Peptidoglycan hydrolysis mediated by the amidase AmiC and its LytM activator NlpD is critical for cell separation and virulence in the phytopathogen Xanthomonas campestris

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SUMMARY
The essential stages of bacterial cell separation are described as the synthesis and hydrolysis of septal peptidoglycan (PG). The amidase, AmiC, which cleaves the peptide side-chains linked to the glycan strands, contributes critically to this process and has been studied extensively in model strains of Escherichia coli. However, insights into the contribution of this protein to other processes in the bacterial cell have been limited. Xanthomonas campestris pv. campestris (Xcc) is a phytopathogen that causes black rot disease in many economically important plants. We investigated how AmiC and LytM family regulators, NlpD and EnvC, contribute to virulence and cell separation in this organism. Biochemical analyses of purified AmiC demonstrated that it could hydrolyse PG and its activity could be potentiated by the presence of the regulator NlpD. We also established that deletion of the genes encoding amiC1 or nlpD led to a reduction in virulence as well as effects on colony-forming units and cell morphology. Moreover, further genetic and biochemical evidence showed that AmiC1 and NlpD affect the secretion of type III effector XC3176 and hypersensitive response (HR) induction in planta. These findings indicate that, in addition to their well-studied role(s) in cell separation, AmiC and NlpD make an important contribution to the type III secretion (T3S) and virulence regulation in this important plant pathogen.

Keywords: amidase, cell separation, LytM protein, peptidoglycan hydrolysis, T3SS, virulence, Xanthomonas.

INTRODUCTION
Gram-negative pathogenic bacteria have evolved various secretion systems, such as the type II (T2S), type III (T3S) and type IV (T4S) secretion systems, which span both the bacterial inner and outer membranes, to transfer virulence factors into host cells and cause disease (Costa et al., 2015). In the periplasmic space between the outer and inner membranes, there is a tight peptidoglycan (PG) layer, which is essential for the preservation of cell integrity by withstanding turgor and maintaining a defined cell shape (Silhavy et al., 2010; Vollmer et al., 2008). However, the PG layer can also be a barrier impeding the assembly of the membrane-spanning apparatuses of the secretion systems. Therefore, the PG layer needs to be locally remodelled by PG-lytic enzymes to provide space and acceptor sites for the secretion apparatus assembly (Burkinshaw and Strynadka, 2014; Koraimann, 2003; Scheuwater and Burrows, 2011). A typical Gram-negative bacterial PG layer is composed of glycan strands of β-1,4-glycosidic bond-linked N-acetylmuramyl-L-glucosamine (GlcnAc) and N-acetylmuramic acid (MurNac) disaccharide, which are cross-linked by short peptides to create the meshwork layer (Silhavy et al., 2010; Vollmer et al., 2008). Bacteria possess two main classes of PG-lytic enzymes: the glycosidases that cleave the glycan backbone and the amidases (or peptidases) that cleave the peptide side-chain (Firczuk and Bochtler, 2007; Frirdich and Gaynor, 2013; Vollmer et al., 2008). Previous studies have revealed that the lytic transglycosylase EtgA of Escherichia coli is required for efficient T3S. Purified recombinant EtgA can degrade PG in vitro and the mutant strain lacking EtgA is attenuated for T3S, suggesting that the lytic transglycosylase EtgA may contribute to PG rearrangement by selectively cleaving the backbone (Burkinshaw et al., 2015; García-Gómez et al., 2011).

It is not yet clear whether the PG amidases are required for the assembly of bacterial secretion systems, such as the T3S system (T3SS), although their significance in cell division has been well established. Previous studies have revealed that the E. coli N-acetylmuramoyl-l-alanine amidases AmiA, AmiB and AmiC are indispensable for daughter cell separation and their PG hydrolytic activity is activated by the LytM factors NlpD or EnvC. Inactivation of the genes encoding the amidases and the LytM factors results in a defect in cell separation, leading to the formation of long cell chains (Heidrich et al., 2001; Uehara et al., 2009, 2010). NlpD and EnvC are members of the LytM family proteins, which contain a LytM (lysostaphin/peptidase M23) domain and are widely distributed in bacteria (Firczuk and Bochtler, 2007). Although E. coli NlpD and EnvC do not seem to have any PG degradation activity themselves (Peters et al., 2013; Uehara et al., 2009), LytM members with PG hydrolytic activity have been found in other bacteria.
(Collier, 2010; Möll et al., 2010; Odintsov et al., 2004; Sycuro et al., 2010).

Nevertheless, despite significant progress, insights into the contribution of LytM family regulators and the amidase enzymes they control, and their link to virulence, remain incompletely understood. To this end, we examined how AmiC and its LytM family regulators contribute to virulence, T3S and cell separation in the important phytopathogen Xanthomonas campestris pv. campestris (Xcc). The pathogenicity of Xcc is dependent on the T3SS as the pathogen translocates effector proteins into host cells to interfere with host cell functions (He et al., 2007; Mansfield et al., 2012; Ryan et al., 2011). The Xcc T3S apparatus is composed of numerous proteins encoded by a cluster of hrp genes, which consists of at least six operons (hrpA to hrpF) (Arlat et al., 1991; Huang et al., 2009).

The aim of this work was to investigate whether these putative LytM factors and amidases are involved in Xcc pathogenicity. Here, we report the finding that one of the LytM proteins and one of the amidases are essential for Xcc virulence, homologous to the E. coli NlpD and AmiC, respectively. Furthermore, we provide evidence showing that the Xcc AmiC1 coupled with its activator NlpD contributes to T3S, in addition to cell division processes.

**RESULTS**

**Xcc encodes an NlpD-like protein that is essential for Xcc virulence**

A SMART (Simple Modular Architecture Research Tool) analysis (http://smart.embl-heidelberg.de) revealed that 10 open reading frames (ORFs) in the genome of Xcc strain 8004 (Qian et al., 2005) encode proteins with an identifiable LytM domain (i.e. peptidase_M23 domain) (Table S1, see Supporting Information). These ORFs are highly conserved in other fully sequenced Xcc genomes (Table S1). We named XC0022 and XC3926 as EnvC and YebA based on their homology with E. coli NlpD and AmiC, respectively. Furthermore, we provide evidence showing that the Xcc AmiC1 coupled with its activator NlpD contributes to T3S, in addition to cell division processes.

