic expression of eukaryotic membrane proteins. *Traffic* 2:99-104.
- Lee, J., Kang, S., Park, S. I., Woo, H.-J., and Kwon, W. 2004. Molecular cloning and characterization of the gene for outer membrane protein H in a *Pasteurella multocida* (D:4) isolate from pigs with atrophic rhinitis symptoms in Korea. *J. Microbiol. Biotechnol.* 14:1343-1349.
- Ma, Q., and Wood, T. K. 2009. OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system. *Environ. Microbiol.* 11:2735-2746.
- Marguerettaz, M., Pieretti, I., Gayral, P., Puig, J., Brin, C., Cociancich, S., Poussier, S., Rott, P., and Royer, M. 2010. Genomic and evolutionary features of the SPI-1 type III secretion system that is present in *Xanthomonas albilineans* but is not essential for xylem colonization and symptom development of sugarcane leaf scald. *Mol. Plant-Microbe Interact.* 24:246-259.
- McBroom, A. J., and Kuehn, M. J. 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol. Microbiol.* 63:545-558.
- Mohan Nair, M. K., and Venkitanarayanan, K. 2007. Role of bacterial OmpA and host cytoskeleton in the invasion of human intestinal epithelial cells by *Enterobacter sakazakii*. *Pediatr. Res.* 62:664-669.
- Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* 67:593-656.
- Pautsch, A., and Schulz, G. E. 1998. Structure of the outer membrane protein A transmembrane domain. *Nat. Struct. Biol.* 5:1013-1017.
- Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M.-A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M., and Rott, P. 2009. The complete genome of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. *BMC Genomics* 10:616.
- Rott, P., and Chagvardieff, P. 1984. Inoculation de la canne à sucre (*Saccharum sp.*) cultivée in vitro avec *Xanthomonas albilineans* (Ashby) Dowson. *Agron. Trop.* 39:89-92.
- Rott, P., and Davis, M. J. 2000. Leaf scald. Pages 38-44 in: A Guide to Sugarcane Diseases. P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally, eds. La Librairie du Cirad, Montpellier, France.
- Rott, P. C., Mohamed, I. S., Klett, P., Soupa, D., de Saint-Albin, A., Feldmann, P., and Letourmy, P. 1997. Resistance to leaf scald disease is associated with limited colonization of sugarcane and wild relatives by *Xanthomonas albilineans*. *Phytopathology* 87:1202-1213.
- Rott, P., Fleites, L., Marlow, G., Royer, M., and Gabriel, D. W. 2011. Identification of new candidate pathogenicity factors in the xylem-invading pathogen *Xanthomonas albilineans* by transposon mutagenesis. *Mol. Plant-Microbe Interact.* 24:594-605.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.
- Sidhu, V. K., Vorholter, F. J., Niehaus, K., and Watt, S. A. 2008. Analysis of outer membrane vesicle associated proteins isolated from the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*. *BMC Microbiology* 8:87-102.
- Singamsetty, V.K., Wang, Y., Shimada, H., and Prasadarao, N.V. 2008. Outer membrane protein A expression in *Enterobacter sakazakii* is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. *Microb. Pathogen.* 45:181-191.
- Singh, S. P., Williams, Y. U., Miller, S., and Nikaido, H. 2003. The C-terminal domain of *Salmonella enterica* serovar Typhimurium OmpA is an immunodominant antigen in mice but appears to be only partially exposed on the bacterial cell surface. *Infect. Immun.* 71:3937-3946.
- Smith, S. G. J., Mahon, V., Lambert, M. A., and Fagan, R. P. 2007. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 273:1-11.
- Sperandio, V., Bailey, C., Giron, J. A., DiRita, V. J., Silveira, W. D., Vettore, A. L., and Kaper, J. B. 1996. Cloning and characterization of the gene encoding the OmpU outer membrane protein of *Vibrio cholerae*. *Infect. Immun.* 64:5406-5409.
- Sugawara, E., and Nikaido, H. 1992. Pore-forming activity of OmpA protein of *Escherichia coli*. *J. Biol. Chem.* 267:2507-2511.
- Schwechheimer, C., Sullivan, C. J., and Kuehn, M. J. 2013. Envelope control of outer membrane vesicle production in gram-negative bacteria. *Biochemistry* 52:3031-3040.
- Von Gabain, A., Belasco, J. G., Schottel, J. L., Chang, A. C. Y., and Cohen, S. N. 1983. Decay of mRNA in *Escherichia coli*: Investigation of the fate of specific segments of transcripts. *Biochemistry* 80:653-657.
- Wang, Y., and Kim, K. S. 2002. Role of OmpA and IbeB in *Escherichia coli* K1 invasion of brain microvascular endothelial cells in vitro and in vivo. *Pediatr. Res.* 51:559-563.
- Wu, H.-H., Yang, Y.-Y., Hsieh, W.-S., Lee, C.-H., Leu, S.-J., and Chen, M.-R. 2009. OmpA is the critical component for *Escherichia coli* invasion-induced astrocyte activation. *J. Neuropathol. Exp. Neurol.* 68:677-690.

AUTHOR-RECOMMENDED INTERNET RESOURCES

pDRAW32 DNA analysis software: www.acaclone.com
 National Center for Biotechnology Information BLAST database: blast.ncbi.nlm.nih.gov
 Institut de Génétique et Microbiologie ARNold database: rna.igmors.u-psud.fr/toolbox/arnold
 Softberry database: linux1.softberry.com