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Techniques

Liberibacter crescens Is a Cultured Surrogate for Functional Genomics of Uncultured Pathogenic '*Candidatus* Liberibacter' spp. and Is Naturally Competent for Transformation

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ABSTRACT

'*Candidatus* Liberibacter' spp. are uncultured insect endosymbionts and phloem-limited bacterial plant pathogens associated with diseases ranging from severe to nearly asymptomatic. '*Ca.* L. asiaticus', causal agent of Huanglongbing or citrus "greening," and '*Ca.* L. solanacearum', causal agent of potato zebra chip disease, respectively threaten citrus and potato production worldwide. Research on both pathogens has been stymied by the inability to culture these agents and to reinoculate into any host. Only a single isolate of a single species of Liberibacter, *Liberibacter crescens*, has been axenically cultured. *L. crescens* strain BT-1 is genetically tractable to standard molecular manipulation techniques and has been developed as a surrogate model for functional studies of genes, regulatory elements, promoters, and secreted effectors derived from the uncultured pathogenic Liberibacters. Detailed, step-by-step, and highly reproducible protocols for axenic culture, transformation, and targeted gene knockouts of *L. crescens* are described. In the course of developing these protocols, we found that *L. crescens* is also naturally competent for direct uptake and homology-guided chromosomal integration of both linear and circular plasmid DNA. The efficiency of natural transformation was about an order of magnitude higher using circular plasmid DNA compared with linearized fragments. Natural transformation using a replicative plasmid was obtained at a rate of approximately 900 transformants per microgram of plasmid, whereas electroporation using the same plasmid resulted in 6×10^4 transformants. Homology-guided marker interruptions using either natural uptake or electroporation of nonreplicative plasmids yielded 10 to 12 transformation events per microgram of DNA, whereas similar interruptions using linear fragments via natural uptake yielded up to 34 transformation events per microgram of DNA.

'*Candidatus* Liberibacter' spp. are fastidious, uncultured, Gram-negative, and phloem-limited α-proteobacteria (order *Rhizobiales*) that are emerging as a versatile group of plant pathogens capable of infecting a wide range of plant hosts. '*Ca.* L. asiaticus' causes Huanglongbing (HLB) or citrus "greening" disease and is arguably the single most devastating disease of citrus worldwide (Gabriel et al. 2019). HLB is also caused—to a much more limited extent—by '*Ca.* L. americanus' in Brazil and '*Ca.* L. africanus' in Africa. '*Ca.* L. asiaticus' is vectored and transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Grafton-Cardwell et al. 2013). HLB is characterized by perturbed assimilate partitioning, progressive decline in productivity, and eventual death of infected citrus trees. '*Ca.* L. solanacearum' is vectored by the potato psyllid *Bactericera cockerelli* Sulc and causes zebra chip disease on potato (Lin et al. 2011). Zebra chip is marked by purplish discoloration and chlorosis of leaves, collapsed stolons, browning and necrotic flecking of vascular tissue in the tubers, and rapid death of infected potato plants. '*Ca.* L. solanacearum' is also capable of infecting several other Solanaceous and Apiaceous crops, causing psyllid yellows in tomato, yellows decline, and vegetative disorders in carrots and celery (Monger and Jeffries 2018).

Only a single isolate of a single species of Liberibacter, *Liberibacter crescens*, has been cultured in vitro (Leonard et al. 2012). The wild-type *L. crescens* strain BT-1 (GenBank accession NC_019907.1) was originally isolated from the sap of Babaco mountain papaya (*Carica stipulata* × *Carica pubescens*) but it nevertheless has no known plant or insect host. Consistent failures in several laboratories to reinoculate BT-1 or derivatives into Babaco and other plants or insects have led to a consensus that BT-1 is both nonpathogenic and noninfectious. Although it is possible that some yet to be discovered strains of *L. crescens* might be parasitic if not pathogenic to plants, *L. crescens* is not listed as a pathogen by the U.S. Department of Agriculture Animal and Plant Health Inspection Service on its U.S. Regulated Plant Pest List. By contrast with cultured *L. crescens* BT-1 and derivatives, all pathogenic '*Ca.* Liberibacter' spp. are uncultured to date.

The sequenced '*Ca.* L. asiaticus' strain gxpsy (NC_020549.1; Duan et al. 2009), '*Ca.* L. americanus' strain Sao Paulo (CP006604.1; Wulff et al. 2014), '*Ca.* L. africanus' strain PTSAPSY (NZ_CP004021.1; Lin et al. 2015), and '*Ca.* L. solanacearum' strain ZC1 (NC_014774.1; Lin et al. 2011) all have highly reduced genome sizes of ~1.2 Mb compared with the slightly larger 1.5 Mb of *L. crescens* BT-1 and the ~6.7 Mb genome of the phylogenetically related *Sinorhizobium meliloti* (NC_003047.1). *L. crescens* BT-1 appears to have diverged earlier during evolution than the pathogenic '*Ca.* Liberibacter' spp. (Nakabachi et al. 2013). Comparative genomics and metabolic pathway analyses of all Liberibacter genomes have revealed a trend for the reduction or complete absence of several biosynthetic pathways, metabolic enzymes, and secretion systems (Wulff et al. 2014).

Attempts to culture '*Ca.* L. asiaticus' in axenic media have resulted only in inconsistent, transient, and "low-titer" (estimated only by

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quantitative PCR) cocultures to date (Davis et al. 2008; Fujiwara et al. 2018; Merfa and De La Fuente 2019; Parker et al. 2014). The inability to culture pathogenic '*Ca.* Liberibacter' spp. or even to reinoculate transient cocultures into insect or plant hosts has severely restricted functional genomic analyses of suspected pathogenicity factors, molecular characterization of host–pathogen interactions, and subsequent development of chemical control methods for HLB and zebra chip. '*Ca.* L. asiaticus' genes that are differentially expressed in plant and insect hosts, and those involved in transcriptional regulation, major metabolic pathways, secretion and transportation systems, motility, and signal transduction, have all been predicted as potential virulence factors (Cong et al. 2012; Yan et al. 2013). Absent cultured pathogenic '*Ca.* Liberibacter' spp., the γ-proteobacterium *Escherichia coli* and the phylogenetically related α-proteobacteria *S. meliloti* and *L. crescens* have primarily been used as surrogate gene expression hosts for specific functional analyses.

