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Article

Towards the completion of Koch's postulates for the citrus huanglongbing bacterium, *Candidatus* Liberibacter asiaticus

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Abstract

Candidatus Liberibacter asiaticus (Las) is one of the causal agents of huanglongbing (HLB), the most devastating disease of citrus worldwide. Due to the intracellular lifestyle and significant genome reduction, culturing Las *in vitro* has proven to be extremely challenging. In this study, we optimized growth conditions and developed a semi-selective medium based on the results of nutritional and antibiotic screening assays. Using these optimized conditions, we were able to grow Las in the LG liquid medium with ca.100- to 1000-fold increase, which peaked after 4 to 6 weeks and were estimated to contain 10^6 to 10^7 cells/ml. The cultured Las bacteria remained in a dynamic state of growth for over 20 months and displayed limited growth in subcultures. The survival and growth of Las was confirmed by fluorescence *in situ* hybridization with Las-specific probes and expression of its metabolic genes. Growth of Las in the optimized medium relied on the presence of a helper bacterium, *Stenotrophomonas maltophilia* FLMAT-1 that is multi-drug resistant and dominant in the Las co-culture system. To recapitulate the disease, the co-cultured Las was inoculated back to citrus seedlings via psyllid feeding. Although the Las-positive rate of the fed psyllids and inoculated plants were relatively low, this is the first demonstration of partial fulfillment of Koch's postulates with significant growth of Las *in vitro* and a successful inoculation of cultured Las back to psyllids and citrus plants that resulted in HLB symptoms. These results provide new insights into Las growth *in vitro* and a system for improvement towards axenic culture and anti-Las compound screening.

Introduction

Huanglongbing (HLB), also known as citrus greening, is the most devastating disease of citrus worldwide. HLB is associated with three species of *Candidatus* Liberibacter [1, 2], a genus containing several prominent plant pathogens in the family Rhizobiaceae. Of the three bacterial species, *Candidatus* Liberibacter asiaticus (Las) is the most widespread globally and is transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri*. Despite HLB being a severe economic issue and even after decades of extensive research, no effective control methods are available for the disease. While extensive work has been done to develop an understanding of Las itself and its interactions with both plant and insect hosts, the full characterization of the pathogen has been hindered by its uncultivability. The first full-genome sequence of Las was published over a decade ago [3], and since then, sequences of additional strains from various areas of the world have followed [4–6]. Comparisons using these sequences have provided some insights into the fastidious nature of the bacteria. The model cultivable relative, *Liberibacter crescens*, has a complete genome of 1 504 659 bp [7], while the genome of Las is significantly reduced (ca. 1.2 Mbp). The smaller size of the Las genome indicates that it may be more reliant upon its hosts for survival and growth since it lacks stringent response-regulator pathways in addition to genes involved in amino acid synthesis, vitamin synthesis, and cell division [8, 9]. For growth *in vitro*, other microbes might be capable of supplying required nutrients, energy, and chemical signals [10, 11], but the described methodologies of previous studies have proven to be difficult to replicate. It remains important to pursue a simple, effective, and repeatable culture method for Las that delivers a stable, reproducible culture that can be used for fulfilling Koch's postulates, understanding host-pathogen relationships, *in vitro* screening for antimicrobial compounds, the development of more targeted and effective control strategies, and certain functional genomic analyses that typically rely on such cultures [12].

Previous work on culturing Las can be divided by strategy [13, 14], such as attempts to replicate the host environmental parameters in culture [15] or development of co-culture systems

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to promote the growth of Las [10, 11, 16]. Through attempts with simplified citrus-juice based media [15], unstandardized media preparations from plant extracts [17], and establishment of biofilms [10], etc. the scientific community has developed a trial-and-error based view of what successful culture might require, as well as demonstrating that some level of *in vitro* survival of Las is possible. In addition to specific nutritional requirements, as well as low oxygen-tension and neutral to alkaline pH, this body of work suggests Las could possibly benefit from the addition of certain antibiotics [11, 18].

Metabolomic and genetic approaches have also identified several parameters for consideration in culturing Las [8, 19, 20]. Genome analysis has been used to compare the reduced Las genome with other *Liberibacter* genomes, often suggesting targeted opportunities to design media and conditions addressing specific growth requirements. When 'omics' approaches are used to compare the metabolomes of plant and vector hosts, the results reveal the commonalities and differences between the hosts that could be utilized for the development of cultural practices. Killiny et al. [20] for example, noted overlap between several constituents including sugars, amino and organic acids, vitamins, and ions in the plant and vector hosts. Additionally, comparative genomics has suggested that the prophages present in Las may be inhibiting its cultivability [19]. Future work must answer the need for a culture or co-culture that is reproducible and contains a titer high enough to be experimentally useful.

Here we report a simple, somewhat effective method for co-culturing Las *in vitro* with one specific helper bacterium and an analysis of the resulting community structure. This culture method establishes the utility of a new semi-selective medium, Las-growth (LG), and contributes to our understanding of Las by elucidating a potential mutualistic relationship between Las and the helper bacterium, *Stenotrophomonas maltophilia* FLMAT-1, as well as providing the first report of using a culture to inoculate hosts in partial fulfillment of Koch's postulates.

Results

Phenotype microarray defines medium additives.

Biolog phenotype microarray (PM) microplates were used to screen for media additives that could benefit Las growth. One advantage of using biolog PM microplates to define putative metabolites is that the output is based upon the reduction of a tetrazolium indicator, and therefore cell division is not a requirement [21]. This system has been used to define conditions that supported the growth of *Coxiella burnetii* in a cell-free system [22] and to identify metabolites that can be utilized by Las, regardless of the ability of media to fully support bacterial cell division and growth. To help determine which of the individual components present within the PM microplates could enhance the growth of *Liberibacter,* we tested inoculum consisting of Las derived from Las-infected periwinkle and inoculum consisting of *Liberibacter crescens* (the only cultured species of the *Liberibacter* genus [9]) for their capacity to utilize the substrates. Analysis revealed 13 different carbon sources and 1 nitrogen source that could be utilized by both *L. crescens* and Las (Table 1). Additionally, it revealed two carbon sources and 1 nitrogen source unique to Las and 3 carbon sources and 1 nitrogen source unique to *L. crescens*. Using the Biolog PM10 microplate, a clear preference for an alkaline pH range for both Las and *L. crescens* growth was demonstrated. This result was consistent with two recent studies [10, 18], but in contrast to a previous report using an acidic media for Las growth [15].

Las-growth liquid medium enables Las growth *in vitro* with preferred source of inoculum.

Based on the results of the phenotype microarrays and referenced to the medium used for growing *L. crescens,* we developed the Las-growth (LG) medium that supported the growth of Las. LG medium consisted of 30% nutrient broth (NB), 1.0% glucuronamide, 0.2% α-ketoglutaric acid, and a phosphate buffer system. NB was prepared from beef extract and expected to provide Las with most of the required nutrients such as amino acids, vitamins, and essential mineral elements. Glucuronamide was chosen as the carbon source for energy metabolism because it was not only identified via the phenotype microarray but also based on the carbon metabolic pathway of Las. Additionally, α-ketoglutaric acid, a known intermediate of the tricarboxylic (TCA) cycle considered to be the regulator of carbon and nitrogen utilization in microbial metabolism [23], was added to the medium.

Growth of Las in LG medium depended on the inoculum source. For the inoculum prepared from psyllids, Las grew well in most cases, but the growth kinetics were not consistent among individual cultures. When analyzed by comparing all cultures from different inoculum sources, trends in the Las titer changes became clear. Compared to the level at Day 0, Las titer increased with time of cultivation and reached a peak within 4 to 6 weeks (Figure 1). After that, the Las titer was maintained at the same or slightly reduced levels for several weeks or months. These Las titer plateaus typically contained 10^5 to 10^7 cells per ml, regardless of the cell population at Day 0. The highest cell number increase was 1025-fold in an individual culture (Figure 1B). We considered those cultures with increased Las titer (cell/ml) of 5-fold or more than the level at Day 0 to be growing well, while those that failed to increase by at least 5-fold were considered to be maintaining, and those that failed to grow or decreased in titer were considered to be dying. According to these criteria, around 60% of cultures with Las inoculation from psyllids were growing well, 35% were maintaining, and 6% were dying (Figure 1). When Las cultures were isolated from psyllids, 94% (of 16 cultures) either grew well or were maintained and only in one culture from psyllids did Las cells decrease (Figure 1B). When using inoculum prepared from plant sources (periwinkle or citrus), Las did not perform, as well as when inoculum from psyllids was used. In most cases, the Las population in those cultures showed a continuous reduction, regardless of whether the Las was extracted from periwinkle or citrus.