NlpD and EnvC are required for Xcc cell morphology and separation

Although the optical density at 600 nm (OD$_{600}$) of cells was very similar during growth in NYG medium, the cell morphology and CFU (colony-forming units) numbers of the ΔnlpD mutant were significantly affected (Figs 2, S3), suggesting that Xcc NlpD plays a similar role in cell separation as E. coli NlpD. To obtain a better insight into the role of NlpD and the other putative LytM proteins in the cell division process, we examined the cell shape and size. For this, strains were cultured in the nutrient-rich medium NYG overnight and bacterial cells were observed under a microscope after Gram staining. As illustrated in Fig. S4 (Supporting Information), most cells lacking NlpD (NK2522) or EnvC (NK0022) developed as unsegmented chains, whereas the other mutants tested displayed a similar phenotype to the wild-type (single cells). This suggested that mutation of nlpD or envC resulted in a defect in cell separation and segmentation. Importantly, complementation partially reverted the ΔnlpD mutant towards the wild-type phenotype (Fig. 2). Furthermore, the number of single cells in the CΔnlpD strain was greater than that of the mutant, although the CΔnlpD strain still formed some unsegmented chained cells (Fig. 2). In addition, the CFU number of the complementary strain CΔenvC exhibited a similar growth rate to the wild-type (Fig. S3), see Supporting Information). The envC deletion mutant strain ΔenvC (Table S2) induced virulence symptoms similar to those of the wild-type (Fig. 1A,B).
number of ΔenvC could be restored to the levels of the wild-type by envC in trans (Figs 2, S3). Although the reason why the phenotype of the mutant strain ΔnlpD could not be completely restored by nlpD in trans remains to be investigated, taken together, the data suggest that NlpD and EnvC are key factors for Xcc daughter cell separation, similar to the role of NlpD and EnvC in many other Gram-negative bacteria.

**NlpD is required for Xcc T3S, but not T2S**

It is well established that T2S and T3S are essential for Xcc virulence to host plants (Ryan et al., 2011). Therefore, to investigate whether the loss of virulence caused by the mutation of nlpD is associated with an impact on these secretion systems, we examined the ΔnlpD strain for impaired T2S and T3S. We assessed the activity of extracellular amylase, endoglucanase and protease as an indicator of T2S function. The results showed that ΔnlpD and the wild-type produced similar activities of these enzymes (Table S3, see Supporting Information), indicating that mutation of nlpD did not affect T2S in Xcc.

Like many other bacterial pathogens, Xcc employs the T3SS to translocate effectors into host cells to overcome plant defences. Inactivation of Xcc T3S results in a loss of the ability to produce...
disease lesions in host plants and to elicit the HR in non-host plants, such as the pepper cultivar ECW-10R (Huang et al., 2009). To ascertain whether NlpD is required for Xcc T3S, we first determined the HR induction ability of the mutant ΔnlpD on the pepper cultivar ECW-10R. The hrcV-deficient mutant strain ΔhrcV was used as a negative control. HrcV is one of the core proteins composing the T3S apparatus and the mutant ΔhrcV is defective in T3S (Hartmann and Büttner, 2013). As shown in Fig. 1C, in contrast with the wild-type strain 8004, which induced distinct HR symptoms at 8 h post-inoculation, the mutant ΔnlpD could not elicit clear HR symptoms up to 16 h post-inoculation. At 24 h post-inoculation, a weak HR symptom was observed at the site of inoculation. These data indicate that deletion of nlpD leads to a delay in HR induction. The result was substantiated by an electrolyte leakage assay. ΔnlpD showed significantly lower electrolyte leakage relative to the wild-type (Fig. 1C,D), suggesting that mutation of envC does not affect the function of the Xcc T3SS.

To further validate the involvement of NlpD in the T3S of Xcc, we evaluated the secretion efficiency of the ΔnlpD strain. Our previous work identified a T3S effector (T3SE) from Xcc strain 8004, which was encoded by the ORF XC_3176 and named XC3176 (Qian et al., 2005). To evaluate the efficiency of T3S, we measured the secretion of the T3SE XC3176 by Western blotting. To this end, the reporter plasmid, named pJXG3176, encoding an XC3176 T3SE fused with a 3× Flag tag at the C-terminus (i.e. XC3176-Flag3) (see Experimental procedures), was introduced into the mutant strains ΔnlpD and ΔhrcV, as well as the wild-type strain 8004, by triparental conjugation. The fusion protein XC3176-Flag3 in strains ΔnlpD/pJXG3176, ΔhrcV/pJXG3176 and 8004/pJXG3176 (Table S2) was extracted and detected by Western blotting after cultivation in the T3S-inducing minimal medium XCM2. As shown in Fig. 3, strain ΔnlpD/pJXG3176 produced a significantly lower amount of XC3176-Flag3 protein in the culture supernatant compared with strain 8004/pJXG3176. As expected,
Xanthomonas campestris pv. campestris (Xcc) strains. The resulting recombinant strains were cultured in XCM2 medium for 12 h. The cells were harvested by centrifugation and the proteins in the cells and in the supernatant fraction were prepared. The proteins obtained were separated in a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and analysed by Western blotting. 8004, ΔnlpD, amiC1::pK18, ΔamiC2, amiC1::pK18ΔamiC2, ΔenvC and ΔhrcV strains were tested.

XC3176-Flag, was undetectable in the culture supernatant from ΔhrcV/pJXG3176. However, all three strains produced a significant amount of intracellular XC3176-Flag protein (Fig. 3). These results revealed that mutation of nlpD could lead to a severe defect in the Xcc T3S mechanism.

As mentioned above, Xcc T3S is encoded by a cluster of hrp genes consisting of hrpA to hrpF operons. It is possible that NlpD regulates the expression of hrp genes. To verify this possibility, we determined the expression level of nlpD in strains carrying the same reporter plasmid (Fig. S5, see Supporting Information). The results showed that there was no significant difference in GUS activity between the mutant ΔnlpD and the wild-type strain 8004 carrying the same reporter plasmid (Fig. S5, see Supporting Information), suggesting that NlpD is not involved in the regulation of hrp gene expression.