E. coli has proved useful in attempts to validate predicted leader peptides encoded by genes cloned from '*Ca.* L. asiaticus' and periplasmic localization of these proteins (Prasad et al. 2016), as well as outer membrane localization of '*Ca.* L. asiaticus' type V autotransporters (Hao et al. 2013). Even though all Liberibacters including *L. crescens* lack the T2SS (Fagen et al. 2014), only *L. crescens* has been used to demonstrate extracellular secretion of '*Ca.* L. asiaticus' enzymes and effectors. The prophage-encoded '*Ca.* L. asiaticus' peroxidase (F489_gp15) was predicted using SecretomeP 2.0 (Bendtsen et al. 2005) to be noncanonically secreted. This prediction was experimentally confirmed by expression of the '*Ca.* L. asiaticus' peroxidase in *L. crescens*, whereas no such secretion was observed using the same clone expressed in *E. coli* (Jain et al. 2015). Similarly, the '*Ca.* L. asiaticus' peroxiredoxin gene (CLIBASIA_RS00445) was cloned and expressed in *E. coli* and functionally characterized as an active peroxiredoxin in vitro (Singh et al. 2017), but the enzyme was not reported as being secreted or having any pathogenicity function. However, nonclassical extracellular secretion of '*Ca.* L. asiaticus' peroxiredoxin was predicted using SecretomeP 2.0 and subsequently demonstrated by expression in *L. crescens* (Jain et al. 2018). Additional characterization of the gene expressed in planta confirmed its likely role in '*Ca.* L. asiaticus' pathogenesis (Jain et al. 2018, 2019b). The use of *L. crescens* to demonstrate extracellular secretion combined with transient expression assays in planta can effectively be used to identify potential '*Ca.* L. asiaticus' and '*Ca.* L. solanacearum' pathogenicity effectors.

S. meliloti has also been used for investigating '*Ca.* L. asiaticus' regulatory genes. For example, LdtR is a multidrug resistance regulator (MarR family transcriptional activator) of both *ldtR* and the immediately downstream *ldtP* (encoding L,D-transpeptidase) that are implicated in osmotic stress tolerance in *S. meliloti* (Pagliai et al. 2014). '*Ca.* L. asiaticus' also encodes *ldtR* and *ldtP* with similar gene organization, and small molecule-mediated inhibition of '*Ca.* L. asiaticus' LdtR (CLIBASIA_RS01145) appeared to reduce '*Ca.* L. asiaticus' titer in planta (Pagliai et al. 2014), possibly owing to a role of '*Ca.* L. asiaticus' *ldtP* (CLIBASIA_RS01140) in preserving '*Ca.* L. asiaticus' envelope and lipid A structure and hence osmotic tolerance, particularly in the sucrose-rich citrus phloem environment (Coyle et al. 2018).

'*Ca.* L. asiaticus' LdtP was found to express unexpected esterase activity that resulted in altered *E. coli* lipid A structure (Coyle et al. 2018). The *E. coli* genome encodes the entire nine-enzyme Raetz pathway, resulting in six hydrophobic acyl chains that anchor lipid A in the bacterial outer membrane (Emiola et al. 2015). By contrast, *L. crescens* lacks the final Raetz pathway enzyme, LpxM (lauroyl-Kdo2-lipid IVA myristoyltransferase), predictably resulting in penta-acylated lipid A. More importantly, all pathogenic (and uncultured) '*Ca.* Liberibacter' spp. also lack LpxL (Kdo2-lipid IVA lauroyltransferase), likely resulting in a tetra-acylated lipid A moiety. Arguably, *L. crescens* seems to be a better choice for functional studies of lipid A genes and structure determination.

L. crescens has been notoriously difficult to culture, and even thought to be recalcitrant to standard genome editing tools (Coyle et al. 2018). However, incremental improvements in media, culture conditions, and molecular techniques have yielded a very practical and reproducible toolkit for making *L. crescens* genetically tractable. The genome content of *L. crescens* is highly similar to and syntenic with the pathogenic '*Ca.* Liberibacter' spp. (Nakabachi et al. 2013). *L. crescens* is clearly the best available surrogate system for functional evaluation of '*Ca.* L. asiaticus' and '*Ca.* L. solanacearum' promoter elements, genes affecting '*Ca.* Liberibacter' spp. membranes, cell structure, and secretion. When combined with transient expression systems in plant hosts, responses to suspected plant effectors can be confirmed. *L. crescens* was shown to colonize the greater wax moth (*Galleria mellonella*), a model insect host (Munoz-Bodnar et al. 2019), raising the possibility of evaluating responses to suspected insect effectors. We provide detailed and highly reproducible protocols for growth, storage, genetic transformation, and site-directed mutagenesis in *L. crescens* strain BT-1. We also demonstrate that *L. crescens* BT-1 is naturally competent for uptake of both linear and circular plasmid DNA and chromosomal integration via homologous recombination, thereby eliminating the need for electroporation.

MATERIALS AND METHODS

Culture conditions for *L. crescens* strain BT-1. Growth of *L. crescens* BT-1 cells (ATCC BAA-2481) was adequately supported on Basal Medium 7 (BM7) consisting of 2 g of α-ketoglutaric acid, 10 g of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer, and 3.75 g of KOH in 550 ml of water, pH 6.9, followed by the addition of 300 ml of filter-sterilized fetal bovine serum (HyClone Laboratories, Logan, UT) and 300 ml of modified Grace's insect culture medium (TNM-FH; HyClone Laboratories; Hink 1970) with gentle shaking at 150 rpm at 28°C. For long term storage, BT-1 was maintained in BM7 plus 25% glycerol (vol/vol) and stored frozen at –80°C. From glycerol stocks, cultures were initiated as streaks on BM7 medium solidified with 1.5% (wt/vol) agar (Difco Agar; BD Diagnostics, VWR, Radnor, PA). From streaks taken from freezer stocks, it usually took between 5 and 6 weeks to isolate single-cell BT-1 colonies for use in liquid cultures or restreaking on solid plates. A marked improvement to achieve higher titers and longer cell survival was to modify BM7 by doubling the ACES buffer (i.e., adding 20 g/liter of medium), termed BM7A medium.

Initially BT-1 colonies on BM7 appeared luminous and transparent, gradually maturing over a period of 10 to 12 days to a dull white appearance. Mature white colonies quickly lost viability within 10 to 12 additional days and had to be transferred to fresh medium during this time. This time was not extendable by refrigeration of plates. Growth of a single mature colony removed from a freshly streaked plate and inoculated in liquid BM7 medium yielded an OD_{600} of 0.65 in 5 days at 28°C, corresponding to ~10^7 CFU/ml. BT-1 cultures were routinely maintained by transferring 100 µl of culture to 2 ml of fresh BM7 or BM7A medium every 5 to 7 days, respectively. *L. crescens* BT-1 stocks and cultures were routinely examined by PCR (Table 1) to authenticate the purity of cultures.

Genomic DNA extraction, PCR amplification, and cloning. Genomic DNA from *L. crescens* cells was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's recommendations. PCR reactions were performed using AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and the amplification products were cloned into pCR2.1TOPO (Invitrogen) and transformed into *E. coli* TOP10 (Invitrogen) cells using the manufacturer's protocol. The plasmids were verified for sequence fidelity.