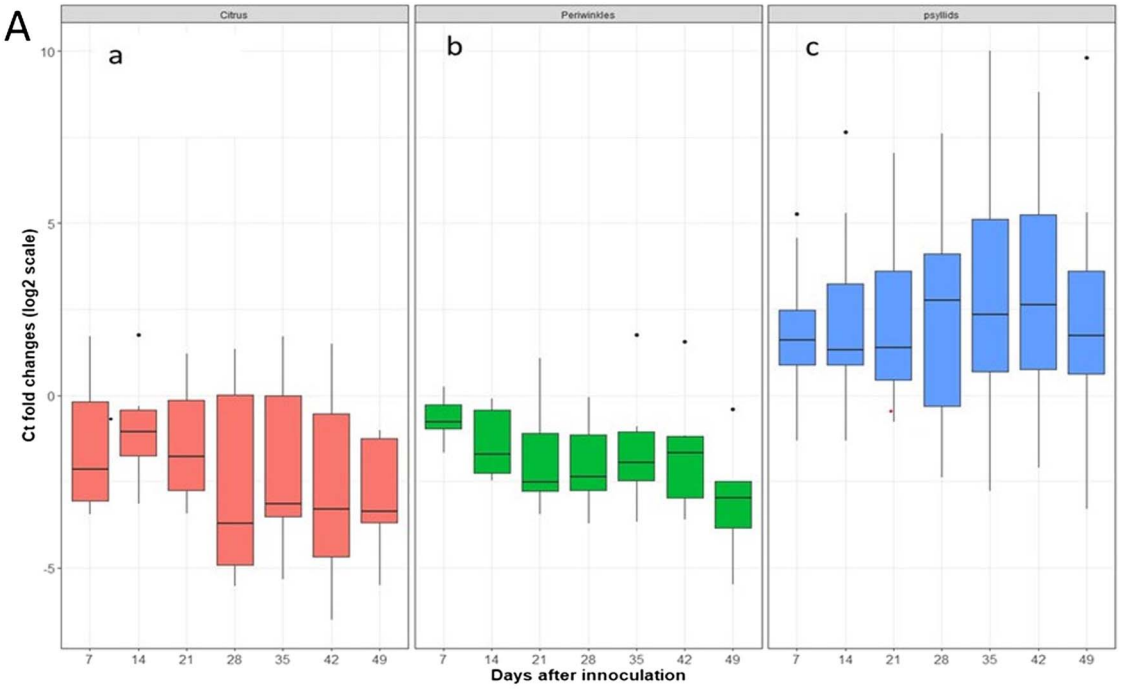
Las cultures in liquid medium were scaled up from 10 ml in Falcon tubes to 50 ml, 75 ml, or 100 ml in glass Erlenmeyer flasks with similar, if not better, results obtained (Figure S1). To date, we have observed only one case where the Las population reached 10^7 cells per ml, which came from two 75-ml cultures on Day 147 (Figure S1B). There were several cases where Las in the medium not only survived but was also maintained at a level between 10^5–10^6 cells/ml for long periods (one for more than one year, and one for two years) (Figure 2) in continuous culture without changing medium or subculturing. Subcultures of Las in liquid medium with 10-fold dilutions were also carried out. In the initial subculture, Las typically grew well, but by the third (in some cases the second) subculture, Las did not show growth and instead maintained its population or declined slightly compared to its Day 0 level (Figure 3).

To confirm the Las growth in LG medium, the morphological and physiological status of Las grown in liquid medium was examined. Individual Las-like elongated cells were observed by scanning electron microscopy both in the Las-infected periwinkle phloem cell (Figure 4A) and Las co-culture (Figure 4B). Two samples from separate cultures were assayed using fluorescence

Table 1. Metabolites Defined by the Biolog Phenotype Microarray

	Unique to Las	Unique to Lc	Both Las and Lc*
Carbon sources	alpha-D-lactose, D-psicose,	3-methyl-glocuse, L-sorbose, D-glocusamine	L-arabinose, D-xylose, D-ribose, L- rhamnose, L-lyxose, glucuronamide, beta-D-allose, D-arabinose, 2-deoxy-D-ribose, palatinose, D-tagatose, 5-keto-D-gluconic acid, dihydroxy acetone
Nitrogen sources	L-cysteine	D-mannosamine	D,L-alpha-amino-caprylic acid

*Las, *Candidatus* Liberibacter asiaticus; Lc, *Liberibacter crescens*



B

Titer fold change	Citrus (6 samples)	Periwinkles (7)	Psyllid (17)
<0	66.7% (4)	57.1% (4)	5.9% (1)
1-5	33.3% (2)	42.9% (3)	29.4% (5)
5-10	0	0	17.6% (3)
10-100	0	0	29.4% (5)
100-1,000	0	0	11.8% (2)
>1,000	0	0	5.9% (1)

Figure 1. Las growth dynamics in LG liquid medium. The Las inoculum derived from infected citrus (a), periwinkle (b), and psyllids (c) with time $0 = 2.08 \times 10^3 \pm 2.0 \times 10^2$, $1.67 \times 10^5 \pm 1.4 \times 10^4$, and $1.05 \times 10^5 \pm 1.2 \times 10^4$, respectively. A) Distribution of log2 fold changes. B) Tiered titer fold changes and the percentage of samples within each tier. The number of individual samples are listed in parentheses. Cultures with Las titers (cell/ml) 5-fold or more above the level measured at Day 0 were well growing, those with an increase between 0-fold and 5-fold were slow growing or maintaining, and those that decrease were not growing or dying.

in situ hybridization (FISH). Live Las cells were visualized via hybridization with a fluorescently labelled Las-specific 16S rRNA probe (LSS). This method confirmed that the Las cells present in the cultures after 2 months of cultivation were viable and that live Las cells within a mixed microbial community can be directly detected by FISH (Figure 4C) despite the low proportion of Las cells.

Las growth *in vitro* requires a helper bacterium

The growth of Las *in vitro* was associated with the inoculum source, which could differ in their Las population level as well as the composition of the associated microbial community. To define the associated microbial community and its relationship with Las growth, metagenomic analysis using 16S rRNA gene sequences was performed on five Las cultures inoculated with inoculum from different sources and cultivated for varying periods. The distribution and relative abundance of bacterial genera from the five Las-associated microbial communities were obtained (Figure 5A). In addition to Las, one or more additional genera were identified at a very high or relatively high abundance in every culture tested (Figure 5A). The growth of Las *in vitro* appeared to be positively correlated with the

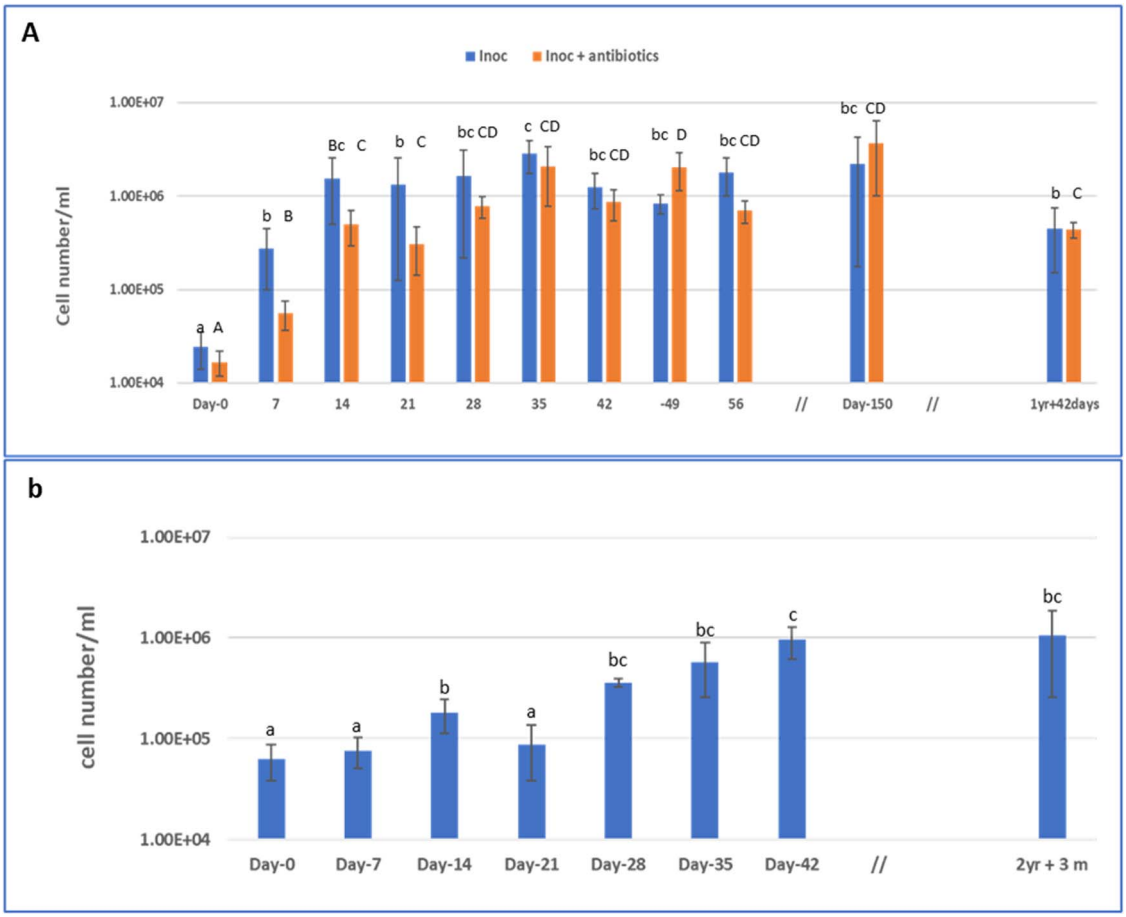


Figure 2. Growth dynamics of Las maintained in LG medium for extended periods of time. A) Las growth for more than one year with inoculum alone, and inoculum plus antibiotics (gentamycin and streptomycin). B) Growth of Las in LG medium for more than two years with inoculum only. The error bars represent the standard error of the mean of three biological replicates. The Las inoculums were isolated from psyllids. Las cell number was calculated using Ct values obtained from qPCR. The statistical analysis using ANOVA revealed significant differences between groups marked with the different lowercase or uppercase letters ($p < 0.05$).

