The lolB gene in Xanthomonas campestris pv. campestris is required for bacterial attachment, stress tolerance, and virulence

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Abstract

Background: Xanthomonas campestris pv. campestris (Xcc) is a Gram‑negative bacterium that can cause black rot disease in crucifers. The lipoprotein outer membrane localization (Lol) system is involved in the lipoprotein sorting to the outer membrane. Although Xcc has a set of annotated lol genes, there is still little known about the physiological role in this phytopathogen. In this study, we aimed to characterize the role of LolB of Xcc in bacterial attachment, stress tolerance, and virulence.

Results: To characterize the role of LolB, lolB mutant was constructed and phenotypic evaluation was performed. The lolB mutant revealed reductions in bacterial attachment, extracellular enzyme production, and virulence. Mutation of lolB also resulted in reduced tolerance to a myriad of stresses, including heat and a range of membrane‑perturbing agents. Trans‑complementation of lolB mutant with intact lolB gene reverted these altered phenotypes to the wild‑type levels. From subsequent reporter assay and reverse transcription quantitative real‑time polymerase chain reaction (RT‑qPCR) analysis, the expression of genes that encode the major extracellular enzymes and the stress‑related proteins was reduced after lolB mutation.

Conclusions: The results in this work contribute to the functional understanding of lolB in Xanthomonas for the first time, and provide new insights into the function of lolB in bacteria.

Keywords: Xanthomonas campestris, Stress tolerance, Virulence

Background

In Gram‑negative bacteria, the outer membrane presents a selectively permeable barrier to the environment and is the first line of defense against harmful chemicals, including detergents and antibiotics [1]. Bacterial lipoproteins are a set of membrane proteins localized on either leaflet of the lipid bilayer and are important components of the Gram‑negative cell envelope [2, 3]. In Escherichia coli, most lipoproteins are considered to be anchored to the inner leaflet of the outer membrane [3, 4]. The Lol (lipoprotein outer membrane localization) pathway is responsible for sorting and localizing lipoprotein [2–5]. The Lol pathway has components in each compartment of the cell envelope: an ATP binding cassette transporter LolCDE in the inner membrane; a soluble chaperone protein LolA in the periplasmic space; and a lipoprotein LolB in the outer membrane [4]. The outer membrane‑directed lipoprotein is extracted from the inner membrane by LolCDE, transferred to LolA, and shuttled to the outer membrane, where LolB receives and then anchors them into the bilayer [2–5]. The Lol proteins of E. coli have been studied in depth and each of the lol genes are considered to be essential for viability of this bacterium [2, 3, 5].
The Lol homologues can be found in many Gram-negative bacteria, suggesting that the pathway is conserved [2, 3, 5]. However, conservation of individual Lol protein encoding genes varies. In most γ-proteobacteria, the LolCDE consists of one copy each of membrane subunits LolC and LolE, and two copies of LolD [3]. LolC and LolE are homologues but cannot functionally substitute each other in E. coli [6]. However, some bacterial genomes contain only one copy of a lolC/lolE gene; the protein product contains sequence motifs of both LolC and LolE and the LolE name was proposed to distinguish such proteins from obvious LolC and LolE homologues [7]. Additionally, phylogenetic analysis suggests that lolB gene is only conserved in β- and γ-proteobacteria [2, 5]. Till now, only the Lol proteins of Pseudomonas aeruginosa have been indicated to involve in the sorting of outer membrane lipoprotein, as in the case of E. coli lipoproteins [8, 9]. Recently, it has been reported that the LolCDE lipoproteins of the pathways of E. coli and P. aeruginosa are interchangeable [10].

Xanthomonas campestris pv. campestris (Xcc), a Gram-negative bacterium, is capable of causing cruciferous plant infections. This pathogen causes black rot disease in the members of Brassica oleracea such as broccoli, cabbage, cauliflower, and radish [11–13]. The virulence of Xcc depends on a number of factors, including biofilm formation, extracellular enzymes (such as cellulase, mannanase, and protease), and extracellular polysaccharides [14, 15]. Four lol genes (lolA, lolB, lolD, and lolF) have been annotated in the fully sequenced Xcc genome [12, 16–18]. Among them, only lolA has been studied. The Xcc lolA has been indicated to play a role in pathogenicity and stress tolerance [19]. The aims of the present work are to characterize lolB in Xcc. The role of lolB in bacterial attachment, extracellular enzyme production, stress tolerance, and virulence was examined in this study.