NlpD is dispersed in cytoplasm, periplasm and outer membrane

It is known that the E. coli NlpD is a lipoprotein with an N-terminal lipid moiety and must be localized to the outer membrane to properly control its activity (Tsang et al., 2017; Uehara et al., 2009). The lipid signal peptide serves as a membrane anchor, and so most Gram-negative bacterial lipoproteins are retained in either the inner membrane or the outer membrane (Narita and Tokuda, 2016; Wilson and Bernstein, 2016). Domain structure analysis revealed that the Xcc NlpD also contains a lipid signal peptide with 22 amino acid residues at the N-terminus (Narita and Tokuda, 2016; Wilson and Bernstein, 2016). Domain structure analysis revealed that the Xcc NlpD contains a lipid signal peptide as well. To determine the subcellular location of the NlpD protein in Xcc, a recombinant strain, named 8004NlpD-Flag (Table S2), was constructed (see below for details). The strain expresses a recombinant NlpD protein with a 3 × Flag tag at the C-terminus (NlpD-Flag). The total, periplasmic, cytoplasmic and outer membrane protein fractions of the recombinant strain 8004NlpD-Flag grown overnight in NYG medium were prepared. Western blotting analysis showed that the expressed recombinant NlpD-Flag protein presented in all of the protein fractions except the inner membrane fraction (Fig. S6, see Supporting Information). These data suggest that, after synthesis in the cytoplasm, the NlpD protein is transported to the periplasm and then anchors to the outer membrane with the N-terminus, supporting the prediction that Xcc NlpD is a lipoprotein.

NlpD functions to bind to PG and modulate AmiC1 activity to degrade PG

To understand the role played by NlpD in affecting T3S in Xcc, we began to biochemically characterize the protein. As described above, bioinformatics analysis revealed that Xcc NlpD carries a PG-binding LysM domain, suggesting that the NlpD protein can bind to PG. To verify this suggestion, an in vitro pull-down binding assay was performed. As illustrated in Fig. 4A, the S1 sample contained only a small amount of His6-NlpD_LN22 protein, whereas

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the S2 sample contained a large amount of His6-NlpDLN22 protein (Fig. 4A), suggesting that most of the His6-NlpDLN22 protein in the mixture was bound to PG to form a complex which was deposited in the pellet after the first centrifugation. These data confirm that the His6-NlpDLN22 protein interacts physically with PG. Importantly, a control reaction using bovine serum albumin (BSA) protein showed that the BSA protein was present in the S1 sample, but not the S2 sample (Fig. 4A), indicating that the BSA protein did not bind PG and that interaction between PG and the His6-NlpDLN22 protein is specific. His6-EnvCLN14 protein was also tested. The result showed that it did not interact with PG in the test conditions (Fig. 4A).

To gain a further understanding of the function of Xcc NlpD, we assessed the ability of the purified His6-NlpDLN22 protein to degrade Xcc PG using a dye release assay (Peters et al., 2013). Purified His6-NlpDLN22 Protein was incubated with Xcc PG labelled with the colorimetric dye remazol brilliant blue (RBB). NlpD degradation activity was determined by measuring the quantity of coloured dye present in the supernatant after stopping the reaction and centrifugation to deposit the intact PG. It was demonstrated that the mutanolysin of Streptomyces globisporus could degrade Xanthomonas PG (Erbs et al., 2008). In the assay, we used mutanolysin (Sigma, St. Louis, Missouri, USA) as a positive control and BSA protein as a negative control. No degradation activity was observed for either His6-NlpDLN22 or BSA, even after 16 h of incubation, whereas mutanolysin displayed obvious PG degradation activity (Fig. 4B). This result indicates that Xcc NlpD cannot hydrolyse PG by itself in vitro.

Like Xcc NlpD, E. coli NlpD does not have PG hydrolytic activity by itself. The effect of E. coli NlpD on daughter cell separation is attained by specific activation of the amidase AmiC to hydrolyse PG (Uehara et al., 2009, 2010). The genome of Xcc strain 8004 encodes four N-acetylmuramoyl-l-alanine amidases, two (XC1816 and XC2472) of which are amidase_3 family members and the others (XC2695 and XC3877) are amidase_2 family members (Qian et al., 2005). A SMART analysis revealed that both XC1816 and XC2472 possess an AMIN domain (Table S4, see Supporting Information), suggesting that both are homologues of E. coli AmiC. We therefore designated XC1816 and XC2472 as AmiC1 and AmiC2, respectively (Table S4). XC2695 and XC3877 were named AmpD and AmiD, respectively, based on both sequence homology and structural similarity (Table S4).

To investigate whether Xcc NlpD affects cell division in a mode similar to that of E. coli NlpD, we first constructed Xcc
mutant strains defective in AmiC1 or AmiC2 and inspected the daughter cell separation of the mutants. As shown in Fig. 2, the amiC1 mutant strain amiC1::pK18 formed unsegmented chained cells, indicating that Xcc AmiC1 is indispensable for regular daughter cell separation. The overwhelming majority of cells of the mutant strain lacking AmiC2 (ΔamiC2) were similar to those of the wild-type, although some short chains consisting of three to four cells were observed (Fig. 2), suggesting that Xcc AmiC2 is not essential, but has a minor effect, on daughter cell separation. A strain deficient in both AmiC1 and AmiC2 (amiC1::pK18ΔamiC2) displayed a more severe chaining phenotype compared with the single mutants alone (Fig. 2), supporting the idea that AmiC2 plays some role in the separation of daughter cells, but is not critical. This was further supported by the observation that the amiC1::pK18ΔamiC2 mutant could be restored by complementation with amiC1, but not amiC2, expressed in trans (Fig. 2). This confirmed that AmiC1 plays a critical role in cell division. Next, we studied the function of AmiC1 on PG degradation. To achieve this, a recombinant 6 × His-tagged truncated AmiC1 protein (His6-amiC1LN22) was created and overexpressed in E. coli. We also made an attempt to overproduce a recombinant 6 × His-tagged truncated AmiC2 protein (His6-amiC2LN22) by the same means, but failed to obtain a soluble form of the fusion protein. The ability of AmiC1 to degrade PG in vitro was assessed using dye release assays. These experiments revealed that the His6-amiC1LN22 protein could only weakly degrade PG (Fig. 4B). However, the presence of His6-NlpDLN22, but not His6-EnvCLN14, could significantly enhance PG degradation by His6-amiC1LN22 (Fig. 4B). These results suggest that Xcc NlpD influences cell division by modulation of AmiC1 activity to hydrolyse PG, similar to E. coli NlpD. The mutant strain envC expressing in trans a recombinant gene encoding the His6-EnvCLN14 protein (strain C2ΔenvC) showed the wild-type phenotype (Fig. S7, see Supporting Information), suggesting that His6-EnvCLN14 is functional, but cannot activate AmiC1.