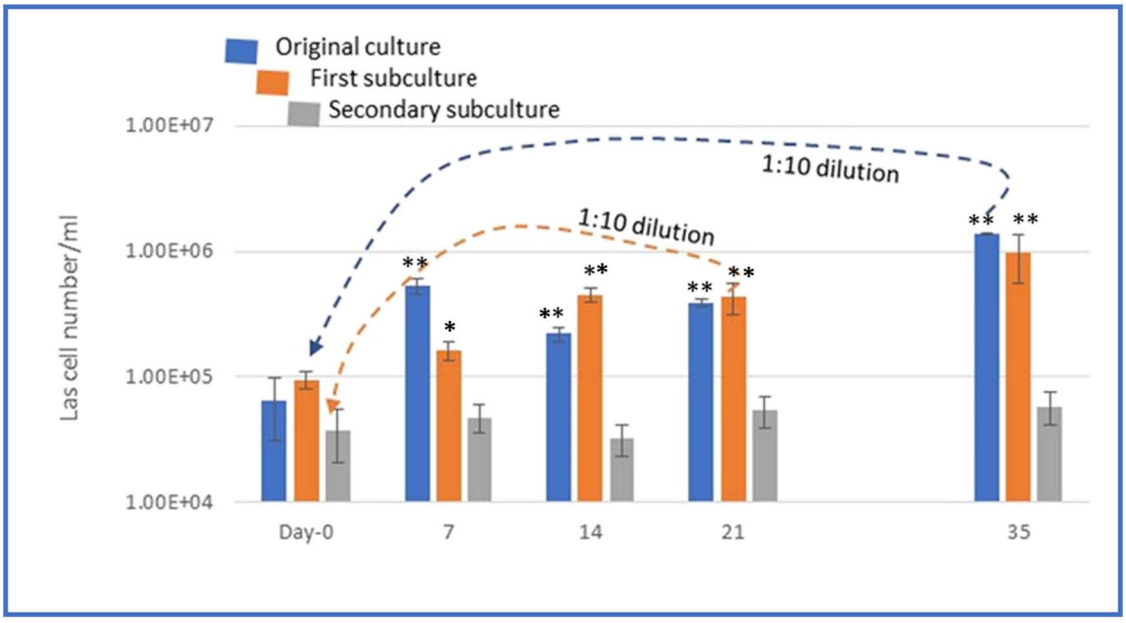


Figure 3. Las co-culture and subcultures in LG medium. In the initial subculture, Las typically grew well, but by the third (in some cases the second) subculture, Las did not show growth and instead maintained its population or declined slightly compared to its Day 0 level. Values shown are the mean of three biological replications with the standard deviation being represented by vertical bars. Single and double asterisks represent statistically significant differences at $p \leq 0.005$ **and** $p \leq 0.001$using Student's t-test compared to Day 0.

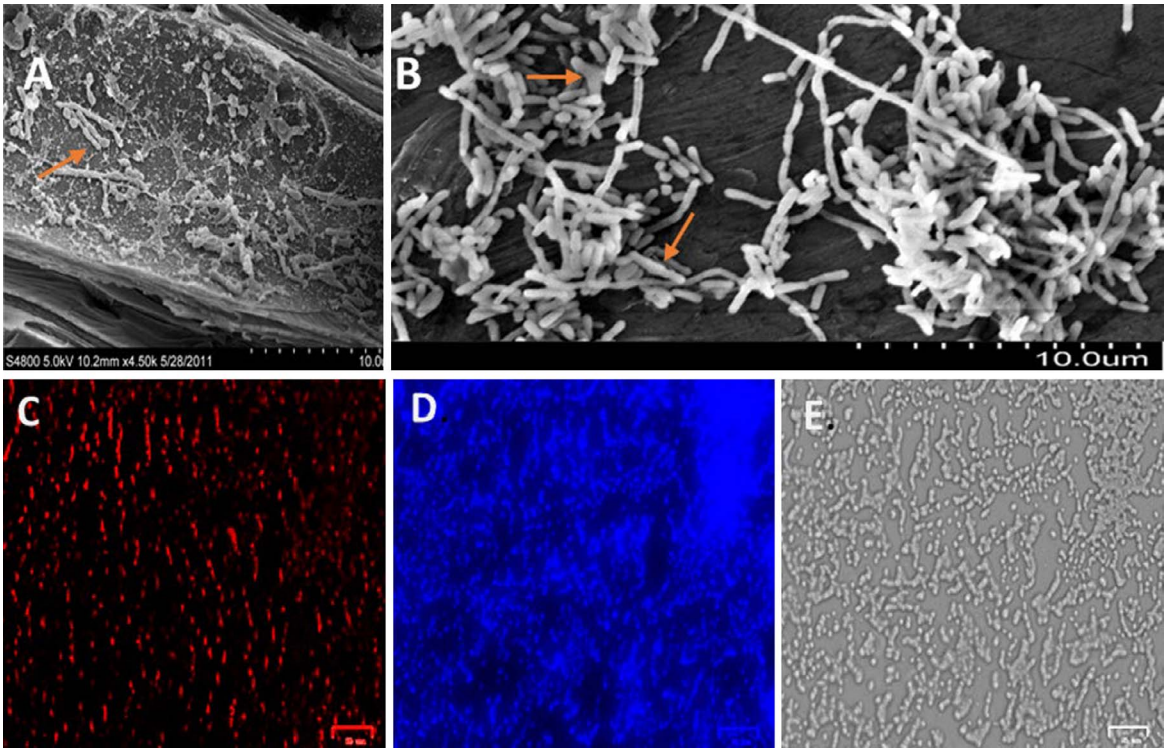


Figure 4. Micrographs of Las cells. A) Scanning electron micrograph of a Las-infected periwinkle phloem cell. B) Scanning electron micrograph of Las co-culture. C) Micrograph of Las labeled by FISH using Las-specific probe. D) Micrograph of Las co-culture stained with DAPI. E) Bright field image. Arrow points to Las-like cells.

abundance of some of these genera, such as *Stenotrophomonas*. For example, cultures 4A1 and 4B1, which utilized the same infected psyllid inoculum, both had a very high abundance (99%) of *Stenotrophomonas* after cultivation for 4 months, and Las in these two cultures also grew well (Figure 5A and B). Culture 4A23, also using the inoculum from infected psyllids, consisted of 96% *Stenotrophomonas*, 2% *Herbaspirillum*, and 1% *Listeria* after 24 days cultivation. Las in this culture also grew well (Figure 5A and B). In comparison, culture 3A1 and 3A4, which both used a preparation from infected periwinkle as inoculum, had a different bacterial profile and relative abundance after cultivation for 100 days. Culture 3A4 had three dominant genera (*Stenotrophomonas* (83%), *Sphingomonas* (11%), and *Leucobacter* (4%), and Las in this culture showed only minor growth with the Ct value remaining slightly lower than the initial level (Figure 5A and B). Culture 3A1 also had three dominant genera (*Rhizobacteria* (55%), *Stenotrophomonas* (28%), and *Leucobacter* (16%), but Las did not grow at all in this culture. Its Ct value showed a significant increase, indicating that Las titer was dramatically reduced (Figure 5A and B).