Preparation of electrocompetent cells of *L. crescens* strain BT-1. *L. crescens* BT-1 cells were transformed by

electroporation partly following a protocol of Guilhabert and Kirkpatrick (2003) useful for *Xylella fastidiosa*, but with several important modifications. A 2-ml starter culture of BT-1 cells was grown at 28°C with orbital shaking in a glass tube (for maximum visibility) at 150 rpm for 5 days. The entire culture was diluted with fresh BM7 or BM7A medium to a final volume of 10 ml and placed in a 220-ml Erlenmeyer flask. The cells were grown at 28°C with orbital shaking for 2 days, an additional 40 ml of BM7 or BM7A was added, and growth continued for 3 more days. The 5-day-old BT-1 cell culture (50 ml, OD_{600} = 0.5) was chilled on ice for 30 min, centrifuged at 4,000 rpm at 4°C, and washed once in 50 ml and once in 20 ml of ice-cold sterile distilled water. The cell pellet was finally washed in 20 ml of ice-cold 10% glycerol (vol/vol) and then resuspended in 1 ml of ice-cold 10% glycerol. Forty-microliter aliquots of competent cells were flash-frozen in liquid nitrogen and stored at –80°C.

Transformation of *L. crescens* strain BT-1 by electroporation. For electroporation, an aliquot of competent cells was thawed on ice and gently mixed with ~5 µl (=1 µg) of plasmid DNA. The mixture of competent cells and DNA was transferred to a 1-mm cuvette prechilled at –20°C and electroporated at 1,800 V, yielding a time constant in the range from ~5.6 to 6.2 ms (Eppendorf Electroporator 2510; VWR). Any transformation mixtures yielding a time constant below 5.6 ms were discarded. The electroporated cells were immediately recovered in 900 µl of BM7 or BM7A broth without antibiotics and transferred to 5-ml sterile tubes. The transformed cells were allowed to recover for 16 h in liquid BM7 or BM7A medium without antibiotics with gentle shaking at 28°C at 150 rpm and finally were plated on 30 ml of BM7 or BM7A medium solidified with 1.5% (wt/vol) agar, with antibiotic selection.

Site-directed mutagenesis in *L. crescens* strain BT-1. *L. crescens* strain BT-1 was marked with a kanamycin (Kn) resistance gene via marker interruption of a nonessential target locus, *lcrRIP* (type I restriction endonuclease subunit R; B488_RS03405) in the chromosome, following the strategy outlined in Castañeda et al. (2005). A partial (769 bp) DNA fragment internal to the *lcrRIP* coding region was amplified using primers MJ01 and MJ02 (Table 1) and the amplicon was cloned into nonreplicative plasmid (in *L. crescens*) pCR2.1-TOPO (pUC ori, Kn^R; Invitrogen). The resulting suicide plasmid pMJ01 (Table 2) was transformed into *L. crescens* strain BT-1 by electroporation.

Natural transformation of *L. crescens* strain BT-1. A 40-µl aliquot of electrocompetent BT-1 cells was mixed gently with 1 µg of linear or closed circular plasmid DNA (either replicative or not) and 50 µl of chitin (1 mM, C-3387; Sigma-Aldrich) or xanthan gum (0.5% wt/vol in water, G-1253; Sigma-Aldrich). The transformation mix was resuspended in 850 µl of liquid BM7A medium, incubated with gentle shaking at 28°C at 150 rpm for 48 h, and finally plated on selective BM7A medium containing gentamycin (Gm; 2.0 mg/liter).

Natural uptake and transformation of *L. crescens* strain BT-1 was also demonstrated by interruption of the target locus *lcrRIP* using either linear or circular "suicide" (narrow host range, *E. coli* replicon) plasmid DNA. The *lcrRIP* internal fragment (2 kb) was amplified with the primer pair CLL02F and CLL05R and cloned into pCR2.1-TOPO (Invitrogen) to yield the suicide plasmid pCLL02 (Kn^R; Table 2). The Gm resistance gene (aminoglycoside-3-*O*-acetyltransferase I gene, *aacC1*; 834 bp) was PCR amplified from pUFR071 (De Feyter and Gabriel 1991) using the primers CLL11F and CLL11R and ligated within the *lcrRIP* fragment using an internal *Sal*I restriction site to yield suicide plasmid pCLL04 (Kn^R and Gm^R). Both pCLL04 (6.765 kb) and the *Bam*HI/*Sma*I-digested linear insert (2.834 kb) were used for BT-1 transformation.

RESULTS

Optimized culture conditions for *L. crescens* strain BT-1. Liquid cultures of *L. crescens* BT-1 grown for longer than 5 days in liquid medium often failed to grow upon transfer to fresh BM7 medium, despite measurably increasing OD_{600} after 5 days. The consistent increase in OD_{600} observed for 8 days after BT-1 growth in BM7 medium was not correlated with bacterial viability (Fig. 1A). In a conventional spot cell count assay, BT-1 viability (in CFU/ml) was significantly reduced after 6 days of culture in BM7, and the cells were completely nonviable by day 8 (Fig. 1B). However, in BM7A medium containing 2× ACES buffer (20 g/liter), liquid cultures of *L. crescens* BT-1 were reliably maintained and transferred to fresh medium for up to 12 days. Higher concentrations (up to 4×) of ACES or substituting 2-(*N*-morpholino) ethanesulfonic acid in BM7 medium decreased the viability of *L. crescens* (data not shown). BT-1 cells in liquid culture also lost viability rapidly (within days) when stored at 4°C.

TABLE 1. Primers used in this study

Target/primer[a]	Sequence (5′→3′)	Reference
L. crescens confirmation		
B488_RS02295_F	TGACGAGATGCTCAGGATTGAGTTTA	This study
B488_RS02295_R	TATCTTCCCGCCATTAACTCTTGCTT	
*gloA*_F	GTTGTGTAGTCTTCAGTATCCCAG	Jain et al. (2017b)
*gloA*_R	GGGTCTCTATGAAGTTGATCGTC	
*sut*_F	TATGCTTGGGAGCATGTTAGG	Jain et al. (2017b)
*sut*_R	CAAGAACCGGAAGAGCGATAG	
Cloning in pCR2.1-TOPO for marker interruption/ exchange and confirmation of transformation events		
*lcrRIP*_internal_F (MJ01)	CCATTGATCTGGTGCTGTTTATC	This study
*lcrRIP*_internal_R (MJ02)	TTGGGCTTGGACGACTTTTGC	
*sut*_internal_F	TATGCTTGGGAGCATGTTAGG	Jain et al. (2017b)
*sut*_internal_R	CAAGAACCGGAAGAGCGATAG	
*lcrRIP*_internal (CLL02F)	GGATCCCCGGCAAAAGTCGTCCAAGC	This study
*lcrRIP*_internal (CLL05R)	CCCGGGTGCGTAGCAAGTTTCTGATG	
aacC1 (CLL11F)	TAGTCGACAGGACAGAAATGCCTCGACT	This study
aacC1 (CLL11R)	TAGTCGACTTAGGTGGCGGTACTTGGGT	
MJ10	ATGCGAGCATCAGCACATCAGGAAAAACA	This study
MJ11	CCACGCAGCAGGTCATAAA	This study
MJ12	ACGTTGTAAAACGACGGCCAGTGAATT	This study
MJ13	CAGGAAACAGCTATGACCATGATTACGCCA	This study
CLL01F	GAGCTCATGCGAGCATCAGCACATCAGGA	This study

[a] F = forward, R = reverse, *gloA* = glyoxalase I (B488_RS02175), *sut* = sugar transporter (B488_RS00965), *lcrRIP* = putative restriction subunit R (B488_RS03405), and *aacC1* = aminoglycoside-3-*O*-acetyltransferase I gene (gentamycin acetyltransferase).