**Methods**

**Bacterial strains and growth conditions**

Table 1 lists the bacterial strains and plasmids used in this study. E. coli and Xcc were grown at 37 °C and 28 °C, respectively. Luria–Bertani (LB) was used as a routinely cultured medium [20]. XOLN was a basal salt medium and contained 0.625 g/L tryptone and 0.625 g/L yeast.

| Table 1  | Bacterial strains and plasmids used in this study |
|----------|--------------------------------------------------|
| **E. coli** | Description | Reference or source |
| ECOS™ 101 | endA1 recA1 relA1 gynA96 hsdR17(rK-, mK+) phoA supE44 thi-1 Δ(lacZYA-argF) U169 q80Δ(lacZ)M15 | Yeastern |
| X. campestris pv. campestris | | |
| XC17 | Virulent wild type strain isolated in Taiwan, Ap<sup>+</sup> | [22] |
| CL17 | XC17-derived mutant with EZ-Tn5 inserted in lolB gene, Ap<sup>+</sup>, Km<sup>+</sup> | This study |
| **Plasmid** | | |
| yT&I | PCR cloning vector, Ap<sup>+</sup> | Yeastern |
| pTlolB | A 790 bp RCR amplified fragment from lolB (nucleotides –133 to +657 relative to the translation start site) and cloned into yT&A | This study |
| pUC19G | Gm<sup>+</sup> cartridge from pUCGM ligated with the blunt-ended AvaI-SspI large fragment from pUC19 | [23] |
| pUClolB | The 790 bp BamHI-EcoRI fragment of the pTlolB cloned into the BamHI and EcoRI sites of pUC19 | This study |
| pUClolBK | pUClolB derivative with Km<sup>+</sup> inserted in the internal region of lolB gene | This study |
| pRK415 | Broad-host-range vector, RK2 ori, Te<sup>+</sup> | [24] |
| pRKlolB | The 790 bp BamHI-EcoRI fragment of the pTlolB cloned into the BamHI and EcoRI sites of pRK415 | This study |
| pFYI3–9 | Promoter-probing vector derived from pRK415, using lacZ as the reporter, Te<sup>+</sup> | [25] |
| pFYengA | The 159-bp fragment, –181–+23 relative to engA translation start site, cloned into the Pstr/Xbal sites of pFYI3-9 | This study |
| pFYmanA | The 360-bp fragment, –372–+13 relative to manA translation start site, cloned into the Pstr/Xbal sites of pFYI3-9 | This study |
| pFYprt1 | The 313-bp fragment, –392–+80 relative to prtI translation start site, cloned into the Pstr/Xbal sites of pFYI3-9 | [26] |
| pFYclpP1 | The 375-bp fragment, –384–+10 relative to clpP translation start site, cloned into the Xbal/Xbal sites of pFYI3-9 | [27] |
| pFYclpX | The 327-bp fragment, –336–+10 relative to clpX translation start site, cloned into the Xbal/Xbal sites of pFYI3-9 | This study |

Ap<sup>+</sup>, ampicillin-resistant; Gm<sup>+</sup>, gentamicin-resistant; Km<sup>+</sup>, kanamycin-resistant; Te<sup>+</sup>, tetracycline-resistant
extract [21]. Glucose or glycerol was added (2%) as required. Liquid cultures were shaken at 180 rpm. Solid media contained 1.5% agar. Growth media was supplemented with antibiotics when required. The added antibiotics and concentrations are: ampicillin (50 μg/mL), gentamycin (15 μg/mL), kanamycin (50 μg/mL), and tetracycline (15 μg/mL).

**Recombinant DNA techniques**

Bacterial genomic DNA and plasmid DNA were purified using the Wizard® Genomic DNA Purification Kit (Promega) and the Gene-Spin™ Miniprep Purification Kit (Protech), respectively. Polymerase chain reaction (PCR) was carried out as previously described [28]. Table 2 lists the primers used in this study. Standard protocols for agarose gel electrophoresis, DNA ligation, restriction digestion, and _E. coli_ transformation were as described previously [29]. Transformation of _Xcc_ was achieved through electroporation [30]. The sequence of DNA fragment was determined by Mission Biotech Co., Ltd. (Taipei, Taiwan).

**lolB mutant construction and complementation**

For the construction of _lolB_ mutant, the 790-bp _BamHI-EcoRI_ fragment containing the upstream 133-bp and the entire coding region of the XC17 _lolB_ was PCR-amplified using primers _lolBF/lolBR_ and cloned into the `yT&A` (Yeastern) to produce pTlolB. After sequence verification, the fragment was excised and cloned into the _BamHI-EcoRI_ sites of pUC19G [23] to generate