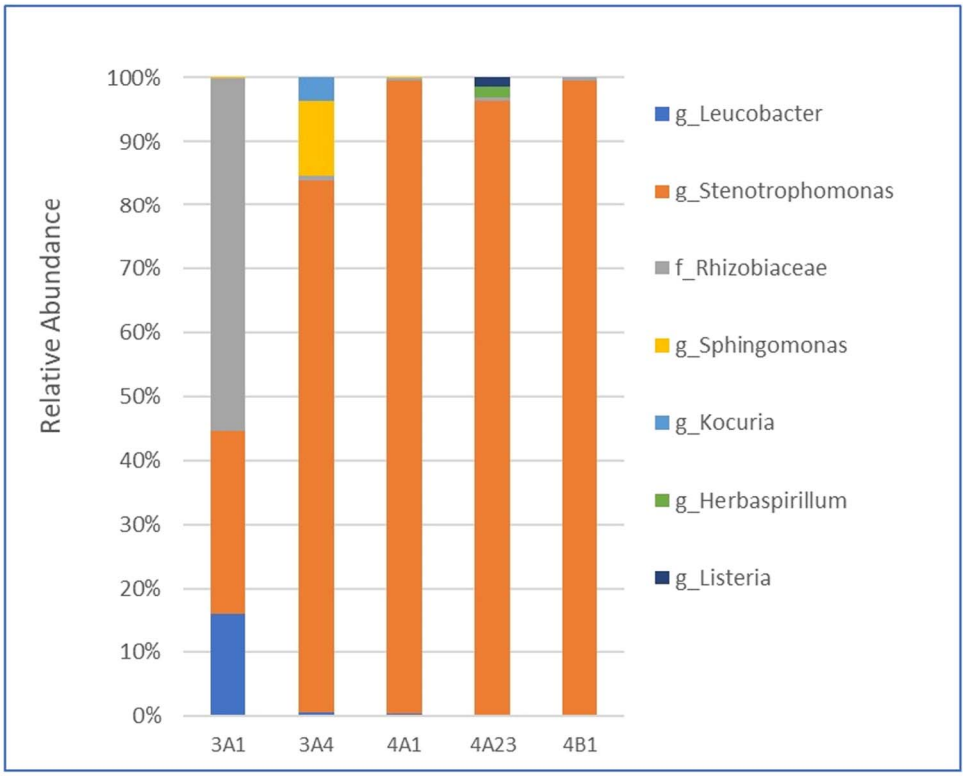
To determine the effect of *Stenotrophomonas* bacteria on Las growth *in vitro*, a single strain from culture 4A1 was isolated in pure culture. Whole-genome sequencing results showed that the entire circular *S. maltophilia* FLMAT-1 genome, without gaps, exhibits a total sequence length of 4 486 066 base pairs (bp). The genome has an average GC content of 66.6%. In terms of coding potential, 4078 coding sequences (CDSs) were predicted in the genome with an average protein length of 329.5 amino acids, accounting for approximately 89.9% of the total genome (Coding Ratio). Additionally, the genome contained 13 ribosomal RNA genes (rRNAs), 75 transfer RNA genes (tRNAs), and two clustered regularly interspaced short palindromic repeats (CRISPRs). The 16S rRNA of *S. maltophilia* FLMAT-1 shared the highest identity (99.48%) with *S. maltophilia* str. IAM_12423 (NR_041577) in the NCBI database. The genome information is available at NCBI database with accession CP140571.We designated this strain as *S. maltophilia* FLMAT-1.

Multidrug resistance of *S. maltophilia* FLMAT-1 enables the use of antibiotics in LG medium

To further characterize *S. maltophilia* FLMAT-1, we tested *S. maltophilia* FLMAT-1 for sensitivity to twelve antibiotics using nutrient agar plates. *S. maltophilia* FLMAT-1 was resistant or tolerant to most of the antibiotics tested except polymyxin B (PMB) and tetracycline (Tet) (Figure S2). Apart from neomycin (Neom), the same 11 antibiotics that were tested against *S. maltophilia* FLMAT-1 were also used to determine their effects on Las growth in liquid cultures with inoculum from psyllids. The results indicated that the cell population level in the polymyxin B (PMB) and tetracycline (Tet) treatments were significantly (ANOVA test, $P \geq 0.001$) lower than those in cultures containing no antibiotics on Day 5. Amikacin (Amik), colistin (Col), and kanamycin (Kan) showed some effect on Las growth, but it was not statistically significant. The remaining antibiotics tested had no effect on Las growth (Figure S3).

To confirm the correlation between the growth of the helper *S. maltophilia* FLMAT-1 and Las and antibiotics *in vitro*, additional co-cultures containing a combination of inoculum from infected psyllids and *S. maltophilia* FLMAT-1, and/or certain antibiotics were carried out. Changes of the Las population were monitored by qPCR. Three scenarios were observed. In the first scenario (Figure 6A), Las did not grow when the culture contained the inoculum alone. Its population continuously decreased; however, in co-culture with *S. maltophilia* FLMAT-1, the Las population continuously increased and reached its maximum in week 4. In

A



B

Sample	3A1	3A4	4A1	4A23	4B1
Inoculum source	PWK	PWK	Psyllid	Psyllid	Psyllid
Age (day)	100	100	120	24	120
Initial Ct	26.1	26.6	25.7	28.1	25.3
Ending Ct	33.8	25.2	22.0	25.4	21.9

PWK=periwinkle

Figure 5. Taxonomic distribution of the bacterial community in Las co-cultures. A) Relative abundance based on 16S rRNA gene sequencing. Only taxonomies with ≥1% read counts have been shown. The 'g' and 'f' indicate taxonomic ranks of genus and family, respectively. The 'f- Rhizobiaceae' failed to be assigned to a specific genus. B) The features of Las cultures used for metagenomic analysis.

the second scenario (Figure 6B), the population of Las gradually increased in the culture with inoculum alone. When *S. maltophilia* FLMAT-1 was co-inoculated, the Las population increased at a faster rate during the first few weeks, but after a longer period, the Las population reached a similar maximum level. In the third scenario (Figure 6C), Las did not grow in the culture with inoculum alone or inoculum plus *S. maltophilia* FLMAT-1. The population in these two cultures decreased in week 1 and week 2 and was then maintained at a lower level. When treated with a mixture of gentamicin (100 μg/ml) and streptomycin (100 μg/ml), the Las population started to increase by week 2. When both antibiotics and *S. maltophilia* FLMAT-1 were added, the Las population was higher than that treated with antibiotics only. It was observed that the growth of Las in liquid culture was only enhanced when living cells of the helper were present, as shown by comparing Las growth in cultures with 1) inoculum alone, 2) supernatants from the *S. maltophilia* FLMAT-1, and 3) co-cultures with live *S. maltophilia* FLMAT-1 cells (Figure 6D).

Co-culture of Las inoculum with the helper *S. maltophilia* FLMAT-1 added, inoculum with antibiotics, or inoculum with both helper and antibiotics all appeared to increase the rate of well-grown cultures, from 60% (inoculum alone) to 77% (inoculum + helper), 100% (inoculum + antibiotics) and 86% (inoculum + antibiotics+ helper). However, there were still cases in which the Las did not grow (2 cases), and cases in which Las grew slowly (5 cases) (Figure 7).

In cultures where the inoculum was derived from infected plants (periwinkle or citrus) but contained 1) *S. maltophilia* FLMAT-1, 2) antibiotics, or 3) *S. maltophilia* FLMAT-1 plus antibiotics, Las growth in all of these treatments was not enhanced when compared to the culture with inoculum alone (data not shown).

Co-cultured Las bacteria multiply in psyllids and citrus plants and cause typical and atypical HLB

Las co-culture suspension was ingested by psyllids after feeding cultures to the 4th to 5th instar of psyllid nymphs [24] and subsequently incubating the Las-fed psyllids on Las-free citrus seedlings for 2–3 weeks. In the past four years, 28 Las-feeding experiments with Las co-cultures have been conducted (Table 2) with a total of ca. 3800 psyllid nymphs fed and ca. 1100 adult psyllids collected from the inoculated citrus seedlings following