Compatible replicative vectors and useful antibiotics for transformation of *L. crescens* strain BT-1. While preparing electrocompetent cells, it was essential to increase the 2-ml starter culture volume in a stepwise manner, increasing to 10 ml and then to 50 ml to obtain reproducible growth (OD_{600} = 0.5) of competent BT-1 cells in 5 days. The transformation competence of prepared cells was compromised significantly if the cells were harvested at $OD_{600} > 0.5$. Given the long incubation time needed for recovering primary transformation events, it was critical that the transformation mix was plated on at least 30 ml of selective medium to avoid desiccation.

To determine whether *L. crescens* BT-1 might be tractable for functional genomics studies, the minimum inhibitory concentrations of several antibiotics commonly used for plasmid selection were determined. BT-1 was quite sensitive to chloramphenicol (Cm; <4 mg/liter), Gm (<1 mg/liter), Kn (<2.5 mg/liter), and tetracycline (<0.3 mg/liter). Wide-host-range shuttle plasmids pUFJ05 (*Bordetella* replicon, Gm^R; Reddy et al. 2007) and pUFR071 (RepW, Gm^R, and Cm^R; De Feyter and Gabriel 1991) (Table 2) were transformed by electroporation at high frequencies into BT-1. The transformation frequencies were estimated to be 3×10^3 transformants/µg of DNA for pUFJ05 and ~20× greater for pUFR071, when selected either on BM7 with Gm (2.0 mg/liter) or Cm (2.5 mg/liter). The recovery of primary transformation events was relatively slow and typically yielded colonies only after 5 to 6 weeks of incubation. In our experience, Cm selection delayed recovery of primary transformants by approximately 2 additional weeks and Gm was the preferred antibiotic for plasmids pUFJ05 and pUFR071 that carry both Gm^R and Cm^R selectable markers. The primary transformed colonies always appeared transparent and luminous in the beginning and required an additional 2 weeks for maturation. It was extremely important to streak only the mature, opaque dull white colonies on fresh plates to preserve their viability. Even for wild-type BT-1 grown on agar media, the initially transparent and luminous colonies failed to grow upon restreaking. After restreaking and growth on selective solid medium, liquid BM7 cultures were initiated with appropriate antibiotic selection from mature and opaque dull white colonies. The transformed BT-1 strains were routinely maintained in liquid BM7A medium as described above following a 5- to 7-day subculture schedule.

High transformation efficiencies were routinely achieved with several wide-host-range shuttle vectors, including pUFR047 (RepW; De Feyter et al. 1993), pBBR1MCS-5 (*Bordetella* replicon; Kovach et al. 1995), and pUFZ075 (RepW) for constitutive expression of green florescent protein (GFP) (Zhang et al. 2009). BT-1 cells transformed with pUFR071 showed only a marginal loss of the plasmid when grown for >70 generations in the absence of Gm (2.0 mg/liter) selection (Fig. 2A). The results are in line with the expected stability of RepW origin in the presence of stabilizing partition locus parA (De Feyter et al. 1990). Plasmid extracted from BT-1 cells transformed with pUFR071 was retransformed into *E. coli* TOP10 cells and appeared from restriction analysis to be unchanged (Fig. 2B). *L. crescens* BT-1 cells were also highly amenable to cotransformation as well as sequential transformation using a combination of compatible plasmids and appropriate selective antibiotics (pUFR071 and pUFJ05, Gm^R and Kn^R; Munoz-Bodnar et al. 2018).

Site-directed mutagenesis for *L. crescens* strain BT-1 via marker interruption. For site-directed mutagenesis, pCR2.1-TOPO (Kn^R) was used in these studies but any "suicide" vector (capable only of replication in *E. coli*) could likely be used. Marker interruption of the nonessential target locus *lcrRIP* (B488_RS03405) in the chromosome was achieved, following the strategy outlined in Castañeda et al. (2005). Electroporation of BT-1 cells with pMJ01, carrying a PCR fragment amplified from the internal coding region of *lcrRIP* cloned in pCR2.1-TOPO, resulted in a single homologous recombination event interrupting the target gene *lcrRIP* and integrating the vector between two incomplete copies of the target gene (Fig. 3A). In a typical transformation experiment for marker interruption at this or any one of several mutated loci (Table 2 and refer below), ~10 transformants/µg of plasmid DNA were obtained in 10 weeks on antibiotic selection (Kn, 4.5 mg/liter). Three independently obtained Kn^R colonies (*lcrRIP*::pMJ01) were analyzed by PCR to confirm interruption of the target gene and integration of the plasmid backbone (Fig. 3B).

Natural transformation, site-directed marker interruption, and marker exchange in *L. crescens* strain BT-1. The pathogenic '*Ca.* Liberibacter' genomes are predicted to possess all structural genes required for assembly of type IV pilus (T4P)

TABLE 2. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics[a]	Reference/source
Strain		
E. coli TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
L. crescens BT-1	Wild-type strain isolated from Babaco mountain papaya	Leonard et al. (2012)
L. crescens LcrRIP-1	*lcrRIP*::pMJ01 derived from BT-1, Kn^R	This study
L. crescens LcrRIP-2	*lcrRIP*::pCLL04 derived from BT-1, Kn^R, Gm^R	This study
L. crescens LcrRIP-3	*lcrRIP*::*aacCI* derived from BT-1, Kn^R	This study
L. crescens Sut-1	*sut*::pCR2.1-TOPO derived from BT-1, Kn^R	Jain et al. (2017b)
Plasmid		
pBBR1MCS-5	Rep *Bordetella*, Mob+, *lacZ*, Gm^R	Kovach et al. (1995)
pCR2.1-TOPO	3.9 kb; PCR cloning vector, Ap^R, Kn^R	Invitrogen
pKLN18	pUC ori, *lacZ*, Ap^R, Kn^R	Reddy et al. (2007)
pMJ01	769-bp internal fragment of *Lcr* restriction subunit R *lcrRIP* (B488_RS03405) in pCR2.1-TOPO, Ap^R, Kn^R	This study
pUFJ05	pBBR1MCS-5 + 978 bp *Kan-2* fragment from pKLN18, Kn^R, Gm^R	Reddy et al. (2007)
pUFR034	RepW, Mob+, Par+, *cos*, *lacZ*, Kn^R	De Feyter et al. (1990)
pUFR040	RepW, ColE1, Mob+, *lacZ*, Par+, Cm^R, Gm^R	De Feyter and Gabriel (1991)
pUFR047	RepW, Mob+, *lacZ*, Par+, Gm^R, Ap^R	De Feyter et al. (1993)
pUFR071	RepW, ColE1, Mob+, *lacZ*, Par+, derivative of pUFR040 lacking *Eco*RI site, Cm^R, Gm^R,	De Feyter and Gabriel (1991)
pUFZ075	P_{tryp}-TIR-GFP cassette in pUFR034, Kn^R	Zhang et al. (2009)
pCLL02	2-kb internal fragment of Lcr restriction subunit R *lcrRIP* (B488_RS03405) in pCR2.1-TOPO, Kn^R	This study
pCLL04	Gm acetyl transferase gene *aacC1* inserted within the *lcrRIP* fragment in pCLL02, Kn^R, Gm^R	This study

[a] Str = streptomycin, *lcrRIP* = putative restriction subunit R (B488_RS03405), Kn = kanamycin, Gm = gentamycin, *sut* = sugar transporter (B488_RS00965), Ap = ampicillin, *Lcr* = *Liberibacter crescens*, Kan = kanamycin, Cm = chloramphenicol, GFP = green fluorescent protein, and *aacC1* = aminoglycoside-3-*O*-acetyltransferase I gene (gentamycin acetyltransferase).