**Table 2** Primers used in this study

| Primer | Sequence (5’ to 3’) a |
|--------|-----------------------|
| **lolB gene (mutant construction, confirmation and complementation)** | |
| lolBF/lolBR | GGA TCC AAA TCG CCG CGC ACG TGG GT/GAA TTC AGG GCG AGA GCG TCC ATT GG |
| **Extracellular enzyme encoding genes (promoter analysis and RT-qPCR)** | |
| engA gene | AACTGCAAGCCTGGGACAGCAGCGCCGCGGAGGGGG/GCTCTAGAGCTGACACCCGAG GCGCGGTTAA |
| engAF/engAR | GTGTTAAGCTGTTGGCTTC/TCTAGCTTCCAGTCCGT |
| manA gene | CATGCATTTGCGCGCGATGGCA/GCTAGACAAAGTGAGGACGGCCAGAC |
| manAF/manAR | AGTGTACATGCGCCGCACAAAC/GCTGTCATGCGAGCGGTGAAA |
| prt1 gene | AACCTCGAGTGTGGTCGCGCCGAGGACTGAC/GCTAGACGGATCCGCTGTTATG |
| prt1F/prt1R | GTATGAC |
| **Stress tolerance related genes (promoter analysis and RT-qPCR)** | |
| clpP gene | CTGCAGGAGGTTCTATGGGACGGCGGCT/GCTAGAGTTGGGACGGCGGCTG |
| clpPF/clpPR | AGATCTGACCTTGGTTCGG/GTTGAGGTGCGGTTTCG |
| clpX gene | CTGCAGGACGGCGGCGATGCGACATCCATCA/GCTGACAGATCCGATCAAGAGTCG |
| clpXF/clpXR | CTGAGCAGACTGAGC/GCTGACAGATCCGATCAAGAGTCG |
| **Putative lipoprotein encoding genes (RT-qPCR)** | |
| 0253F/0253R | GCAATTCACCAGCTGCGCTCT/TTGTTGACATCGTCAAGCG |
| 0677F/0677R | GGCGACTCTAATGCTTACG/GCACCAGTTCATCAAGCG |
| 0679F/0679R | GCCGATTCTACCAGCGGAGCCA/TTTGTTGGCTGGGAAAGGCT |
| 0707F/0707R | GGTTACGCCATCGATCGTCC/GGCGACTCTACGAGATCGG |
| 1519F/1519R | GCCCTGATGGAAAGCGAAC/CCGTTCTGCTGACATCGT |
| 1584F/1584R | CGACAGACGCTTGACTAGGAAG/TATGAGCGGCTGCAAGG |
| 3831F/3831R | TGAAGATCCACTTGGGCGT/TTTGTTGCTGCTGCTG |
| 4152F/4152R | CGAATTTGCTACATCGTCA/GTCTAGAGTTGGGACGGCGGCT |
| **16S rRNA gene (RT-qPCR)** | |
| 16SF/16SR | GTAAAGCCTGGCTAGGTGTG/GCTGACAGATCCGATCAAGAGTCG |

*a* : Added restriction enzyme sites are underlined
pUCloB. The EZ-Tn5™<KAN-2> Transposon (Km®, 1221 bp) was randomly inserted into pUCloB using the EZ-Tn5™<KAN-2> insertion kit according to the manufacturer's instructions (Lucigen). One plasmid, pUCloBK, with the transposon inserted into the lolB-coding sequence at 326 bp from the translational start site was used for mutant construction. This plasmid was then introduced into the Xcc wild-type XC17 by electroporation, allowing for double crossover, and transformants were selected on LB medium supplemented with kanamycin (transposon selection marker). Insertion of transposon into lolB gene was confirmed by PCR. The confirmed lolB mutant was designated as CL17.

For complementation of the lolB mutant, the 790-bp BamHI-EcoRI fragment of pTloB was excised and inserted into the BamHI-EcoRI sites of pRK415 [24]. The generated plasmid pRKloB was transferred into the lolB mutant strain CL17 by electroporation, giving the complemented strain CL17(pRKloB). For phenotypic comparison, the empty vector pRK415 was introduced into XC17 and CL17, giving transformants XC17(pRK415) and CL17(pRK415) in parallel.

Assays of bacterial attachment and pathogenicity

The bacterial attachment was evaluated by examining the ability of cells to adhere to the 96-well polystyrene microtiter plates (Nunc) and cabbage leaves surface as the previously described method [31]. The experiments were done at least three times. The pathogenicity of Xcc to host plant cabbage was tested by leaf-clipping method [26] and the disease symptoms in cabbage were photographed and lesion lengths were measured 14 days after inoculation. Testing was performed in three independent experiments, each with six replicates.

Extracellular enzyme activity analysis

Extracellular enzyme activity was analyzed by spotting 3 μl of overnight culture (OD550=1) onto XOLN agar plates containing the appropriate substrates. The substrates used are: carboxymethyl cellulose (0.5%, substrate for cellulase), locus bean gum (0.2%, for mannanase), and skimmed milk (1%, for protease). After 2 days (cellulase and mannanase) or 3 days (protease) of incubation, enzyme activity was determined as described previously [20]. Each test was carried out at least three replicates.

Stress tolerance assay

Stress tolerance was tested by inoculating overnight culture into fresh XOLN medium containing glycerol to obtain an initial OD550 of 0.35 in the absence or presence one type of stress condition. The stresses and their concentrations used were as following: EDTA (0.2 mM), H2O2 (0.005%), polymyxin B (2 μg/mL), puromycin (10 μg/mL), and sodium dodecyl sulfate (SDS, 0.0075%). The growth of each strain was determined by measuring the OD550 values after shaking (180 rpm) at 28 °C for 24 h. The method for temperature tolerance assay was according to previously study [31]. Each stress test was repeated at least three times.