AmiC1 is essential for Xcc virulence and T3S

The above results demonstrate that Xcc NlpD affects cell division by activating AmiC1 PG degradation and is involved in virulence and T3S. It is possible that the influence of NlpD on virulence and T3S is also accomplished via the modulation of AmiC1 activity. If so, removal of AmiC1 should result in attenuated virulence and T3S. To assess this possibility, we first examined the virulence of the mutant strain amiC1::pK18. Indeed, the virulence of the mutant in the host plant Chinese radish was severely diminished. At 10 days post-inoculation, the wild-type strain caused disease symptoms with a mean lesion length of approximately 11 mm, whereas the mutant induced very weak disease symptoms with a mean lesion length of only about 2 mm (Fig. 5A). The HR induction ability of the mutant was then determined in the non-host plant pepper cultivar ECW-10R using an electrolyte leakage assay. As shown in Fig. 5B, the leaf tissues inoculated with the mutant had a much lower electrolyte leakage compared with those inoculated with the wild-type, suggesting that AmiC1 is important for Xcc HR induction. Consistent with the effect on cell division, deletion of AmiC2 only slightly affected the virulence and electrolyte leakage (Fig. 5B). Furthermore, the deletion of AmiC2 in the AmiC1-deficient background (amiC1::pK18ΔamiC2 double mutant) further depressed the virulence and electrolyte leakage (Fig. 5B). Interestingly, AmiC1, but not AmiC2, was able to restore the virulence of the amiC1::pK18ΔamiC2 double mutant (Fig. 5).

To further verify the idea that NlpD influences T3S via the activation of AmiC, the T3S efficiency of the mutant amiC1::pK18 was determined by Western blot analysis using the effector fusion protein XC3176-Flag3. As with the previous experiments detailed above, the XC3176-Flag3-encoding plasmid pJXG3176 was introduced into the mutant strains amiC1::pK18, ΔamiC2 and amiC1::pK18ΔamiC2. The resulting recombinant strains amiC1::pK18/pJXG3176, ΔamiC2/pJXG3176 and amiC1::pK18ΔamiC2/pJXG3176 were used to examine the secretion of XC3176-Flag3 protein by Western blot assay. As shown in Fig. 3, a large amount of XC3176-Flag3 protein was present in the cells of all the strains tested and in the culture supernatants of the strain ΔamiC2/pJXG3176, but a very small amount was present in the supernatants of the strains amiC1::pK18/pJXG3176 and amiC1::pK18ΔamiC2/pJXG3176. These results suggest that AmiC1 is critical for T3S of Xcc. The extracellular amylase, endogluca nase and protease activities of the mutant strains amiC1::pK18, ΔamiC2 and amiC1::pK18ΔamiC2 were similar to those of the wild-type (Table S3), indicating that AmiC1 and AmiC2 are not involved in Xcc T25.

DISCUSSION

N-Acetylmuramoyl-l-alanine amidases and their LytM activation factors are broadly distributed in bacteria, with members of this family in each species. In E. coli, the amidases AmiA, AmiB and AmiC affect cell division via the cleavage of PG bonding and their hydrolytic activities are stimulated by the LytM activation factors EnvC (AmiA and AmiB) and NlpD (AmiC) (Heidrich et al., 2001; Uehara et al., 2009, 2010). Here, we show, for the first time, that AmiC and its LytM family activator NlpD, as well as being important for cell separation, play a role in T3S and virulence in the plant pathogen Xcc. Unlike E. coli, Xcc does not possess AmiA and AmiB homologues, but encodes two proteins homologous to AmiC (AmiC1 and AmiC2) (Table S4). In addition to LytM factor NlpD, Xcc also encodes EnvC. Interestingly, Xcc strains lacking NlpD, EnvC or AmiC1 displayed a severe defect in daughter cell separation, whereas the AmiC2-deficient mutant showed only a slight defect in cell division (Figs 2, S4). Purified recombinant proteins His6-NlpDLN22 and His6-EnvCLN14 could interact individually.
with purified Xcc PG in vitro, but could not degrade PG by themselves. In addition, the in vitro PG degradation activity of the purified His6-AmiC1LN32 protein was weak, but could be strongly activated by His6-NlpDLN22, but not His6-EnvCLN14 (Fig. 4). Taken together, these observations suggest that the LytM factors EnvC and NlpD, as well as the amidase AmiC1, are essential for Xcc daughter cell separation and NlpD functions via the modulation of the amidase activity of AmiC1. These findings closely mirror the observations in Vibrio cholerae, which encodes only a single amidase, AmiB, and two LytM factors, EnvC and NlpD, both of which can activate AmiB to cleave PG and affect cell division (Möll et al., 2014).

Although Xcc encodes an EnvC homologue, its role in cell separation is still unclear. This work showed that the purified His6-EnvC1LN14 protein could neither degrade PG itself nor activate His6-AmiC1LN32. Given the different phenotypes observed on daughter cell separation of the mutants deficient in EnvC or AmiC2, and the fact that His6-EnvC1LN14 could not activate His6-AmiC1LN32, Xcc EnvC may influence cell division by an as yet unknown mechanism, which is probably independent of AmiC1, although we cannot exclude the possibility that Xcc EnvC can activate AmiC2. Mutation of amiC2 resulted in a small portion of cells forming short chains and inactivation of amiC2 in the amiC1-deficient background potentiated the daughter cell separation defect. These results indicate that AmiC2 is also associated with cell division, but its influence is much weaker than that of AmiC1. Unfortunately, our attempt to study the potential enzymatic activity of AmiC2 failed because of our inability to express a soluble form of AmiC2 protein.