(Andrade and Wang 2019). In addition to predicted T4P genes, the *L. crescens* genome also encodes the *Vibrio cholerae* homologs (Seitz and Blokesch 2014) needed for natural DNA uptake and transformation: *comF* (B488_RS05095), *comEA* (B488_RS00175), and *comEC* (B488_RS05330) for DNA uptake; *ssbB* (B488_RS01885) and *dprA* (B488_RS04595) for DNA protection; and *recA* (B488_RS02195) for recombination. To determine whether *L. crescens* could naturally take up exogenously supplied DNA, the 9.4-kb shuttle vector pUFR071 (used for electroporation) and chitin or xanthan gum were mixed with thawed electroporation competent *L. crescens* cells, but this time without the use of electroporation. The transformation mix was incubated for 48 h with 850 µl of liquid BM7A medium, resulting in ~900 transformation events. By comparison, 6 × 10^4 transformed *L. crescens* cells were obtained via electroporation of the same plasmid with similarly prepared competent cells. Natural transformation was dependent on the addition of chitin or xanthan gum to the *L. crescens* cells/DNA transformation mix. Cells not washed repeatedly with water and 10% glycerol (as described in the Materials and Methods) did not appear competent for natural transformation.

To determine whether *L. crescens* could integrate naturally acquired exogenously supplied DNA into its genome, marker interruption of the nonessential target locus *lcrRIP* was performed. Both linear and circular plasmid vector DNA carrying an internal fragment(s) of *lcrRIP* plus the Gm acetyltransferase (*aacC1*) were used for natural uptake and homology-guided integration by *L. crescens* cells. The schematic design of pCLL04 used for demonstration of homology-directed integration and marker interruption of *lcrRIP* is illustrated in Figure 4A. Nonreplicative plasmid pCLL04 reliably yielded ~10 to 12 transformation events. Similar transformation efficiencies were routinely obtained with electroporated circular suicide plasmids for insertional mutagenesis of target loci (Jain et al. 2017b; Naranjo et al. 2019). A single homologous recombination event within the target locus *lcrRIP* resulted in integration of the vector backbone carrying the Kn resistance gene in between two incomplete copies of the target while also knocking in the Gm resistance gene in the recipient strain *lcrRIP*::pCLL04 (Fig. 4C). *L. crescens* transformants (*lcrRIP*::pCLL04) were also verified for their ability to grow on either of the two antibiotics (Kn and Gm).

Natural transformation was also achieved by providing homologous linear DNA fragments via two homologous recombination events (marker exchange). For example, *Bam*HI/*Sma*I digestion of pCLL04 released a 2.834-kb insert with the Gm resistance gene (*aacCI*) flanked by 1-kb regions homologous with *lcrRIP* on each side (Fig. 4B). This fragment was gel purified and incubated with

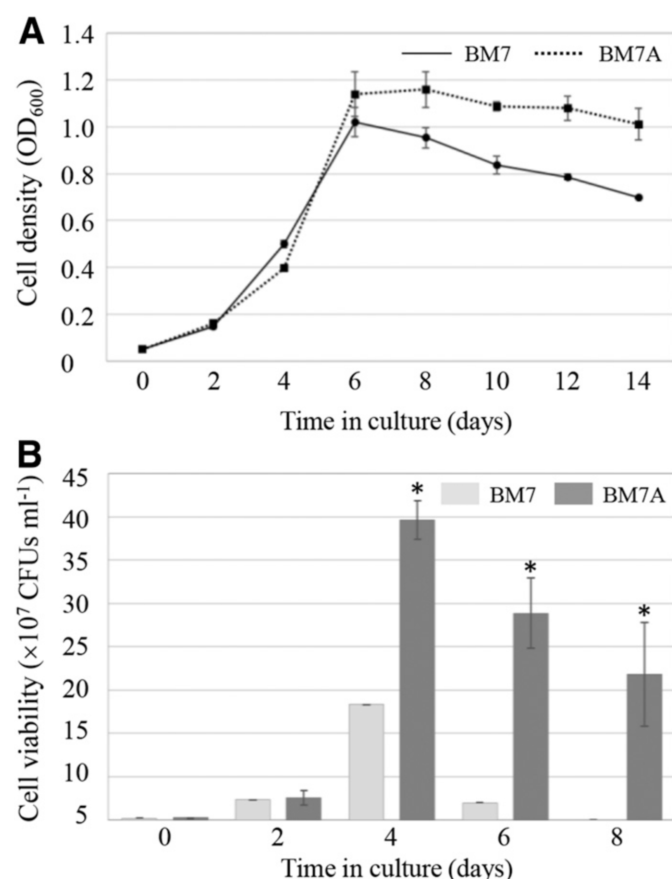

Fig. 1. Effect of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer concentration on viability of *Liberibacter crescens* strain BT-1 cells in culture. Growth of *L. crescens* cells in Basal Medium 7 (BM7) or BM7A (containing 10 or 20 g/liter of ACES, respectively) was monitored for **A,** an increase in absorbance (OD_{600}) and **B,** cell viability (in CFU/ml). For cell viability assays, 100 µl of *L. crescens* cells was withdrawn at the indicated time points, serially diluted, and enumerated for viable cell counts. The data represent means ± SD of three experiments with three replicates each. Asterisks indicate significant differences between the treatment means as determined by the Student's *t* test ($P < 0.05$).