Cell membrane integrity analysis

The integrity of Xcc cell membrane was examined by the SYBR Green I/propidium iodide (PI) viability assay as the previously described methods [33, 34] with some modifications. Briefly, the cultured bacteria were harvested by centrifuging at 12,000 rpm for 2 min and washed twice then resuspended in sterile 0.85% NaCl. The final cell suspension was adjusted to an OD550 = 1. Then, the bacterial cells (100 μL) were stained with SYBR Green I (2X, 100 μL) and PI (250 mg/mL, 10 μL). The samples were incubated for 40 min in dark at room temperature. After staining, the samples were washed twice and resuspended in 50 μl 0.85% NaCl, and 5 μl of this sample was trapped in between coverslip and glass slide. The slide was viewed under a fluorescence microscope.

Reporter plasmid construction and promoter activity analysis

Reporter constructs (pFYengA, pFYmanA, and pFY-clpX) were generated by cloning the PCR-amplified upstream regions of engA, manA, and clpX into pFY13–9 [25], with lacZ as the reporter. Briefly, the upstream region of each gene was amplified by PCR using primers 844pstF/1002xbaR for the engA gene, 61pstF/420xbaR for manA gene, and 114xhoF/440xbaR for the clpX gene. Then, the PCR fragments were cloned into pFY13–9, giving rise to pFYengA, pFYmanA, and pFYclpX. Reporter constructs pFYprtI and pFYclpP containing the upstream regions of prtI and clpP, respectively, were obtained as previously described [26, 27]. Xcc strains harboring these constructs were grown overnight and inoculated into fresh media to obtain an initial OD550 of 0.35, after which growth was allowed to continue. Samples were taken in triplicate at designated intervals and the β-galactosidase activity was assayed as previously described, with the enzyme activity expressed in Miller units [20].

RNA isolation, reverse transcription (RT), and quantitative real-time PCR (qPCR)

Total RNA was isolated from bacteria grown to the mid-exponential phase (OD550=0.6) in XOLN medium supplemented with 2% glycerol using the RNeasy Mini Kit (Qiagen) according to provided instructions. The isolated RNA (1 μg) was reverse-transcribed to cDNA using the iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD).
qPCR was performed using iQ™ SYBR® Green Supermix in a CFX96 Real Time PCR system (BIO-RAD). The PCR amplification conditions were as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 30 s at 72 °C. Table 2 lists the sequences of primer sets of the tested target genes. The Xcc 16S rRNA gene was used for normalization. All qPCRs were performed at least three times. The fold change for transcript was calculated by the $2^{-\Delta\Delta Ct}$ method.

**Statistical analysis**
Each experiment was carried out at least three repeats. Values are the averages of three replications per experiment. Student's t test was used to evaluate the statistical significance of differences between averages. A p value < 0.05 was considered statistically significant.

**Results**
Disruption of lolB leads to decrease bacterial attachment
In the genome of Xcc strain XC17, the locus_tag AAW18_RS04315 is annotated to encode lipoprotein insertase outer membrane protein LolB (Gen-Bank accession no. NZ_CP011946) [16]. The XC17 lolB open reading frame is 657 bp in length and is located in the genome sequence at positions 1,003,373–1,004,029 [16]. The XC17 lolB gene are found in several sequenced Xcc strains, such as ATCC33913, 8004, and B100 [12, 17, 18]. Through sequence comparison, it was found that the coding product of XC17 lolB was identical in both amino acid sequence and size to LolBs from Xcc strains ATCC33913 and 8004. The orthologous gene of lolB was also highly conserved in other members of Xanthomonas, such as X. campestris pv. raphani 756C [35], X. campestris pv. vesicatoria 85–10 [36], X. citri subsp. citri (formerly X. axonopodis pv. citri) 306 [18], X. hortorum pv. gardneri ICMP 7383 [37], and X. oryzae pv. oryzae KACC10331 [38] (Table 3). Although the lolB gene has been found in several members of Xanthomonas, none of them has been characterized with regard to function, and no relevant studies were found in the literature.

To explore the physiological role of lolB in Xcc, the lolB mutant and its complemented strain were generated. Biofilm formation was tested on polystyrene microtiter plate (Fig. 1a), and leaf surface (Fig. 1b). As depicted in Fig. 1, it was indicated that the lolB mutant exhibited reduced attachment ability compared with the parental strain, and complementation of lolB mutant with plasmid pRKlolB (with intact lolB gene cloned in pRK415) could restore the adhesion ability to the wild-type level.

The lolB gene is required for the full virulence of Xcc
To determine whether mutation of lolB caused loss of pathogenicity, lolB mutant was used to infect host plant cabbage by leaf-clipping method. At 14 days post inoculation, typical V-shaped black rot symptoms were found on leaves inoculated with the wild-type strain (Fig. 2a) and the lesion lengths were about 1.84 cm (Fig. 2b). However, the lolB mutant showed reduced virulence compared with the wild-type strain (Fig. 2a) and the lesion lengths caused by the mutant were about 0.45 cm (Fig. 2b). The complementation of lolB mutant with pRKlolB partially restored the virulence of the mutant (Fig. 2a). Although the complementary strain cannot fully restore pathogenicity, its consequent mean lesion length (0.93 cm) was significantly longer than that inoculated with the mutant strain (Fig. 2b). These results indicated that lolB is important for host virulence of Xcc.