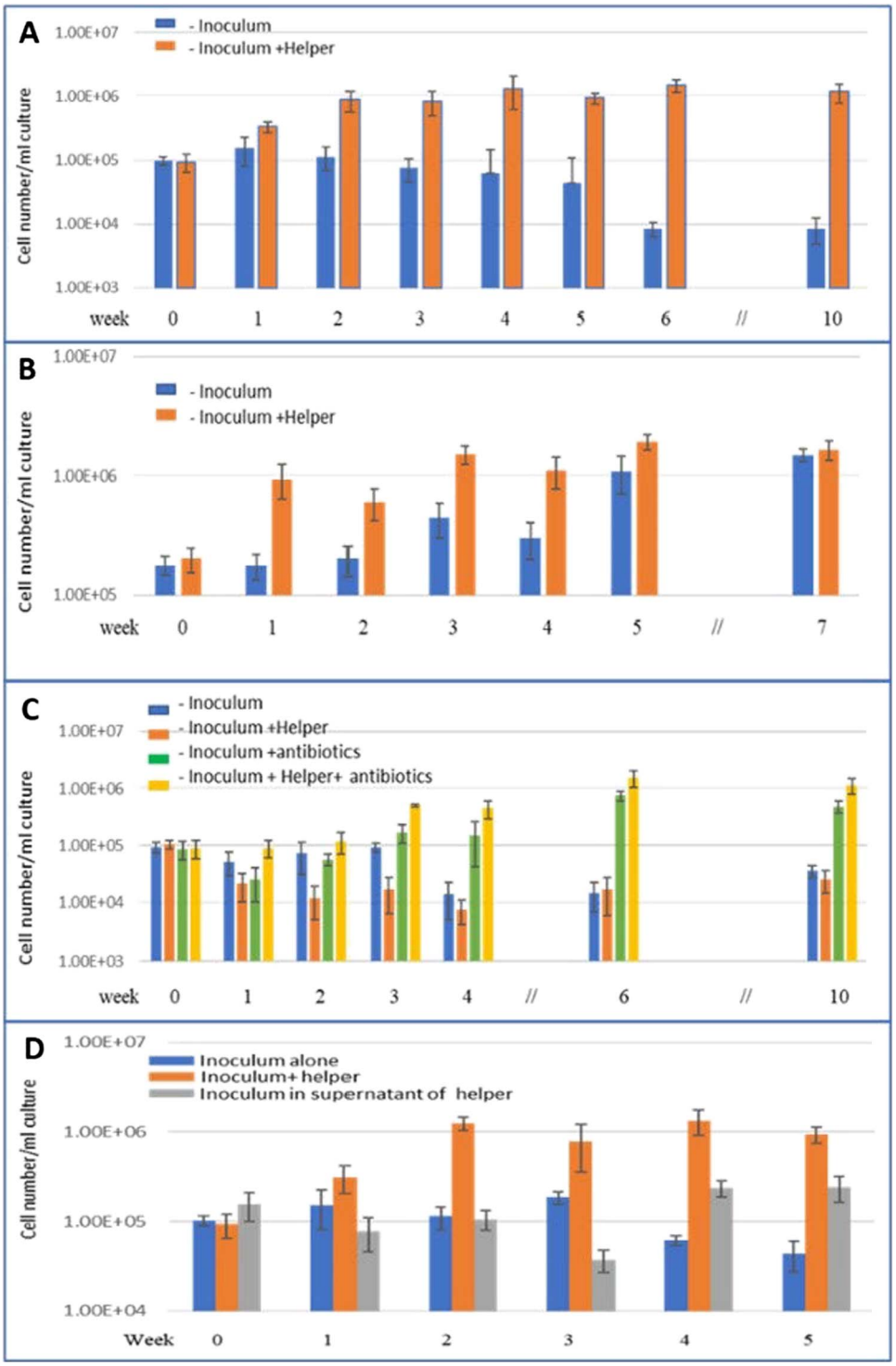
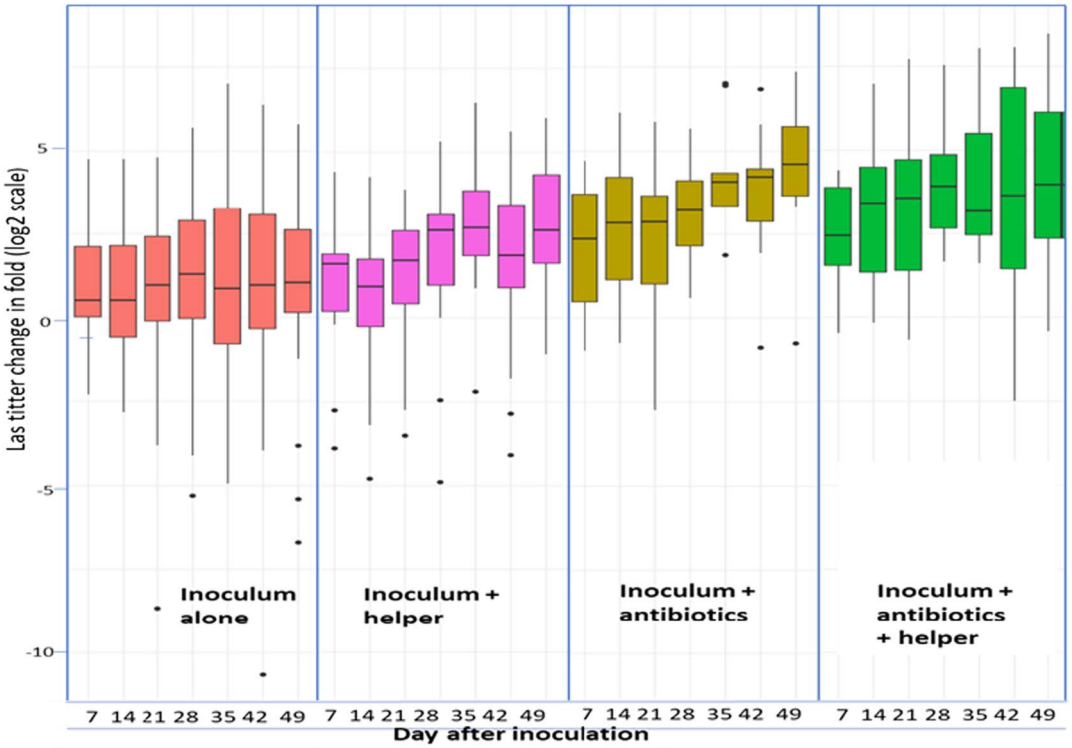


Figure 6. Effects of helper, *Stenotrophomonas maltophilia* FLMAT-1 on Las growth *in vitro*. A) and B) Las growth with inoculum alone, inoculum plus helper, C) Inoculum plus helper and/or antibiotics (gentamycin and streptomycin) and D) Effects of living cells of helper *S. maltophilia* FLMAT-1 and its supernatant on Las growth. The supernatant was made from the culture of *S. maltophilia* FLMAT-1 growing in LG medium. The same volume of supernatant was used as medium for Las growth. The error bars represent the standard error of the mean of three biological replications.

feeding. Overall, the survival rate was low, averaging 30%. The Las-positive rate of the fed psyllids collected was around 23.2%, which was similar to the results of nymphs on excited leaf of infected citrus [25] and nymphs on Las-infected plants for one week [26]. Of the 191 Las-free citrus seedlings inoculated with Las-fed nymphs, qPCR detected Las in 18 seedlings, resulting in a Las-positive rate in psyllid-inoculated citrus plants of 9.4% (Table 2).

The acquisition of Las by psyllid nymphs from the co-cultured cell suspensions was not correlated with the Las titer when cell suspensions were in the range of 10^5-10^6 and > 10^6-10^7 cells/ml (Figure 8A). The Las-positive rate of psyllids fed with the Las growing *in vitro* from inoculum alone was higher than those from inoculum co-cultured with the helper bacteria with or without antibiotics (Figure 8B). The Las-positive rate of citrus plants

A



B

Titer change in fold (cell number/ml)	Inoculum alone (N=30)	Inoculum + helper* (N=22)	Inoculum + antibiotics** (N=12)	Inoculum +antibiotics +helper(N=15)
<0	10.0% (3)	9.1% (2)	0	0
1-5	30.0% (9)	13.6% (3)	0	13.3% (2)
>5-10	30.0% (9)	27.3% (6)	8.3% (1)	13.3% (2)
>10-100	26.7% (8)	50.0% (11)	75.0% (9)	40.0% (6)
>100-1,000	3.3% (1)	0	16.7% (2)	33.3% (5)

*= *S. maltophilia* FLMAT-1, **= $gentamicin_{100}$ + $streptomycin_{100}$

Figure 7. Comparison of Las population dynamics during growth *in vitro*. The Las inoculums used for the cultures were from psyllids. Las cell number was calculated using Ct values obtained from qPCR. The Las cultivation had 4 treatments that were inoculum alone, inoculum plus antibiotics, inoculum plus helper added, and inoculum plus both. A) Distribution of log2 fold changes. B) Percentage of Las titer fold changes. N = number of trials.

showed the same correlation with these two factors mentioned above as the Las-positive rate of psyllids (Figure 8E and F). The age of Las co-cultures showed a clear correlation with the Las-positive rate of psyllids. Psyllids more readily acquired and propagated Las from younger Las co-cultures than older ones when used for feeding (Figure 8C), although the age of the culture did not affect the transmission of Las from the psyllid to citrus plants (Figure 8G). We noticed that the amount of time psyllids were maintained in the cage after feeding (rearing time) was negatively correlated with the Las-positive rate of the fed psyllids; the shorter the rearing time, the higher the Las-positive rate of psyllids (Figure 8D). However, the rearing time appeared positively correlated with Las-positive rate of citrus; the longer the rearing time, the higher the Las-positive rate of citrus (Figure 8H). This suggests that Las cultures reduced the life span of psyllids and the longer that a Las-positive psyllid remained feeding on citrus, the more Las was transmitted into the citrus plant. Among the Las-positive citrus plants, three showed severe HLB symptoms and only two harbored a high titer of Las bacteria (Ct = ~20.0). All others showed atypical HLB symptoms, such as small leaves and growth retardation with a low titer of Las (Ct >32) (Figure 9 and 10). Graft transmission of the two high-titer Las-infected plants to healthy Duncan grapefruit seedlings reproduced typical HLB symptoms and high Las titers with an over 80% success rate of transmission. While sticks of low titer Las-infected plants were grafted onto healthy Duncan grapefruit seedlings, low titers of Las were obtained with no visible symptoms. It is worth noting that yellow leaves and symptoms of whitened vein necrosis were both observed in citrus plants with low titers of Las, and high titers of Las were found in the graft transmitted HLB plants (Figure 10B, C, D and E). In general, the infection rate of the co-cultivated Las appeared to be low, and low titer infections comprised the majority of the culture-fed psyllid-infected plants from the 28 trials.