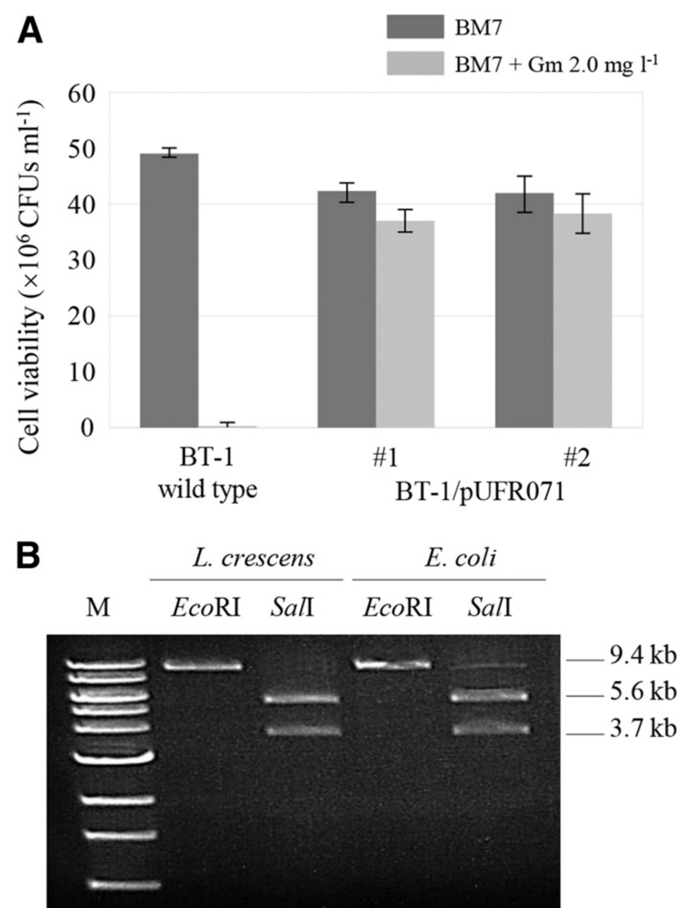

Fig. 2. A, Growth of *Liberibacter crescens* strain BT-1 wild-type and pUFR071-transformed cells on Basal Medium 7 (BM7) with and without antibiotic selection. Cell density (in CFU/ml) was determined on plain and selective (gentamycin [Gm], 2.0 mg/ml) BM7. Two independently transformed colonies, numbers 1 and 2, were grown for 70 generations in BM7 liquid medium without antibiotic selection, serially diluted, and plated on plain and selective BM7 plates ($n = 3$). **B,** Diagnostic agarose gel (0.8%) electrophoresis of *Eco*RI/*Sal*I-digested plasmid DNA extracted from pUFR071-transformed *L. crescens* BT-1 and *Escherichia coli* Top10 cells. pUFR071 was extracted from transformed BT-1 cells and retransformed in *E. coli*. A single 9.4-kb *Eco*RI fragment and two 5.6- and 3.7-kb *Sal*I fragments were visualized by ethidium bromide staining (M, 1-kb DNA ladder).

electrocompetent *L. crescens* cells (plus either chitin or xanthan gum) to yield three to four transformation events per microgram of DNA. Integration of *aacCI* within the target locus (*lcrRIP*::*aacCI*) was mediated through two recombination events and was confirmed as a marker exchange product by PCR analysis (Fig. 4C). The efficiency of marker exchange appeared dependent on the length of the flanking homologous regions. Homologous flanking regions of 250, 500, 750, and 1,000 bp each were evaluated, and the flanking region length of 750 bp on each side was optimal, yielding ~34 transformation events for marker exchange of *lcrRIP*.

DISCUSSION

Extensive genome reductive evolution has rendered the plant-pathogenic and insect-endosymbiotic '*Ca*. Liberibacter' spp. strictly dependent on intracellular lifestyles (Hartung et al. 2011; Jain et al. 2017b). Lack of axenic culture methods has severely restricted functional genomic analyses and subsequent development of chemical control methods for pathogenic '*Ca*. Liberibacter' spp. Despite high genomic similarity and syntenic context (Nakabachi et al. 2013), the core Liberibacter genomes share only 658 genes and most of the species-specific genes encode proteins of unknown function (Fagen et al. 2014; Wulff et al. 2014). Bioinformatic analysis also revealed that 37% of the functionally annotated genes in the *L. crescens* genome are species specific compared with 17% in '*Ca.* L. asiaticus' and 20% in '*Ca.* L. solanacearum' (Wulff et al. 2014). As described below, molecular characterization of some of the functionally annotated '*Ca.* L. asiaticus' genes using *L. crescens* as a surrogate host has already provided valuable insights into both the bacterial requirements for free-living growth in axenic culture and host–pathogen interactions.

L. crescens was first used for functional genomics analyses of '*Ca.* L. asiaticus' genes to investigate the repression of a strong constitutive '*Ca.* L. asiaticus' phage holin (F488_gp16) promoter (Fleites et al. 2014). A small protein encoded by the genus *Wolbachia* (a primary endosymbiont of *D. citri*) was found to permeate *L. crescens* cells, bind to the promoter, and repress holin expression, thereby providing the first example of protein-mediated interspecies bacterial communication not related to quorum sensing (Jain et al. 2017a). Two '*Ca.* L. asiaticus' plant effectors, one a prophage-encoded peroxidase and the other a chromosomally encoded peroxiredoxin, were also functionally characterized using *L. crescens* as a surrogate host to confirm extracellular secretion and enzymatic activity; transient expression assays were then used to confirm suppression of both reactive oxygen species-mediated systemic and localized defense signaling cascades in planta (Jain et al. 2015, 2018, 2019b).

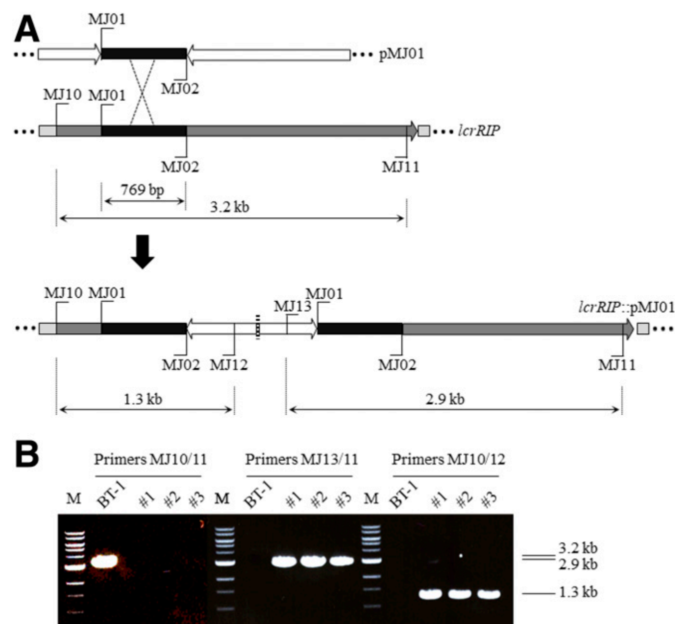

Fig. 3. A, Schematic representation of a single homologous recombination event leading to duplication of the cloned homology region (filled black box) and integration of suicide vector pMJ01 (empty white box) in between two incomplete copies of the target locus (*lcrRIP*) in *Liberibacter crescens* BT-1 cells (*lcrRIP*::pMJ01). **B,** Agarose gel (0.8%) electrophoresis showing the indicated PCR products obtained using primer pairs MJ10/11 for amplification of the target regions in wild-type *L. crescens* BT-1, and MJ13/11 and MJ10/12 for amplification of the vector-interrupted target regions in three independently transformed *lcrRIP*::pMJ01 strains. Directional positions of different primers and sizes of the PCR-amplified products are indicated in A. Lanes: M = 1-kb DNA ladder, and #1, #2, and #3 = three independently transformed *lcrRIP*::pMJ01 clones.