The lolB gene is involved in extracellular enzyme production
It has been indicated that extracellular enzymes and extracellular polysaccharides contribute to the virulence

| Bacteria                     | Gene ID         | Predicted product                                      | Size (aa) | Identities (%) |
|------------------------------|-----------------|--------------------------------------------------------|-----------|----------------|
| X. campestris pv. campestris ATCC33913 | XCC0870         | Outer membrane lipoprotein precursor                   | 218       | 100            |
| X. campestris pv. campestris 8004   | XCC_3360        | Outer membrane lipoprotein precursor                   | 218       | 100            |
| X. campestris pv. campestris B100  | Xccb100_3479    | Outer membrane lipoprotein receptor LolB               | 218       | 99.1           |
| X. campestris pv. raphani 756C    | XCR_1061        | Outer membrane lipoprotein receptor LolB               | 218       | 98.6           |
| X. campestris pv. vesicatoria 85–10| XCV0978         | Outer membrane lipoprotein receptor LolB               | 217       | 84.4           |
| X. axonopodis pv. citri 306       | XAC0947         | Outer membrane lipoprotein precursor                   | 217       | 85.3           |
| X. hortorum pv. gardneri ICMP 7383| B137_05530      | Lipoprotein localization factor LolB                    | 218       | 85.6           |
| X. oryzae pv. oryzae KACC10331   | XOO3605         | Outer membrane lipoprotein precursor                   | 217       | 84.4           |

*: X. campestris pv. campestris ATCC33913 (GenBank accession number: AE008922); X. campestris pv. campestris 8004 (CP000050); X. campestris pv. campestris B100 (AM920689); X. campestris pv. raphani 756C (CP002789); X. axonopodis pv. citri 306 (AE008923); X. hortorum pv. gardneri ICMP 7383 (CP018731); X. campestris pv. vesicatoria 85–10 (AM039952); X. oryzae pv. oryzae KACC10331 (AE013598)

**: According to a BLASTP search
Fig. 1 Effects of mutation of lolB on cell attachment to polystyrene plates (a) and cabbage leaf surfaces (b) in Xcc. Strains to be tested were grown overnight, washed, and diluted using fresh XOLN medium supplemented with glucose, and were assayed as described in the Material and methods section. XC17v: wild-type strain XC17 carrying empty vector pRK415; CL17v: lolB mutant CL17 carrying pRK415; CL17c: complemented lolB mutant; Blank: XOLN medium supplemented with glucose without inoculation of bacteria. Values presented are the mean ± standard deviation (n = 3). Significance was determined using the Student t test (* indicates significance at p < 0.05).

Fig. 2 Effects of mutation of lolB on virulence of Xcc in cabbage. (a) Black rot symptoms caused by Xcc strains on inoculated leaves of host cabbage plant. After 14 days inoculation, the photographs were taken. (b) Mean lesion lengths caused by different Xcc strains. Values shown are the average ± standard deviation from three repeats, each with six leaves. Significance was determined using the Student t test (* indicates significance at p < 0.05).
of Xcc [39, 40]. The reduced virulence of the lolB mutant (Fig. 2) suggested that the lolB gene has roles in the production of these pathogenicity-related determinants. The activity of extracellular hydrolytic enzymes (including cellulase, mannanase, and protease) was first tested. The results showed that the levels of extracellular cellulase and mannanase were reduced in the lolB mutant and could be restored by complementation (Fig. 3). In the protease assays, the diameters of the hydrolysis zones formed by the lolB mutant were significantly smaller than those found for the wild type and complementary strains. As the colony diameter of wild type are larger than those of lolB mutant and the complementary strain, lolB might be not involved in protease production, although small effects could not be excluded. Next, extracellular polysaccharide production was tested. The extracellular polysaccharide yields produced by the mutant were similar to those of the wild type (data not shown).

The lolB mutant displays increased sensitivity to various stresses

As several factors reported to influence bacterial attachment also have roles in stress tolerance in Xcc [19, 31, 41, 42], we aimed to determine whether the lolB gene was required for stress adaptation of Xcc. To examine whether lolB contributes to stress tolerance, the sensitivity of the lolB mutant together with the wild-type and complementary strains was evaluated under a range of stresses, including heat, EDTA, H$_2$O$_2$, polymyxin B, puromycin, and SDS. At physiological temperature (28 ºC), the tested strains plated at all densities grew normally (Fig. 4a, left). When bacterial strains grew at elevated temperature (37 ºC), the growth of lolB mutant was inhibited and this growth deficiency was restored by genetic complementation (Fig. 4a, right). When bacterial strains were exposed to EDTA, H$_2$O$_2$, polymyxin B, and SDS, the lolB mutant exhibited significant growth reduction compared to the wild type and complementary strains (Fig. 4b). These data indicated that lolB is involved in stress in Xcc.