Table 2. Infection of insect host (psyllid) and plant host (citrus) with co-cultivated Las

Trial	Las			Fed nymphs	Psyllids In cage	Collected psyllids			Infected citrus plants		
	Inoculum source	Titer (cell/ml)	Age * (day)	Num	Day	Num	Las +	Las +%	Num	Las +	Las +%
1	psyllid	7.70E+05	31	120	15	34	12	35.3	6	1	16.7
2	psyllid	4.70E+05	42	150	36	37	15	40.5	7	0	0
3	periwinkle	7.50E+04	17	130	25	12	10	83.3	7	0	0
4	psyllid	1.00E+05	22	120	19	28	19	67.9	6	1	16.7
5	psyllid	9.90E+05	92	150	21	48	13	27.1	6	0	0
6	psyllid	1.60E+06	42	130	19	27	4	14.8	4	1	25
7	psyllid	4.80E+05	14	60	18	18	8	44.4	2	1	50
8	psyllid	1.00E+06	10	125	21	32	11	34.4	4	1	25
9	psyllid	1.10E+06	14	125	21	27	12	44.4	5	1	20
10	psyllid	2.10E+06	60	125	18	23	17	73.9	8	2	25
11	psyllid	2.90E+06	85	150	48	11	3	27.3	6	0	0
12	psyllid	1.30E+06	14	100	14	12	6	50	4	0	0
13	psyllid [a]	1.20E+06	28	100	13	30	16	53.3	6	0	0
14	psyllid	6.80E+05	35	120	20	59	8	13.6	5	1	20
15	psyllid [a]	2.80E+05	49	75	18	48	12	25	4	0	0
16	psyllid [a]	2.90E+05	21	125	33	30	8	26.7	4	1	25
17	psyllid	2.70E+06	35	150	14	97	9	9.3	8	0	0
18	psyllid	8.80E+05	42	200	18	58	7	12.1	8	0	0
19	psyllid [a]	8.30E+06	28	200	14	78	10	12.8	10	0	0
20	psyllid [a]	2.90E+06	48	150	15	69	36	52.2	10	1	10
21	psyllid [a]	5.60E+06	58	150	21	45	9	20	10	1	10
22	psyllid [a]	7.10E+06	92	200	32	70	9	12.9	10	2	20
23	psyllid [a]	4.80E+06	200	160	28	48	7	14.6	8	0	0
24	psyllid	2.50E+06	42	110	20	13	4	30.8	8	0	0
25	psyllid [a]	2.50E+06	42	160	25	21	0	0	8	0	0
26	psyllid	1.00E+06	28	200	21	18	5	4.2	8	0	0
27	psyllid [a]	1.50E+06	60	120	17	24	0	0	6	0	0
28	psyllid [a]	2.00E+06	63	120	29	45	0	0	13	6	46.2
Sum				**3825**		**1162**	270		**191**	18	
Average								**23.20%**			**9.40%**
Survival rate						**30.40%**					

*The time of Las growing in vitro before used for feeding. [a]Inoculum plus helper and or antibiotics when growing Las in vitro Num, Number

Las employs the pentose-phosphate pathway and part of the glycolytic pathway for its carbon utilization

Four Las co-culture samples with different growing times and statuses (growing faster or slower, Figure 11) from different cultures were used for gene expression profiling using RT-qPCR analysis. According to genomic information, Las has an incomplete glycolytic pathway due to the absence of the gene encoding phosphor-glucose isomerase (PGI). Carbohydrate metabolism in Las occurs via the pentose-phosphate pathway and part of the glycolytic pathway. In Las, glucose can be converted into glyceraldehyde-3-phosphate, enter the glycolytic pathway, and be further used for pyruvate synthesis. The complete set of seven genes in the pentose-phosphate pathway were targeted in this study. The RT-qPCR analysis showed expression of all seven genes with higher expression in faster growers than in slower growers except for ribose-5-phosphateisomerase in samples-I (Figure 11). These results demonstrated that the pentose-phosphate pathway in Las cells grown *in vitro* was active and complete. Compared to the Las residing within psyllids, the gene encoding 6-phosphogluconolactonase was expressed at a much lower level in all Las samples from *in vitro* co-cultures (Figure 11A), indicating that the Las in culture had utilized the glucuronamide in the LG medium as its main carbon source for growth by converting the compound to 6-phophate-gluconate in the pentose-6-phosphate pathway. The low expression of the gene encoding ribose-phosphate pyrophosphokinase in the samples with a shorter growing time (45 days in this study) indicated that their metabolic flux was mainly forwarded to glycolysis by converting ribose-5-phosphate into glyceraldehyde-3-phosphate and then pyruvate (Figure 11A). However, in the samples with a longer growing time (84 days in II-A and 70 days in II-B), the metabolic flux was mainly forwarded to nucleotide biosynthesis because the expression of the gene encoding transketolase was low (Figure 11B).

Discussion

Although Las is an intracellular plant pathogenic α-proteobacterium with a significantly reduced genome, we have demonstrated that it can grow outside plant or insect cells with the help of other bacteria in co-cultures. The growth rate of Las in LG liquid medium depended on the source of inoculum. The inoculum from psyllids grew better and more consistently than those from plants. We originally hypothesized that the Las transcriptome and/or metabolomes may change when Las switches from an insect to a plant niche. The differences that may contribute to Las growth in LG medium co-cultures included: 1) Las chromosomal deletion mutations [27], 2) the number of prophages and their activities [28], and 3) the more than 100 differentially expressed genes in citrus versus ACP [29]. On the other hand, inoculum prepared in the study either from the insect vector or from a plant host always contained a microbial community. To survive and grow under *in vitro* conditions, Las may need to adapt to the new environmental

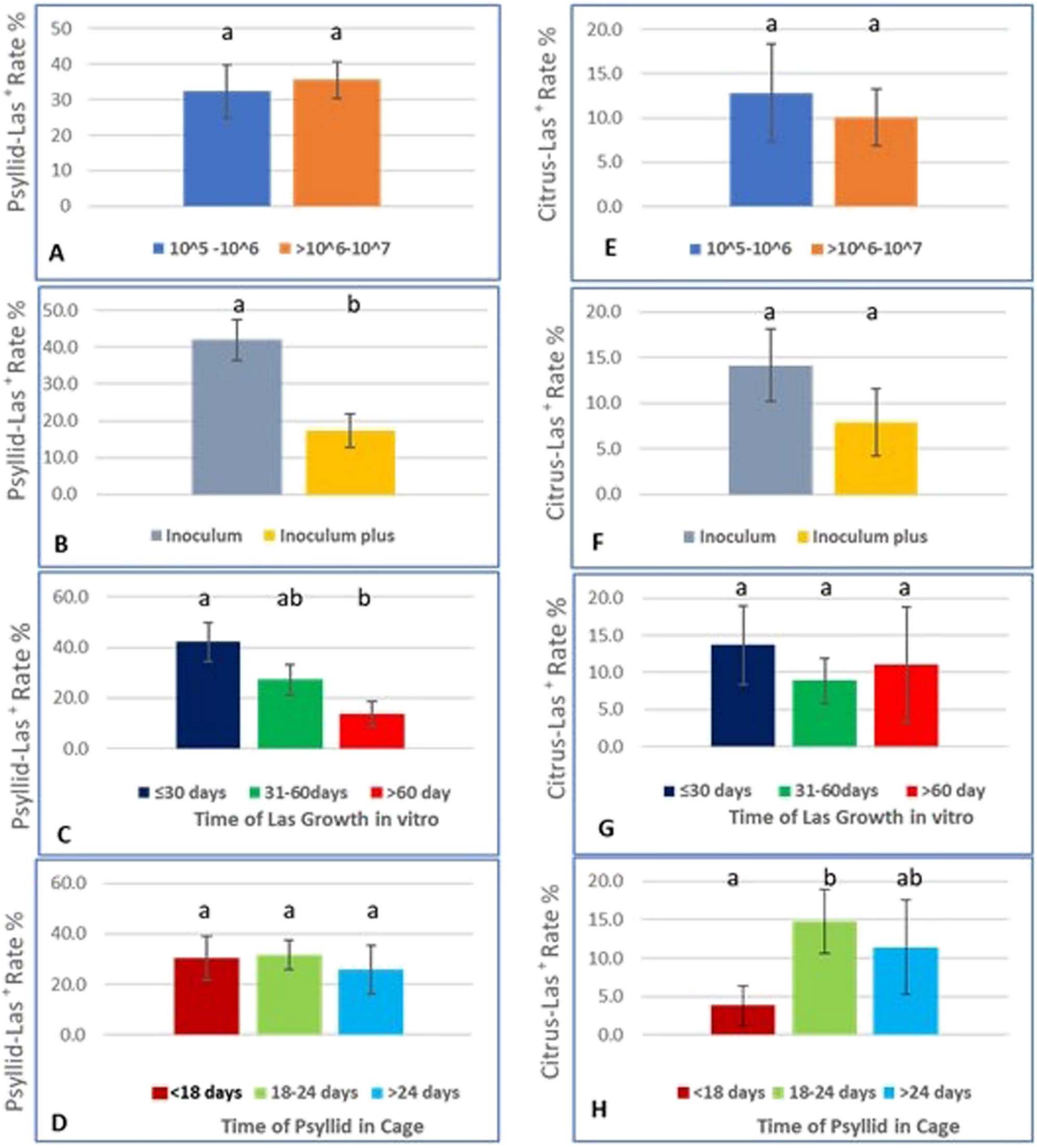


Figure 8. Factors that affect growth of the co-cultivated Las in psyllids and citrus. A and E) Las titers were determined before inoculation with 10^5–10^6 and 10^6–10^7 (cell/ml culture). B and F) Cultivated Las used for feeding from inoculum alone and inoculum plus antibiotics. C and G) Time of Las grown *in vitro* before used for feeding. D and H) Time of fed-psyllids rearing on citrus before being collected. The statistical analysis using ANOVA revealed significant differences between groups marked with different lowercase letters ($p < 0.05$).

conditions and may directly interact with other members of the community. To this point, we found *S. maltophilia* was able to aid Las growth *in vitro* in a series of co-culture tests, but only when isolated from psyllids. Analysis of these experiments revealed some important findings: 1) Las growth *in vitro* required a co-cultured helper, 2) the helper was always present in the inoculum, whether the inoculum was prepared from Las-infected psyllids or plants, and 3) *S. maltophilia* FLMAT-1 could not be eliminated in the Las co-cultures due to its multidrug resistance and extremely dominant growth in LG medium. The inability of Las to grow in supernatants derrived from *S. maltophilia* FLMAT-1 suggests that the Las growth *in vitro* requires live cells of the helper in the medium.