Systemic, circulative, and propagative colonization of the psyllid hosts by '*Ca.* L. asiaticus' and '*Ca.* L. solanacearum' depends on attachment to the midgut cells, traversing through it and biofilm formation on the outer midgut surface and likely involving active motility within the hemolymph. All '*Ca.* L. asiaticus' T4P genes examined were upregulated in the psyllid host by comparison with the citrus host (Andrade and Wang 2019). Despite numerous observations of '*Ca.* L. asiaticus' in citrus and psyllids, neither flagella nor pili has been observed in electron micrographs of '*Ca.* L. asiaticus'; however, surface appendages were observed in micrographs of '*Ca.* L. solanacearum' within infected potato psyllids (Cicero et al. 2016). By contrast, both flagella and pili were observed in micrographs of *L. crescens*, swimming and twitching motility were readily confirmed in culture, and knockout mutations of *L. crescens* flagellar genes *flgK* (B488_RS04530) and *flgF* (B488_RS00930) resulted in demonstrably reduced bacterial swimming motility (Cai et al. 2019).

Visibly marked bacterial strains can be useful for following movement and localization in host tissues. GFP-marked *L. crescens* has been used to demonstrate biofilm formation in microfluidic chambers and investigate environmental conditions that may be relevant to '*Ca.* L. asiaticus' biofilm regulation (Naranjo et al. 2019). Colonization of Malpighian tubules of the greater wax moth *G. mellonella* by GFP-marked *L. crescens* was demonstrated (Munoz-Bodnar et al. 2019), thereby providing a potential surrogate insect model host for *L. crescens* infection. This experimental model used in combination with microfluidic chambers may be useful for screening mutants and transformants affecting motility, secretion, and biofilm formation. The *Galleria* infection model may even prove useful in evaluating suspected '*Ca.* Liberibacter' effectors cloned, expressed, and secreted from *L. crescens* that are suspected of involvement in psyllid endosymbiosis and pathogen transmission.

L. crescens has also been used to characterize a few key genetic differences that likely determine both lack of culturability and pathogenicity of '*Ca.* Liberibacter' spp. Knockout mutations of the *L. crescens* sugar transporter gene *sut* (B488_RS00965) combined with expression analysis of the '*Ca.* L. asiaticus' ATP/ADP translocase gene *nttA* (CLIBASIA_RS01005) in *L. crescens* were used by Jain et al. (2017b) to confirm the proposed hypothesis (Hartung et al. 2011) that '*Ca.* L. asiaticus' is an intracellular energy scavenger in both plant and psyllid hosts (as '*Ca.* L. asiaticus' lacks a *sut* ortholog and *L. crescens* lacks a *nttA* translocase). As opposed to the cultured *L. crescens*, in addition to a truncated glycolytic pathway, all of the pathogenic '*Ca.* Liberibacter' spp. have also lost glyoxalase I (lactoylglutathione lyase; EC 4.4.1.5), the key enzyme required for detoxification of methylglyoxal, a cytotoxic byproduct of glycolysis (Jain et al. 2017b). Finally, characterization of *L. crescens* lipid A structure has proven quite useful in comparing and predicting differences from lipid A of pathogenic '*Ca.* Liberibacters' spp. As previously mentioned, all pathogenic and uncultured '*Ca.* Liberibacter' spp. lack LpxL, whereas the predicted *L. crescens lpxL* (B488_RS04675) encoded enzyme is homologous to the *S. meliloti* LpxXL catalyzing the very long chain fatty acid