Mutation of lolB influences the expression of genes encoding extracellular enzymes and involved in stress tolerance

Since mutation of lolB leads to reductions in bacterial attachment, extracellular enzyme production, and stress tolerance, lolB might be involved in expression of genes related to these phenotypes. Five genes (engA, manA, prt1, clpP, and clpX) were selected based on the alternated mutant phenotypes mentioned above. Among them, engA (encodes major cellulase) [28, 40], manA (encodes major mannanase) [39, 43], and prt1 (encodes major protease) [40, 44] have been implicated as virulence factors. Both clpP and clpX (encode the proteolytic core and ATP-binding subunit of Clp protease, respectively) were known to play a role in extracellular enzyme production, stress tolerance, and virulence [27, 31]. In addition, the clpX gene was also reported to be involved in bacterial attachment [31]. To evaluate the involvement of lolB in expression of these virulence-related genes, reporter constructs containing the upstream regions of these genes (pFY-engA, pFY-manA, pFY-prt1, pFY-clpP1, and pFY-clpX) were introduced into XC17 (wild type) and CL17 (lolB mutant), and the resultant strains were subjected to β-galactosidase assays. As depicted in Fig. 5a, the β-galactosidase levels of CL17 harboring pFY-engA, pFY-manA, pFY-prt1, pFY-clpP1, and pFY-clpX were 64%, 49%, 47%, 68%, and 71% of the levels of XC17 carrying the same constructs. The effect of lolB mutation on the expression of these genes was also evaluated by RT-qPCR. The results indicated that all of the tested genes were significantly reduced in the lolB mutant when compared with wild type (Fig. 5b). Taken together, both sets of expression results from the reporter assay and RT-qPCR analysis suggested lolB mutation affects the expression of engA, manA, prt1, clpP, and clpX.

![Fig. 3](image-url) Effects of mutation of lolB on extracellular enzyme production in Xcc. The extracellular enzyme activity was evaluated using the substrate-supplemented plate assay as described in the Material and methods section. Values under each photograph are the diameter of hydrolysis zone (in cm) (mean ± standard deviation) from three independent experiments. Different letters following the values indicate significant difference (Student t test, p < 0.05)
Mutation of lolB influences the expression of genes encoding putative lipoproteins and the integrity of cell membrane

The findings showing reduced bacterial attachment and resistance to several membrane-perturbing compounds in lolB mutant, compared to the wild-type strain, might be due to an altered outer membrane lipoprotein profile and change in cell membrane integrity. Till now, the key amino acid residues involved in lipoprotein localization in Xcc remain unknown. According to DOLOP, a database of bacterial lipoproteins, 101 lipoproteins in the genome sequence of Xcc strain 8004 were identified [45]. To test the effects of lolB mutation on the expression of predicted lipoproteins, eight putative lipoprotein encoding genes are randomly selected, and the expression of these genes is evaluated by RT-qPCR. Table 4 shows that three genes, including XC_0707, XC_1584, and XC_4152, were not expressed differently in the lolB mutant and wild-type strains. However, the expression of five genes was significantly upregulated in the lolB mutant compared to the wild-type strain; they were genes encoding a dipeptidyl aminopeptidase (XC_0253), a methanol dehydrogenase (XC_0679), an alkaline phosphatase (XC_1519), and two hypothetical proteins (XC_0677 and XC_3831).

For examining the cell membrane integrity, SYBR Green I and PI were used for double staining of nucleic acids. SYBR Green I is a green permeable dye that stains all live cells, whereas PI is a red impermeant dye that stains only dead or damaged cells with a compromised cell membrane [34]. Thus, live bacteria with intact membranes fluoresce green, while bacteria with damaged membranes fluoresce red. As shown in Fig. 6, it can be clearly seen that the wild type appeared predominantly green (indicating cells with intact membranes); whereas the lolB mutant appeared substantially red (demonstrating cells with damaged membranes).

Discussion

In the fully sequenced Xcc genome, four lol genes have been annotated to encode proteins constituted to form Lol system [12, 16–18]. Till now, only lolA has been studied [19]. The goal of the present study was to characterize the function of Xcc lolB. Through genetic complementation and phenotypic evaluation, it was demonstrated that in Xcc, lolB is involved in various cellular processes, including bacterial attachment, extracellular enzyme production, pathogenesis, tolerance to a range...
of environmental stresses, and the maintenance of cell membrane integrity.

The LolB homologues have been found in multifarious bacteria, and there are 1435 sequences with LolB domain (Pfam03550) are listed in the Pfam family database [46]. Among them, only the LolB of *E. coli* has been characterized in detail. In *E. coli*, lolB is an essential gene and deletion of lolB is lethal and causes accumulation of lipoprotein localization intermediates in the periplasm [47, 48]. Via mutagenesis analysis, five conserved Trp residues (at positions 18, 52, 117, 148, and 183) of LolB were determined to affect membrane localization.