Although NlpD, AmiC1 and EnvC have a similar effect on cell division, their significance on virulence is very different. The strains lacking either NlpD or AmiC1 almost completely lost virulence, but the mutant lacking EnvC still demonstrated levels of virulence comparable with those of the wild-type (Figs 1, 5, S1). These data, together with the observation that nlpD-, amiC1- and envC-deficient mutants displayed a chained cell phenotype, indicate that the formation of long-chained cells does not impact the pathogenicity of Xcc. The fact that the mutants of nlpD and amiC1, but not envC, showed a severe defect in T3S suggests that...
the loss of virulence for npd and amiC1 mutants is associated with deficiency in the T3SS rather than the defect in daughter cell separation. The bacterial T3S apparatus is a complex macromolecular nanomachine made up of more than 20 proteins, which can be divided into three parts: an extracellular pilus or needle-like appendage, a membrane-spanning basal body and the peripheral inner membrane cytoplasmic components. The basal body supports the pilus or needle appendage by anchoring it on the bacterial membranes (Abrusci et al., 2014; Büttner, 2012; Deng et al., 2017; Galán et al., 2014; Portaliou et al., 2016). Although great progress has been made in the understanding of the mechanism by which the bacterial cells assemble the T3S machinery, there are still some crucial issues to be addressed. One of the issues is how the basal body crosses the bacterial cell wall during the assembly process (Deng et al., 2017; Scheurwater and Burrows, 2011). It is understood that the PG mesh becomes an impediment to the basal body crossing and therefore needs to be remodelled during T3S assembly (Burkinshaw et al., 2015; Demchick and Koch, 1996; Meroueh et al., 2006). Given that, in the PG mesh, the first peptide of the peptide side-chains is L-alanine, which is attached to MurNAc in the glycan strands, the N-acetylmuramoyl-L-alanine amidases are capable of releasing the peptide side-chains from the MurNAc residue by specifically cleaving the amide bond between MurNAc and L-alanine, which would free space (Frirdich and Gaynor, 2013; Vollmer et al., 2008). In E. coli and other bacteria, the enzymatic activities of the N-acetylmuramoyl-L-alanine amidases are activated by LytM family regulators; our data confirm a similar system in Xcc by showing that AmiC1 is activated by the LytM factor NlpD. This fact, coupled with the observation that the Xcc mutant cells in the absence of either AmiC1 or NlpD were defective in T3S, suggests that the cleavage of the amide bond by AmiC1/NlpD is associated with T3S. Therefore, it is reasonable to deduce that the cleavage of the peptide side-chain by AmiC1/NlpD is a crucial action to remodel the PG layer for facilitation of the T3S apparatus assembly in Xcc. This is also consistent with our observations showing that NlpD is dispersed in the cytoplasm, periplasm and outer membrane (Fig. S6).

This work also shows that the T2S system (T2SS) is regularly functional in the mutants lacking either AmiC1 or NlpD, as well as in the strains lacking both AmiC1 and AmiC2. This finding indicates that the effect of AmiC1/NlpD on the Xcc T3SS is specific. The T2SS is composed of more than a dozen proteins. The outer membrane secretin (GspD) is a core component of the T2S architecture, which penetrates the PG layer (Gu et al., 2017). It is thought that, in Aeromonas and Vibrio species, a complex formed by two different proteins (GspA and GspB) is involved in the modification of the PG layer to allow the assembly of the GspD secretin (for a review, see Gu et al., 2017). However, no direct evidence has been reported to date to show that a PG-lytic enzyme is involved in T2SS assembly. There is no doubt that the cleavage of PG by the amidase enzymes must be controlled in a timely and spatially correct manner, otherwise it will be destructive to bacterial cells. Previous studies have demonstrated that, during cytokinesis in E. coli, PG hydrolysis at the division site leading to daughter cell separation is precisely regulated to avoid uncontrolled cell lysis. The amidases AmiB and AmiC, as well as their activators EnvC and NlpD, are recruited to the division site, and the localization of AmiC, AmiB and NlpD requires the essential divisome protein FtsN (Bernhardt and de Boer, 2003; Peters et al., 2013; Uehara et al., 2010). Of course, there are many unanswered questions, but one that is outstanding is: ‘What is the mechanism by which Xcc cells coordinate the cleavage of PG by AmiC1/NlpD during T3S apparatus assembly?’ This is a topic that merits further investigation.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used are described in Table S2. Escherichia coli strains were grown in Luria–Bertani (LB) medium (Miller, 1972) at 37 °C, and Xcc strains were grown in NYG rich medium (3 g/L yeast extract, 5 g/L peptone, 20 g/L glycerol, pH 7.0) (Daniels et al., 1984a). XCM2 induction medium (20 μM succinic acid, 0.15 g/L casamino acids, 7.57 mM (NH₄)₂SO₄, 0.01 mM MgSO₄, 60.34 mM KH₂PO₄, 33.07 mM K₂HPO₄, pH 6.6) and MMX minimal medium (5 g/L glucose, 1 g/L sodium citrate, 2 g/L (NH₄)₂SO₄, 0.2 g/L MgSO₄, 4 g/L K₂HPO₄, 6 g/L KH₂PO₄, pH 7.0) (Daniels et al., 1984b) at 28 °C. Antibiotics were used at the following final concentrations: ampicillin (Amp), 100 μg/mL; gentamicin (Gm), 10 μg/mL; kanamycin (Kan), 25 μg/mL; rifampicin (Rif), 50 μg/mL; spectinomycin (Spc), 50 μg/mL; tetracycline (Tc), 15 μg/mL for E. coli and 5 μg/mL for Xcc.

**Determination of the transcriptional start site**

The transcriptional start site of the gene XC_2522 encoding the LytM protein NlpD was determined by 5’ RACE analysis (Fig. S2). Total cellular RNA was extracted by RNAiso Blood (TaKaRa, Dalian, China) and treated with RNase-free DNase (Promega, Madison, Wisconsin, USA), followed by a second purification. The cDNA fragments were obtained using the 5’ RACE kit (Invitrogen, Carlsbad, California, USA). RNA was reverse transcribed using the XC_2522 sequence-specific primer 2522GSP1 (Table S5, see Supporting Information). An anchor sequence was then added to the 3’ end of the cDNA using terminal deoxynucleotide transferase, followed by direct amplification of tailed cDNA using the nested gene-specific primers 2522GSP2 and 2522GSP3 (Table S5) and the anchor-specific primer provided. Polymerase chain reaction (PCR) products were then cloned into the pGEM-T Easy vector (Promega) and sequenced (Table S6, see Supporting Information).