The need for a helper to aid *in vitro* growth may also help explain the results observed using inoculum derived from different sources (e.g. plant versus psyllids) in the LG medium. Figure 1

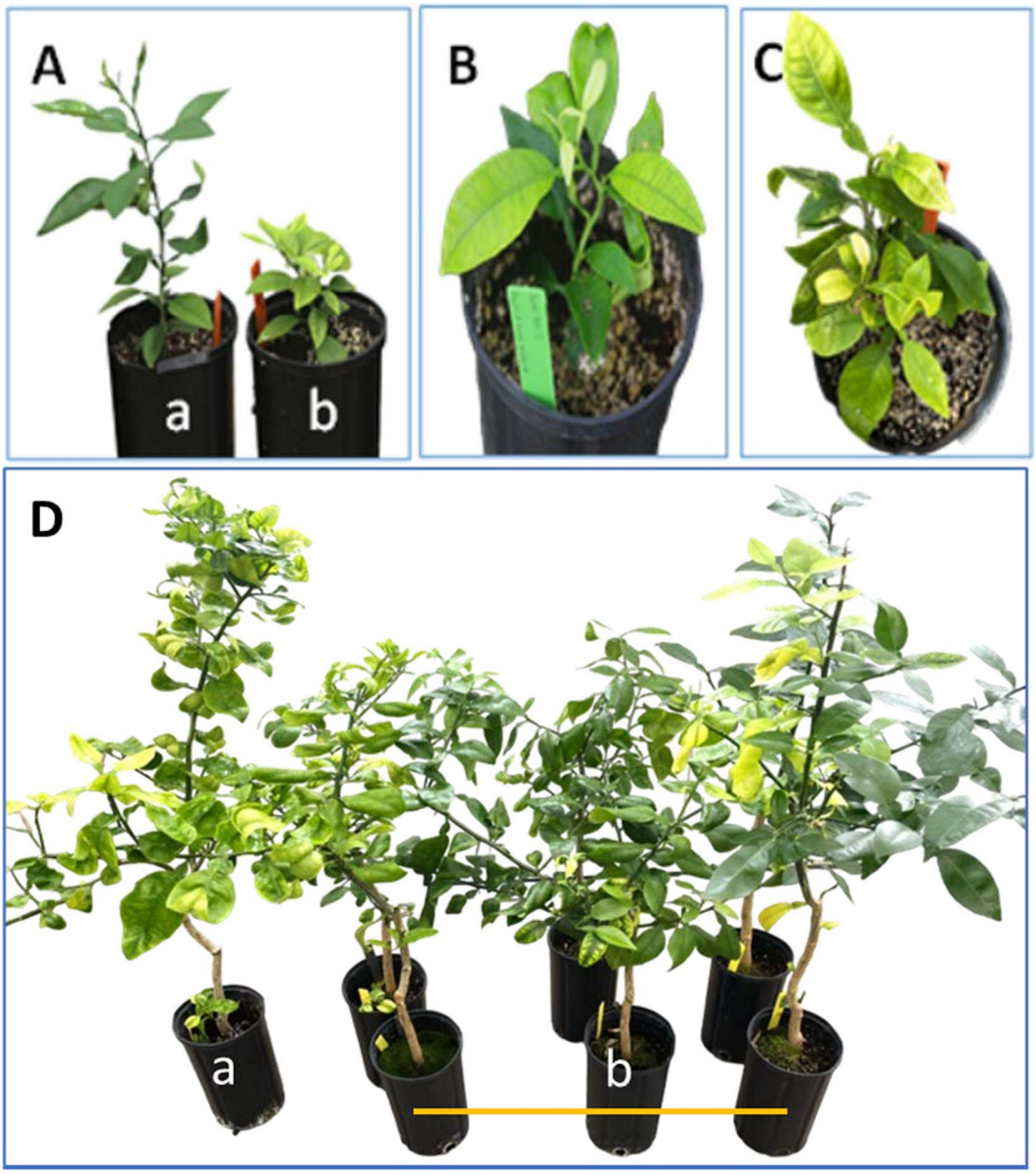


Figure 9. HLB caused by cultivated Las and its subsequent graft transmission. A), B), and C) Cultivated Las-infected plants displaying HLB symptoms. Aa, Healthy control, Ab Las-infected plant. B) typical HLB with high Las titers at 2 months after inoculation (MAI). C) Atypical HLB symptoms with extremely low titers of Las at 39 MAI. C) Cultivated Las-infected plant displaying typical HLB symptoms and high Las titers (a) at 36 MAI. All the other HLB-affected plants (b) by graft-transmission from plant (a) displaying typical HLB symptoms and high Las titers at 23 MAI.

demonstrated enhanced *in vitro* growth using inoculum from psyllids compared to either citrus or periwinkle. Comparisons made using metagenomic studies investigating gut endosymbionts within Las-affected ACPs [30] and the epiphytes and endophytes associated with Las-affected citrus trees [31] show vast differences amongst the most abundant genera present. Even at the phyla level, only four of the ten most abundant phyla overlap between Las-infected ACP and citrus trees and these include Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes. Taken together, these data suggest that the helper may not always be present in a particular inoculum. Although *S. maltophilia* is not listed within either of these microbiome manuscripts, it is highly possible they are only initially present in low numbers and therefore fall within the 'other' category; whereas the LG medium used in this study acts to enrich it.

Exactly what Las would acquire from the helper for its growth *in vitro* is currently unknown. Based upon its genome [3], Las may acquire several essential nutrients and energy in the form of ATP from the living cells of a helper bacterium. We hypothesize the acquisition of ATP by Las because of the unique ATP translocase it encodes, which allows the direct transport of ATP into the Las cell and is not found in free-living *L. crescens* [32]. This ability to acquire ATP from living host cells could provide Las a source of energy when other metabolites are not readily available. Genome comparisons can also help predict additional putative essential metabolite(s) that Las may acquire from the helper. For example, pantothenate and folate are likely both essential metabolic compounds needed by Las for growth. Pantothenate (also referred to as vitamin B5) is the key precursor for the biosynthesis of coenzyme A, which is an essential cofactor for cell growth and is involved in many metabolic reactions [33]. In Las