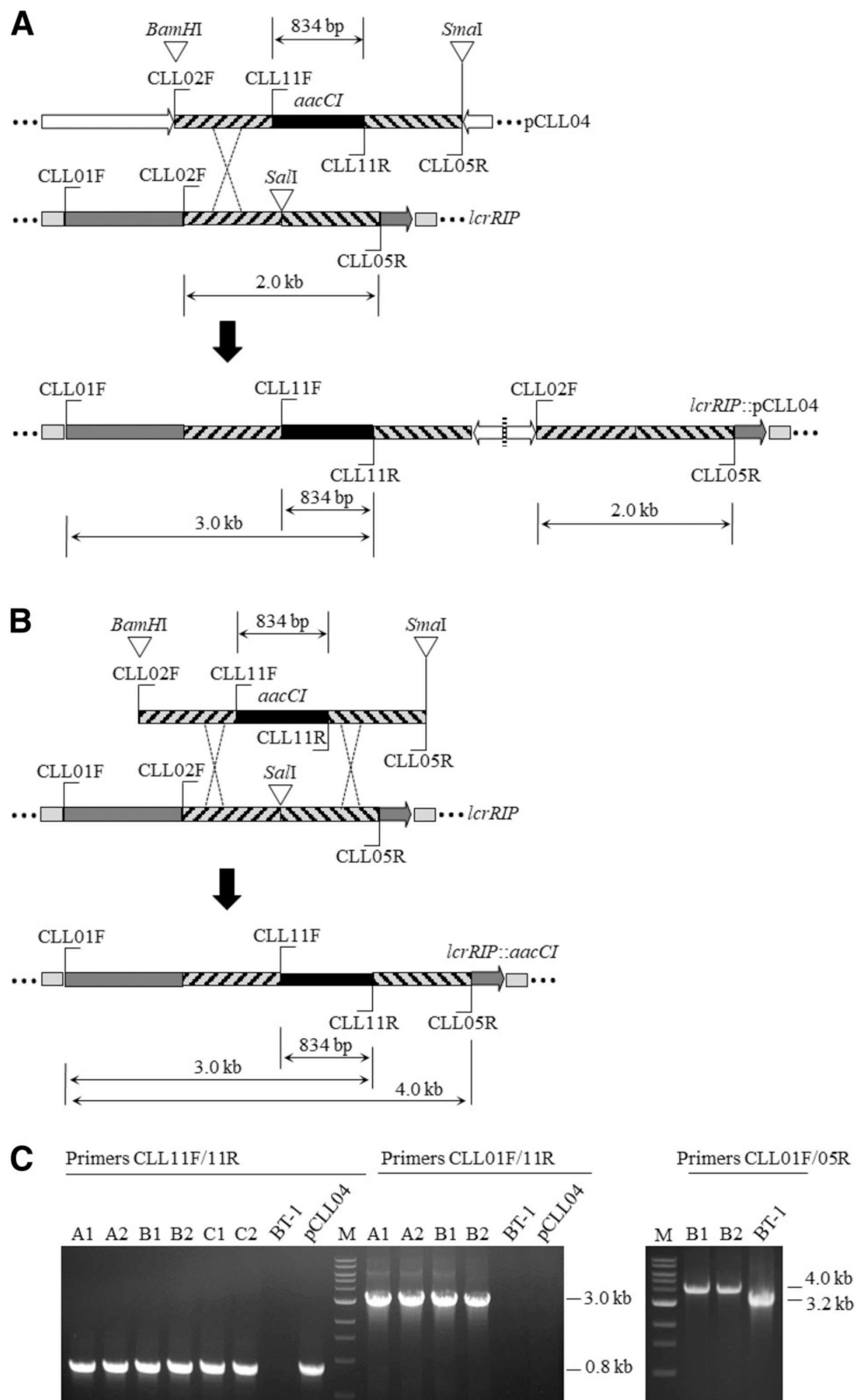

Fig. 4. A, Schematic design for construction of plasmid pCLL04 for marker interruption and marker exchange of the target locus *lcrRIP* in *Liberibacter crescens* BT-1 cells using a gentamycin acetyltransferase (*aacC1*) gene. A single homologous recombination event results in marker interruption, characterized by the duplication of the cloned homology region (hashed gray boxes) and integration of suicide vector pCLL04 (empty white box) in between two incomplete copies of the target locus in *L. crescens* BT-1 cells (*lcrRIP*::pCLL04). **B,** Double homologous recombination resulting in marker exchange, characterized by the insertion of the *Bam*HI/*Sma*I released linear 2.834-kb fragment carrying *aacC1* (filled black box) into the target locus in *L. crescens* BT-1 cells (*lcrRIP*::*aacCI*). **C,** Agarose gel (0.8%) electrophoresis showing indicated PCR products obtained using primer pairs CLL11F/11R, CLL01F/11R, and CLL01F/05R for amplification of *aacCI* and the marker-interrupted target locus, respectively. Directional positions of different primers and sizes of the PCR-amplified products are indicated in A and B. Lanes: M = 1-kb ladder, A1 and A2 = *L. crescens* BT-1 cells transformed with pCLL04 via marker interruption (*lcrRIP*::pCLL04), B1 and B2 = *L. crescens* BT-1 cells transformed via marker exchange (*lcrRIP*::*aacCI*), and C1 and C2 = *L. crescens* BT-1 cells transformed with pUFR071 (BT-1/pUFR071).

(VLCFA) modification of lipid A. VLCFA-modified lipid A is a unique feature of several bacteria such as *S. meliloti* and *Brucella abortus* that form chronic intracellular infections within legumes and mammalian hosts (Ferguson et al. 2004). *L. crescens* lipid A has a penta-acylated chitobiose backbone, containing two ester-linked C16:0 (3-OH) and two amide-linked C14:0 (3-OH) fatty acid residues and a C28:0 (26-OH) VLCFA (Jain et al. 2019a). Although knockout mutations of various *L. crescens* genes are readily obtained (Cai et al. 2019; Jain et al. 2017b; Naranjo et al. 2019), mutations in the *L. crescens lpxL* gene appeared to be lethal, raising the speculation that uncultured '*Ca.* Liberibacter' spp. may require VLCFA-modified lipid A for free-living growth in culture.

L. crescens was naturally competent for direct uptake and chromosomal integration of both linear and circular plasmid DNA. Natural DNA transformation is a horizontal gene transfer mechanism for bacterial uptake of DNA through the base of T4P, followed by its stable integration in the recipient genome (Johnsborg et al. 2007; Mell and Redfield 2014). Natural transformation drives bacterial genome plasticity and adaptability and has been demonstrated in several bacterial species such as *Streptococcus pneumoniae*, *Helicobacter pylori*, *X. fastidiosa*, *V. cholerae*, *E. coli*, *Agrobacterium tumefaciens*, and *Pseudomonas fluorescens*. The '*Ca.* Liberibacter' genomes are predicted to possess all of the structural genes required for assembly of T4P (Andrade and Wang 2019) and ultrastructural evidence for T4P has been documented in *L. crescens* (Cai et al. 2019) and '*Ca.* L. solanacearum' (Cicero et al. 2016). T4P-mediated natural uptake of DNA by *L. crescens* appeared dependent on the presence of xanthan gum or chitin with the DNA. Chitin has been shown to be essential for natural transformation of *V. cholerae* (Udden et al. 2008).

Unlike *Neisseria* spp. that synthesize their competence proteins in a constitutive manner, it is likely that most naturally transformable bacteria regulate competence gene expression in response to certain cellular and/or environmental signals (Johnsborg et al. 2007). Natural competence for environmental DNA uptake likely fulfills a requirement of some bacteria for nutrients (i.e., the DNA is used as food, both nucleotides and phosphate), but for other bacteria, the competence involves both uptake for food and transformation for the acquisition of novel genetic material for repair or adaptation. The confirmation of natural competence in *L. crescens* for transformation is also significant for the context of food and is consistent with previously reported lack of enzyme systems for purine and pyrimidine metabolism in the genomes of '*Ca.* Liberibacter' spp. (Hartung et al. 2011). Uptake of environmental DNA likely fulfills the nucleotide requirements, highlighting the need for adding nucleotides to overcome nutritional bottlenecks for successful culturing of '*Ca.* Liberibacter' spp. in axenic media. '*Ca.* L. asiaticus' appears to lack full-length versions of ComF, DprA, and ComEC, which are needed for the import and protection of intact DNA for transformation but are not essential for the import of DNA for food (Hovland et al. 2017; Seitz and Blokesch 2014).

'*Ca.* L. asiaticus' has been transiently cocultured to maximum titers of ~20,000 cells/ml (Merfa and De La Fuente 2019). Such low-titer cultures are inadequate for typical transformation and electroporation methods and '*Ca.* L. asiaticus' may not be amenable to natural transformation owing to a lack of critical genes. Extracts of '*Ca.* L. asiaticus'-free psyllids were used to obtain plaque-like lytic zones on agar plated lawns of *L. crescens* expressing the '*Ca.* L. asiaticus' Outer Membrane Protein (*ompA*) gene (CLIBASIA_RS00965; Sena-Velez et al. 2018), thus providing a possibility of phage transduction of '*Ca.* L. asiaticus' at low titer levels. Isolating bacteriophages able to infect '*Ca.* L. asiaticus' may provide novel molecular tools to genetically modify even transient and low-titer '*Ca.* L. asiaticus' to allow axenic cultures, as well as open avenues for reintroduction of '*Ca.* L. asiaticus' to plants and psyllids to explore more effective HLB control.

In the absence of culturable pathogenic Liberibacters (for a recent comprehensive review of culturing attempts, refer to Merfa et al. 2019), *L. crescens* has been developed into a genetically tractable proxy for the '*Ca.* Liberibacter' spp. *L. crescens* has been transformed by replicative and integrative vectors carrying multiple '*Ca.* L. asiaticus' genes, and it has served as a gene expression and secretion host to characterize potential secreted effectors and even to approximate the lipopolysaccharide of the pathogenic '*Ca.* Liberibacter' spp. The ability to genetically transform *L. crescens* and infect a model insect host may make it easier to turn *L. crescens* into a cultured pathogen than to culture a pathogenic '*Ca.* Liberibacter' spp.

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