**Construction of strains for protein expression**

For the overproduction of a truncated NlpD protein lacking the N-terminal 22-amino-acid signal peptide, a 720-bp fragment was amplified using the primer pairs E2522-F(B)/E2522-R(E) (Table S5) designed according to the
sequence of the nlpD ORF Xc_2522 reported in the Xcc strain 8004 (Qian et al., 2005). The amplified fragments were cloned into the vector pET30a to generate the recombinant plasmid pET-NlpDLN22. The plasmid pET-NlpDLN22 was introduced into the E. coli strain BL21(DE3) to create the strain His6-NlpDLN22 (Table S2), which was used for protein production. The strains His6-EnvCLN14, His6-AmiC1LN32 and His6-AmiC2LN23 (Table S2) for the overproduction of 6×His-tagged truncated EnvC, AmiC1 and AmiC2 proteins, respectively, were constructed, and expressed in E. coli in accordance with the above method.

Mutant construction and complementation

The mutants of the genes (XC_0022, XC_0463, XC_0921, XC_1250, XC_1354, XC_1857, XC_2522, XC_3502, XC_3926 and XC_4282) encoding proteins with a LytM domain, named NK0022, NK0463, NK0921, 182E08, NK1354, 032B12, NK2522, 209A07, NK3926 and 024A02, respectively (Table S2), were obtained from the mutant library of the Xcc strain 8004, which was constructed by transposon (Tn5) insertion or homologous suicide plasmid (pK18mob) insertion or homologous suicide plasmid (pK18mob) integration mutagenesis in our laboratory.

To construct the deletion mutant of nlpD, the region from 718 bp upstream to 722 bp downstream of the Xc_2522 ORF was amplified by the primer pairs D2522-LF(B)/D2522-LR(X) and D2522-RF(X)/D2522-RR(H) (Table S5), and fused into the suicide plasmid pk18mob8c8 to create the recombinant plasmid pkD2522. The plasmid pkD2522 was transferred into the Xcc wild-type strain 8004 by triparental conjugal conjugation, followed by the selection of colonies on NYG plates containing Rif and Kan. The nlpD deletion mutant was obtained by further selection on an NYG plate supplemented with Rif and 10% sucrose. The mutant colonies were then checked for Kan sensitivity and were further confirmed by PCR. The confirmed mutant was named ΔnlpD. Other deletion mutants were constructed in accordance with this method.

To construct the plasmid integration mutant of amiC1 (ORF XC_1816), a 378-bp internal fragment of Xc_1816 was amplified by the primer set NK1816-F(B)/NK1816-R(X) (Table S5). The confirmed sequenced DNA fragment was cloned into the suicide plasmid pk18mob (Table S2) to create the recombinant plasmid pk1816 (Table S2). The plasmid pk1816 was introduced from E. coli DH5α into the Xcc wild-type strain 8004 by triparental conjugation. The transconjugants were screened on a NYG plate supplemented with Rif and Kan, and the amiC1 mutant obtained was confirmed by PCR and named amiC1::pk18 (Table S2). Confirmation PCR was performed using the total DNA of the amiC1 mutant cells obtained as the template and the primers pkmob18Con (located in pk18mob) and D1816R (located downstream of the amiC1 gene) (Table S5). The amiC2 deletion mutant AmiC2 (Table S2) was constructed using the above deletion mutant construction method. The amiC1 and amiC2 double mutant amiC1::pk18AmiC2 (Table S2) was constructed in the background of the mutant ΔamiC2.

For complementation of the nlpD mutant, a 1359-bp DNA fragment containing the nlpD coding region and extending from 491 bp upstream of the 5′ end to 40 bp downstream of the 3′ end of the ORF was amplified using the primer set C2522-F(B)/C2522-R(H) (Table S5), and the amplified DNA fragment was cloned into the plasmid pLaFR6 (Table S2) to generate the recombinant plasmid pLCnlpD (Table S2). The recombinant plasmid was transferred into the deletion mutant of nlpD by triparental conjugation, resulting in the complemented strain CΔnlpD (Table S2). Other complementary strains were constructed in accordance with the above method, except for the use of pLaFR6 instead of pLaFR6.

Virulence assay

The virulence of Xcc strains in Chinese radish manshenhong was tested by the leaf clipping method, as described previously (Dow et al., 2003). The bacterial cultures were grown overnight at 28 °C with shaking at 200 rpm in NYG medium and the concentration was adjusted to an OD600 of 0.001. The Chinese radishes were planted in a glasshouse with natural light and temperatures of 25 to 30 °C. Five-week-old seedlings with four fully expanded leaves were inoculated by clipping the vein endings of the expanded leaves with sterilized scissors dipped in the bacterial cultures. At least 60 leaves were inoculated for each strain, and the experiment was repeated three times independently. After inoculation, the plants were maintained in 80% humidity for the first 24 h, and then in the conditions described above. The lesion lengths of the inoculated leaves were measured at 10 days post-inoculation, and the data were analysed by t-test.

Detection of HR

For HR test, seedlings of the Xcc non-host plant pepper (Capsicum annuum cv. ECW-10R) were grown in a glasshouse with a 12-h day and night cycle with illumination by fluorescent lamps at temperatures of 25–28 °C. The bacterial cells of Xcc strains from overnight cultures were washed and resuspended in sterile water. The bacterial resuspensions were diluted to an OD600 of 0.01 in double-distilled H2O and infiltrated into the pepper leaf tissues at the stage of four fully expanded leaves using a needleless syringe. After infiltration, the plants were grown at 28 °C and 80% relative humidity. HR symptoms were photographed at 8, 16 and 24 h post-inoculation. At least three plants were inoculated in each experiment, and each experiment was repeated at least three times. For the electrolyte leakage assays, measurements were carried out as described previously (Castaneda et al., 2005). Essentially, for each sample, three leaf discs were removed with a 0.7-cm-diameter cork borer, submerged in 10 mL of double-distilled H2O and vacuum infiltrated. Then, the net leakage after 1 h was measured with a conductivity meter. Three samples were taken for each measurement in each experiment, and the experiments were repeated at least three times.

Microscopic assays

For the light microscopy assay, bacterial cells of Xcc strains from overnight cultures were diluted in distilled water. The diluted bacterial suspension was spread on slides, stained with Gram staining solution and observed using an Olympus microscope BX41 (Tokyo, Japan).