![Fig. 5](image)

**Fig. 5** Effects of mutation of lolB on the expression of genes coding for extracellular enzymes or products associated with stress tolerance. (a) β-Galactosidase activities of XC17 (wild type, black bar) and CL17 (lolB mutant, gray bar) carrying pFYengA, pFYmanA, pFYprtl, pFYclpP1 and pFYclpX were determined. Tested strains were cultured in XOLN medium containing glycerol for 24 h. Significance was determined by the Student t test (*) indicates significance at *p* < 0.05). (b) The expression level of extracellular enzyme genes (engA, manA, and prtl) and stress tolerance-related genes (clpP and clpX) in the wild-type strain XC17 and lolB mutant strain CL17 was examined by RT-qPCR. The relative expression level of each test gene in XC17 and CL17 was normalized to its 16S rRNA content. Values shown are the average ± standard deviation (*n* = 3).

### Table 4: Comparison of expression of putative lipoprotein encoding genes in the wild type XC17 and the lolB mutant CL17 by RT-qPCR

| Gene ID  | Description                              | Predicted lipoprotein signal | Fold change (CL17/XC17)  | *p* value |
|----------|------------------------------------------|------------------------------|---------------------------|-----------|
| XC_0253  | Dipeptidyl aminopeptidase                | MQRLLLASSLLLAL**LSAC**LDSK  | 3.1015 ± 0.1651           | 0.0361    |
| XC_0677  | Hypothetical protein                     | MKYLLSALCVAALSGCTDRE        | 6.9535 ± 0.4812           | 0.0312    |
| XC_0679  | Methanol dehydrogenase                   | MHQSSCRARGGVMLMLALSAV**LACG**KDT | 5.3956 ± 0.3450         | 0.0348    |
| XC_0707  | Rare lipoprotein A                       | MNSITGPKWLPAALMLGLACSSLAP  | 3.0953 ± 0.2584           | 0.0733    |
| XC_1519  | Alkaline phosphatase                     | MPMRYRLPALLTLTV**VAAC**ASTA | 1.7876 ± 0.0305           | 0.0177    |
| XC_1584  | Cyanogoglobin                            | MMTRWRLYSSLVLLTL**LACG**ATQQ | 3.0530 ± 0.3839           | 0.0615    |
| XC_3831  | Hypothetical protein                     | MKHWAVLACATLL**LACG**ORQ   | 2.2654 ± 0.1078           | 0.0056    |
| XC_4152  | Cytochrome c biogenesis protein          | MARRFPWLLGL**LACG**LVA    | 3.4164 ± 0.3353           | 0.0581    |

*a*: Gene ID is based on *X. campestris pv. campestris* strain 8004  
*b*: According to the DOLOP database. The predicted lipobox with invariant cysteine is bold and underlined  
*c*: The relative expression level of each test gene in XC17 and CL17 was normalized to its 16S rRNA content. Values shown are the average ± standard deviation (*n* = 3)
of *E. coli* lipoproteins [49], and Leu-68 in the protruding loop of LolB was also revealed to play critical roles in the membrane anchoring activity [50]. The *Xcc* LolB protein deduced from the gene contained 218 amino acids, with a typical *N*-terminal lipoprotein signal peptide, and the predicted signal peptidase II cleavage site was at LSG^{20–21}V as predicted by signal P software [51]. Conserved domain search showed that it has a LolB domain located at residues 58–214 (bit score: 138.90; E-value: 4.2e-37). The *Xcc* LolB had 25% identity and 41% similarity to *E. coli* LolB (encoded by *b1209* gene of *E. coli* K-12). Sequence analysis displayed that the aforementioned amino acid residues essential for the function of *E. coli* LolB were not fully conserved in *Xcc* LolB. The conserved amino acid residues included Trp-81, Trp-147, and Trp-214, which corresponding to Trp-52, Trp-117, and Trp-183 in *E. coli* LolB. The residues in comparable positions for Trp-18, Leu-68, and Trp-148 in *E. coli* LolB were substituted by Val-39, Val-96, and Ile-179 in *Xcc* LolB, respectively. The role of these residues in *Xcc* LolB function remains to be elucidated.

Apart from the observations that mutation in the lolB gene of *E. coli* affected the localization of lipoproteins, nothing is known about the role of lolB in cellular processes of bacteria. Here, we find that lolB has multifaceted biological functions in *Xcc*. We first demonstrate that mutation in lolB gene affects bacterial attachment of *Xcc* on abiotic surfaces and host leaves (Fig. 1). As biofilm has been characterized as a virulence trait in many phytopathogenic bacteria [52], our phenotypic evaluation showing the involvement of lolB in bacterial attachment prompted us to determine whether the lolB gene is associated with pathogenicity of *Xcc*. Further, regarding to the general role of biofilm formation in promoting bacterial survival against stresses and protecting bacteria from harsh environment, we reasoned that lolB inactivation might impair the growth ability of *Xcc* under stress treatment. Therefore, the roles of lolB in pathogenicity, virulence factor production, and stress tolerance were evaluated. We demonstrate that mutation in lolB results in a substantial reduction in virulence (Fig. 2). The attenuation in virulence of the lolB mutant may result, at least partially if not entirely, from the reduction in extracellular enzyme (including cellulase and mannanase) production (Fig. 3). In addition, the lolB mutant grew much slower under heat stress and in the presence of agents that influence integrity of cell membrane compared with that of the wild-type strain (Fig. 4). Because mutation of lolB led to reductions in extracellular enzyme production and stress tolerance, it seems reasonable to predict that LolB might influence the expression of genes related to these phenotypes. The reporter assay and RT-qPCR analysis (Fig. 5) revealed that mutation in lolB reduced the expression of genes known to be essential for extracellular enzyme production, stress tolerance, and virulence (*engA, manA, prt1, clpP*, and *clpX*). It is implying that lolB might affect the expression of these genes transcriptionally and that the decrease in extracellular enzyme production, bacterial attachment, as well as stress tolerance of the lolB mutant may be attributable to the reduced transcription of these genes.

Lipoprotein is a crucial structural component of the outer membrane, and is central to the physiology of the Gram-negative cell envelope. It is essential for maintaining cellular integrity, envelope stability, and nutrient acquisition, and also plays roles in bacterial pathogenic mechanisms such as attachment, colonization, and invasion [1, 53]. Bacterial lipoproteins also play an important role in growth and survival of bacteria [54]. Correct localization of lipoproteins is essential for their function, and the Lol system is required for lipoproteins localization [2, 3, 5, 55]. Although the involvement of *Xcc* LolB...
in lipoprotein localization was not experimentally demonstrated, we discerned that several putative lipoprotein encoding genes were significantly up-regulated in the lolB mutant (Table 4) and mutation of lolB decreases the integrity of cell membrane (Fig. 6). Hypothetically, we reasoned that the declined biofilm formation attributable to lolB inactivation is resulted from changed cell membrane integrity that subsequently affects adhesion ability. It is possible that the lolB mutant has impacts in lipoprotein localization and subsequently the outer membrane lipoprotein profile was altered due to lolB mutation, thereby sensitizing cells to membrane perturbing compounds such as SDS and EDTA. The findings showing impaired tolerance against heat stress and several membrane-perturbing compounds in lolB mutant could be a plausible explanation for the altered membrane integrity seen in lolB mutant and suggests that Xcc LolB might possess potential role in lipoprotein localization. The mechanism by which the LolB protein acts on lipoprotein outer membrane localization in Xcc remains to be experimentally elucidated.

Bacterial lipoproteins contain a characteristic consensus sequence [LVI][ASTV][GAS]C known as a lipobox [2, 45]. Overall, the putative lipoprotein encoding genes tested in this study showed a typical lipobox (Table 4). None of these putative lipoprotein encoding genes had been characterized with respect to lipid modification and membrane localization. The involvement in Xcc attachment, virulence, and stress tolerance of these lolB regulated genes is still not known and remains to be explored. It is intriguing that the lolB mutant exhibits an increased expression of several putative lipoprotein encoding genes. Together with the above observations, it is suggested that the inactivation of lolB might alter the outer membrane lipoprotein profiles and such alterations subsequently stimulate the compensatory pathway(s) to increase the expression of lipoprotein related genes to keep lipoprotein homeostasis. It is also pertinent to note that the Xcc LolB possibly affects the transcription of these tested genes indirectly, as the gene product encoded by lolB is not belonging to a regulatory protein. LolB likely influences these genes through an unknown regulatory pathway in Xcc. Further investigation of the potential genes that encoding the unknown regulatory trail that is activated after lolB mutation is needed to confirm the possibility.

Conclusion
Here, we characterize the lolB gene in Xcc. By the use of genetic complementation and phenotypic evaluation, we acquired conclusive genetic evidence demonstrated that the lolB plays relevant roles in bacterial attachment, extracellular enzyme production, stress tolerance, as well as virulence of Xcc. Consistent with phenotypic alterations, the reporter assay and RT-qPCR analysis displayed that the genes encoding major extracellular enzymes, and genes previously reported to be associated with adhesion, stress tolerance, and virulence were reduced in the lolB mutant compared with the wild type. To the best of our knowledge, this is the first work to provide insights of the lolB physiological roles in multifarious cellular processes, including pathogenicity-related functions and environmental stress adaptation.

Abbreviations
LB: Luria–Bertani; Lol: lipoprotein outer membrane localization; qPCR: quantitative real-time polymerase chain reaction; RT: reverse transcription; Xcc: Xanthomonas campestris pv. campestris.

Acknowledgements
Not applicable

Authors’ contributions
CT and YM managed the grants, supervised the laboratory work, and conceived and design the experiments. CE, HC, CH, and YC performed the experiments. CT and YM analyzed the experimental results, interpreted the data, and wrote the paper. All authors read and approved the final manuscript.

Funding
This study has been supported by Ministry of Science and Technology of Taiwan (grants No. MOST107-2313-B-166–001-MY3 and MOST110-2313-B-166–001) to YWH, and Central Taiwan University of Science and Technology (grant No. CTU109-P-102) to CTL. The authors declare no conflict of interest.

Availability of data and materials
The data generated and/or analyzed during the current study are included in this article.

Declarations
Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interest.

Received: 5 July 2021 Accepted: 6 December 2021
Published online: 07 January 2022

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