Flag epitope-tagging of NlpD

For Flag-tagging of NlpD, two fragments containing 798 bp upstream and 730 bp downstream, respectively, of the nlpD termination codon were amplified and EcoRI, BamHI, XbaI and HindIII restriction sites were introduced. The amplified fragments and 3 × Flag oligonucleotides (custom made by Invitrogen) with BamHI and XbaI restriction sites were fused
together and cloned into the EcoRI and HindIII restriction sites of the suicide plasmid pK18mobsacB. The recombinant plasmid obtained was introduced into the Xcc wild-type 8004 by triparental conjugation and the sequence of the wild-type nlpD gene on the chromosome was replaced with nlpD-3 × Flag. The resulting strain was named 8004NlpD-Flag and was used to investigate the subcellular localization of NlpD (NlpD-Flag) by Western blot analysis. For this purpose, protein samples were separated in 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, Massachusetts, USA). After blocking, the 1 : 2000 diluted anti-Flag-tag rabbit monoclonal antibody (Sigma) was used as the primary antibody, and the 1 : 5000 diluted horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Pierce, Rockford, Illinois, USA) was used as secondary antibody.

**Preparation of total, periplasmic and membrane proteins**

The bacterial total and periplasmic proteins were prepared by the method reported previously (Zang et al., 2007). Preparation of the outer and inner membrane proteins was carried out as described by Chen et al. (2010). Briefly, 100 mL of Xcc cells were grown in NYG medium at 28 °C overnight and collected by centrifugation at 3,488 g for 10 min. The cells were suspended in cold TM buffer (10 mM Tris, pH 8.0, containing 8 mM MgSO4), washed three times, resuspended in 4.0 mL of the same buffer and broken by sonication. The unbroken cells and the cell debris were removed by centrifugation at 14,000 g for 30 min. The supernatants were further centrifuged at 135,000 g at 4 °C for 1 h and the supernatants containing cytoplasmic and periplasmic proteins were retained. The pellets containing membranes and ribosomes were suspended in 1.0 mL of cold TM buffer by gentle aspiration and ejection using a 25-gauge needle attached to a 1-mL syringe, and the resuspended samples were centrifuged at 135,000 g as before. The pellets were resuspended with 1.0 mL of cold TM buffer, resuspended in 3.9 mL of 0.25% (w/v) sarkosyl and loaded into 3.9-ml ultracentrifuge tubes. After incubation at room temperature for 1 h, the tubes were centrifuged at 135,000 g for 1 h, as above. The supernatants containing the sarkosyl-soluble inner membrane fraction were retained. The sarkosyl-insoluble pellets containing the outer membrane fraction were washed twice with 1.0 mL of 0.25% (w/v) sarkosyl, incubated at room temperature for 1 h and centrifuged at 135,000 g as described above. The pellets containing the outer membrane fraction were suspended in 40 μL of cold TM buffer.

**Purification of recombinant proteins**

The overnight cultured E. coli strains for protein overexpression were transferred into 200 mL of LB medium and cultivated at 37 °C to an OD600 of 0.4. Then, 0.5 mM IPTG (Isopropyl β-D-thiogalactoside) was added and the strains were cultivated at 18 °C for 5 h. In particular, the strain His6− EnvCLN14 was induced with 0.125 mM IPTG and grown at 18 °C for 3 h. The cells were harvested and resuspended in 10 mL of lysis buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0). After ultrasonication, the cells were broken and the cell debris was removed by centrifugation at 12,000 g for 15 min at 4 °C. The supernatant was incubated with Ni-agarose resin (CWBio, Beijing, China) pre-equilibrated with lysis buffer for 1 h at 4 °C. Then, the column was washed with 50 mL of wash buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant proteins were eluted with elution buffer (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 8.0). The imidazole was removed by ultrafiltration in PBS (10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl, pH 7.4) with 10% glycerol. The proteins were detected in 12% SDS-PAGE gel and stained with Coomassie blue.

**Purification of PG**

The PG of Xcc was extracted and purified from dried cells as described previously (Glauner, 1988; Kuhner et al., 2014). Briefly, 300 mL of Xcc overnight culture was suspended in ice-cold water and added drop-wise to boiling 8% SDS and boiled for 30 min. After cooling to room temperature, the SDS-insoluble material was collected by centrifugation. The pellet was washed several times with double-distilled H2O until no SDS could be detected. Five millilitres of solution B (15 μg/mL DNase and 60 μg/mL RNase in 0.1 M Tris/HCl, pH 6.8) were added and incubated for 60 min at 37 °C in a shaker. Then, 5 mL of solution C (50 μg/mL trypsin in double-distilled H2O) were added and incubated for an additional 60 min at 37 °C. The suspension was incubated at 100 °C for 3 min to inactivate the enzymes before centrifugation (5 min at 9,600 g). After washing with double-distilled H2O, 1 M HCl was added and incubated for 4 h at 37 °C in a shaker. The PG in the suspension was spun down (5 min at 9,600 g) and resuspended in 1 mL of double-distilled H2O after washing several times with double-distilled H2O until the pH was 5–6. The purified PG can be stored at −20 °C.

**PG binding assay**

The PG binding experiment was carried out as described by Rocaboy et al. (2013). Ten micrograms of purified protein were incubated for 2 h at room temperature with 10 μL of purified PG in binding buffer (30 mM Tris, pH 6.8, 50 mM NaCl, 10 mM MgCl2) in a total volume of 100 μL. The sample was centrifuged for 20 min at 14,000 g and the supernatant (S1) was analysed by 12% SDS-PAGE. The pellet was washed twice with 150 μL of binding buffer and then resuspended in 40 μL of 2% SDS solution and incubated for 1 h at room temperature. The resuspension was centrifuged for 20 min at 14,000 g and the supernatant (S2) was analysed by SDS-PAGE. The protein in SDS-PAGE was determined by staining with Coomassie blue.

**Preparation of PG sacculi labelled with RBB**

PG sacculi were labelled with RBB as described previously (Peters et al., 2013). The sacculi were incubated with 20 μM RBB (Sigma) in 0.25 mM NaOH overnight at 37 °C. Then, the preparation was neutralized with HCl, and RBB-labelled sacculi were pelleted by centrifugation for 20 min at 12,000 g. The labelled sacculi were washed several times with water until the supernatant was clear. The final pellet was resuspended in water and stored at 4 °C.

**PG cleavage assay**

The PG hydrolysis activity of proteins was detected as described previously (Peters et al., 2013). RBB-labelled sacculi (10 μL) were incubated with
protein (4 μL) at 37 °C in 60 μL of PBS buffer for 16 h and 30 μL of ethanol were added to terminate the reaction, followed by centrifugation at 21,000 g for 20 min at room temperature. An Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA) was used to measure the absorbance of the supernatant at 595 nm.

### GUS activity assay

The overnight cultured Xcc strains in NYG medium were collected and cultivated for 20 h after suspension in XCM2 to an OD₆₀₀ of 0.1. For determination of the total GUS activity, 1 mL of culture for each strain was mixed with 60 μL of merylphenol and vortexed. The supernatant was then taken for GUS activity assay. The GUS activity assay was performed by measurement of the OD₄₅₄ using p-nitrophenyl-β-D-glucuronide as substrate, as described previously (Jefferson et al., 1986).

### Detection of Xcc T3S efficiency by Western blot analysis

To assess the T3S efficiency of Xcc strains, a recombinant plasmid, named pJXG3176 (Table S2), was constructed by cloning the T3SE XCC3176-encoded ORF without its stop codon into the vector pJXG, which was generated by cloning a DNA fragment containing the 3 × Flag-tag coding sequence into the Pst and HindIII restriction sites of pLAFR1 (Table S2). In pJXG3176, the 3’ end of the XCC3176-encoding ORF was fused to the 3 × Flag-tag coding sequence to ensure the expression of an XCC3176 protein with a 3 × Flag-tag at its C-terminus. The recombinant plasmid pJXG3176 was introduced into Xcc strains by triparental conjugation. The recombinant strains obtained were cultured in NYG medium overnight and cells were collected by centrifugation. After adjusting to OD₆₀₀ = 0.1, the cells were transferred into hpr-inducing medium XCM2 and incubated for 12 h. The cells were harvested by centrifugation at 3488 g for 15 min. The proteins in the cells were isolated by resuspending the pellet in PBS buffer to OD₆₀₀ = 0.2 and ultrasonication for 1 min, followed by filtration. The proteins secreted in the supernatant fraction were filtered, precipitated with chilled trichloroacetic acid to a final concentration of 10% on ice overnight, washed with acetone and resuspended in rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS (3-(3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate). The proteins obtained were separated in 12% SDS-PAGE gel and analysed by Western blotting as described above.

### Extracellular enzyme activity assay

The activities of the extracellular enzymes amylase, endoglucanase and protease were assessed quantitatively as described previously (Chao et al., 2008). Briefly, for endoglucanase activity quantification, 10 μL of cytoplasmatic extract was added to 200 μL of the indicated buffer containing 1% (w/v) carboxymethylcellulose (CMC). Reactions were carried out for 30 min at 28 °C. The released reducing sugars were measured as β-glucose equivalents. One unit (U) of endoglucanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute. Amylase activity quantification was conducted in the same manner as the endoglucanase measurement, except that the substrate 1% (w/v) CMC was replaced by 1% (w/v) starch solution. The quantification of protease activity was performed as described by Swift et al. (1999).

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:
0.05 (for MMX) and then incubated at 28°C with 200 rpm. The bacterial density was determined by measuring the OD_{600} values at several time points post-inoculation (a, c). The cell number in the culture was determined using the dilution plate counting method, and the number of colony-forming units (CFUs) was counted after incubation at 28°C for 2 days (b, d). Data displayed are the means ± standard deviations from three replicates. The experiment was repeated three times and similar results were obtained.

**Fig. S4** Xanthomonas campestris pv. campestris (Xcc) XC_2522 (nlpD) and XC_0022 (envC) have a crucial effect on cell separation. Bacterial cells of Xcc strains cultured overnight in NYG medium were collected by centrifugation at 13 800 g for 30 s and resuspended in double-distilled H₂O. Five microlitres of bacterial resuspension were placed on a slide and dried at room temperature. After Gram’s staining, bacterial cells were observed by light microscopy. Representative micrographs are shown.

**Fig. S5** Mutation of nlpD does not affect the expression of hrp genes. The β-glucuronidase (GUS) activities of hrpG, hrpX, hrpB and hrpF promoter-gusA reporters in nlpD deletion (∆nlpD) and wild-type (Xcc 8004) backgrounds were determined. Xanthomonas campestris pv. campestris (Xcc) strains were cultured in the minimal medium XCM2 for 20 h, and the GUS activities were then determined by measurement of the absorbance at 415 nm using p-nitrophenyl-β-D-glucuronide as substrate. Data are the means ± standard deviations of triplicate measurements. The experiment was repeated three times, and similar results were obtained.

**Fig. S6** Subcellular localization of Xanthomonas campestris pv. campestris (Xcc) NlpD by Western blot analysis. The bacterial total and periplasmic proteins, as well as the outer and inner membrane proteins, were prepared from 100 mL of Xcc cells grown in NYG medium overnight. Thirty micrograms of each protein sample were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Anti-Flag-tag was used to detect the presence of the recombinant protein NlpD-Flag. NlpD-Flag, protein from recombinant strain 8004_nlpD.Flag; WT, protein from the wild-type strain 8004.

**Fig. S7** The mutant strain ∆envC expressing in trans a recombinant gene encoding His_6-EnvCLN14 protein (strain C2_∆envC) shows the wild-type phenotype. Bacterial cells of Xcc strains cultured overnight in NYG medium were collected by centrifugation at 13 800 g for 30 s and resuspended in double-distilled H₂O. Five microlitres of bacterial resuspension were placed on a slide and dried at room temperature. After Gram staining, bacterial cells were observed by light microscopy. Representative micrographs are shown.

**Table S1** The predicted LytM proteins in Xanthomonas campestris pv. campestris (Xcc) and their homologues in Escherichia coli.

**Table S2** Bacterial strains and plasmids used in this work.

**Table S3** Quantification of the extracellular enzymes produced by Xanthomonas campestris pv. campestris (Xcc) strains.

**Table S4** The predicted N-acetylmuramoyl-L-alanine amidases in Xanthomonas campestris pv. campestris (Xcc) and their homologues in Escherichia coli.

**Table S5** Primers used in this work.

**Table S6** 5’ Rapid amplification of cDNA ends (5’ RACE) sequencing result.