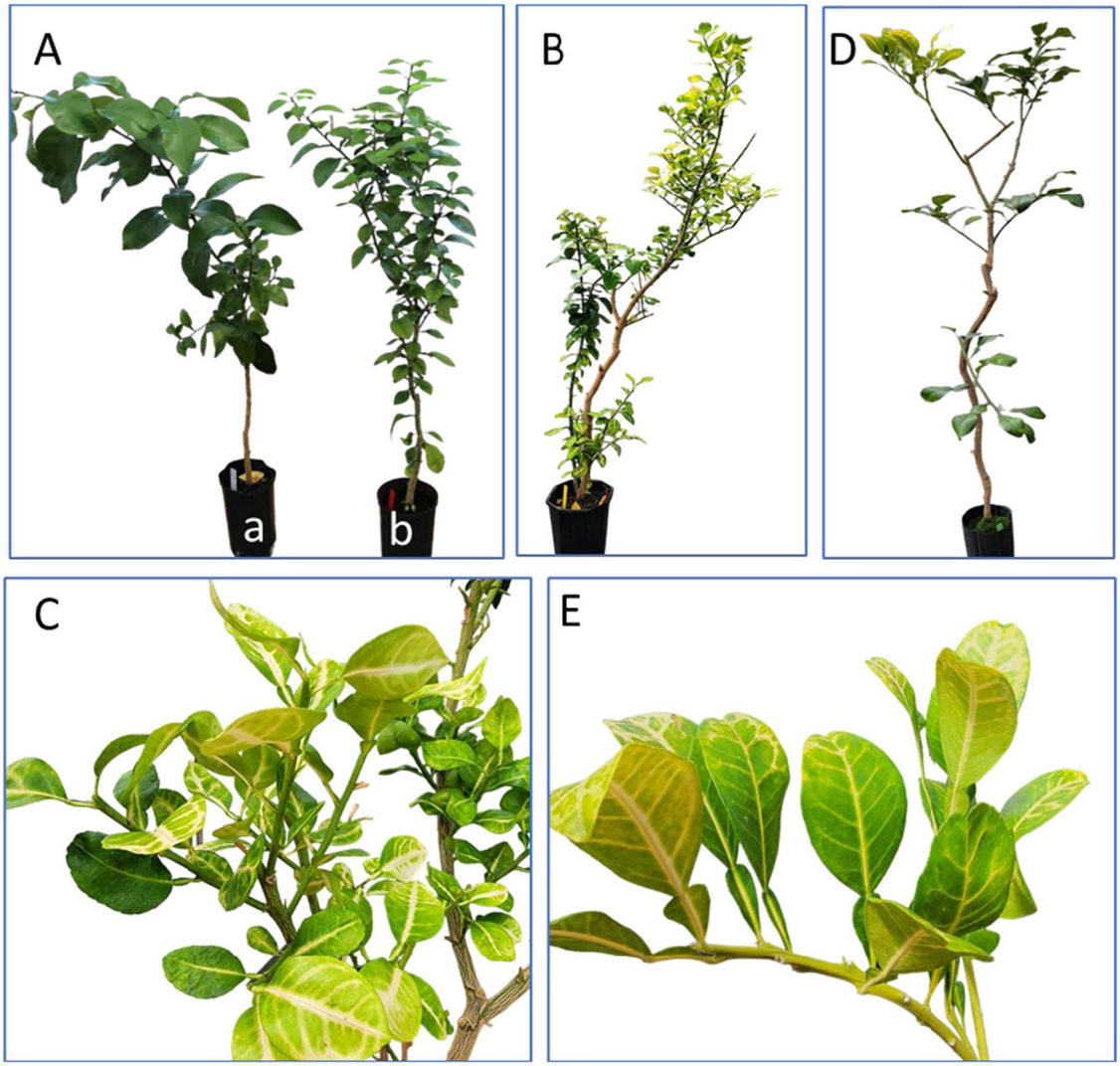


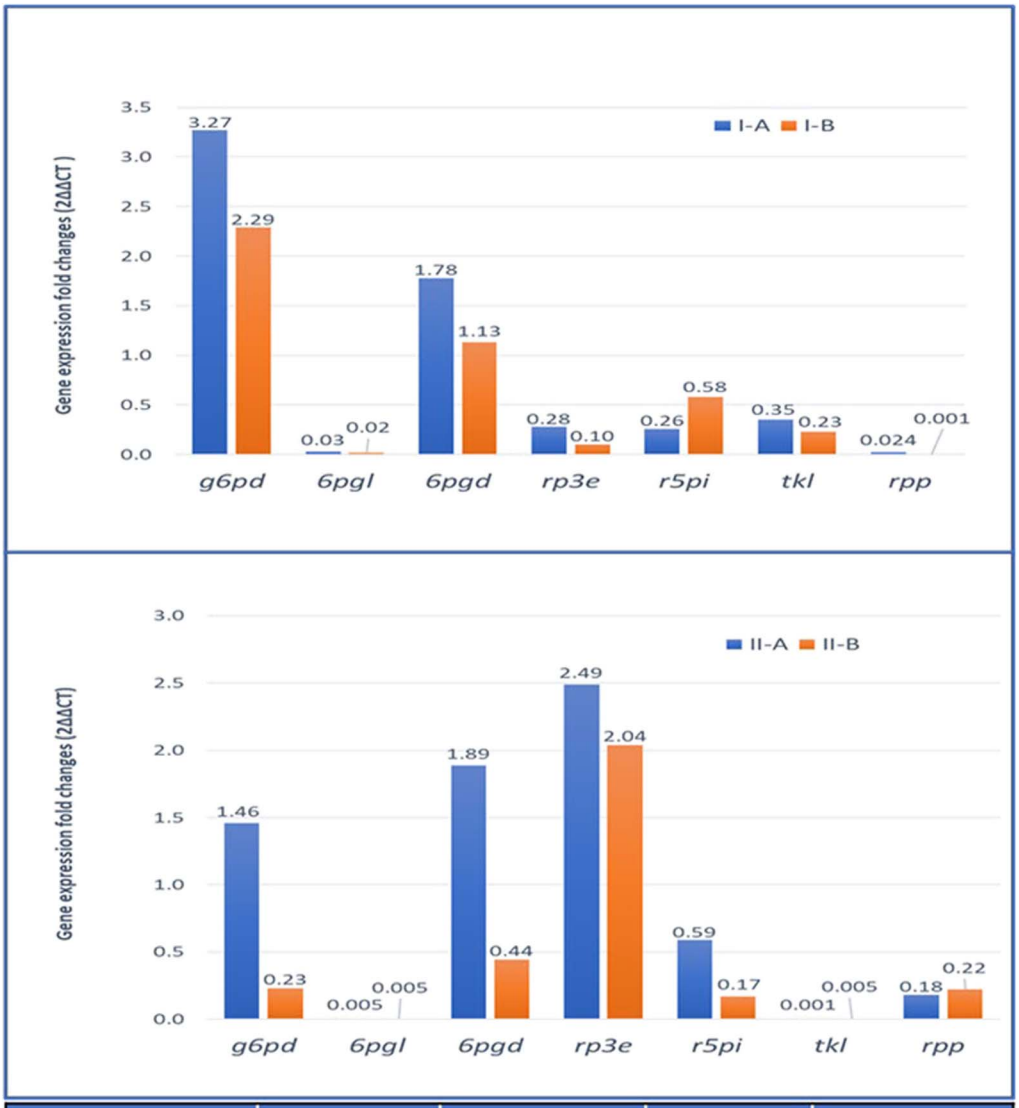
Figure 10. HLB-like symptoms caused by low titers of cultivated Las. Aa) control Duncan grapefruit. Ab) Small leaves and growth retardation with low titer of Las (Ct = 34.51) at 23 months after inoculation. B and C) Small yellow leaves and vein necrosis with Low titer of Las (Ct = 34.68) at 59 months after inoculation, C was the closeup of B. D and E) HLB affected plant with high titers of Las, displaying small yellow leaves and vein necrosis, E was a closeup of D.

and all other *Candidatus* Liberibacter species, the entire biosynthetic pathway for pantothenate, which consists of four genes, was lost and no gene encoding a pantothenate-specific transporter has been found. Therefore, Las may acquire pantothenate from cells of the helper, possibly in a contact-dependent manner. Folate (vitamin B9) is likely essential for all sequenced bacteria except *Mycoplasma hyopneumoniae* [34]. Except for Las, all other species in the *Liberibacter* genus identified so far possess a complete pathway for *de novo* folate synthesis and therefore should be able to synthesize folate themselves. Las however lacks three genes, *folB*, *folK*, and *folP*, in the folate synthesis pathway [9] that result in the inability to produce folate. Las may acquire folate from helper cells as well. *Spiroplasma citri*, another citrus phloem-limited bacterial pathogen, also is not able to synthesize pantothenate and folate by itself but possesses specific transporters for both.

Las is an obligate intracellular bacterium that is limited to the phloem tissue in plants and primarily accumulates and propagates within vacuoles inside psyllids [35]. These *in vivo* intracellular environments are different from *in vitro* culture. The locations of Las *in vivo* suggest the existence of a stable, specific, and less complex microbial community (intracellular bacteria) in which no member may achieve a very high abundance. Therefore, Las *in vivo* may not need a helper to acquire the essential compounds that Las itself does not produce. Abu Kwaik and Bumann have suggested that hosts deliver nutrients to intracellular pathogens [36], and there is evidence to suggest the participation of host solute carrier (SLC) transporters (membrane proteins) in the host. For Las in hosts (psyllid and citrus), certain essential compounds such as pantothenate and folate could be provided by the host via specific SLCs. In the case of ATP, the ATP translocase may allow uptake of ATP directly from either the psyllid or plant host and bypass the need for a helper *in vivo* [32].

In this study, we were able to experimentally demonstrate for the first time that Las synthesizes pyruvate via the pentose-5-phosphate pathway and part of the glycolytic pathway. We also elucidated the mechanism of ATP production and regulation in this pathway. The pentose phosphate pathway allows for the conversion of glucose, gluconate, xylose, or other carbohydrates to ribose-5-phosphate and allows it to enter the glycolytic pathway

A.



B.

Sample	I-A	I-B	II-A	II-B
Inoculum source	Psyllids	periwinkle	Psyllid	Psyllid
Age (day)	45	45	84	70
Initial Ct	26.37	27.34	26.43	28.79
Ending Ct	25.13	29.09	23.49	26.69

Figure 11. Gene expression profiles of the Las pentose-phosphate pathway during growth *in vitro*. A) Relative expression fold changes ($2^{-\Delta\Delta CT}$) of the genes *in vitro* in compared with those from psyllids; I, II-A, faster growers and I, II-B, slower growers. *g6pd*, glucose-6-phosphate dehydrogenase; *6pgi*, 6-phosphogluconolactonase; *6pgd*, 6-phophogluconate dehydrogenase; *rp3e*, ribulose-phosphate 3-epimerase; *r5pi*, ribose-5-phosphate isomerase; *tkl*, transketolase; *rpp*, ribose-phosphate pyrosphokinase. B) Features of the Las co-culture samples used for gene expression profiling using RT-qPCR.

or be involved in nucleotide biosynthesis (Figure 12). We observed that the metabolic flux was mainly toward glycolysis in the samples with shorter growing times (Figure 11A, I-A and I-B. 45 days), while in samples with longer growing times (Figure 11B, II-A, 84 days, and II-B, 70 days) the metabolic flux was mainly toward nucleotide biosynthesis based upon the gene expression levels of ribose-phosphate pyrophosphokinase and transketolase (Figure 11). In the case of the metabolic flux mainly forwarded to glycolysis, Las produces ATP not less than that via a complete glycolytic pathway (with PGI presence). However, when the metabolic flux is mainly forwarded to nucleotide biosynthesis, much less ATP is produced. Control of these metabolic flux switches is likely associated with the physiological status of Las. Therefore, the effects of ATP from an extracellular source via the unique ATP/ADP transporter may occur only under a certain physiological status of Las.

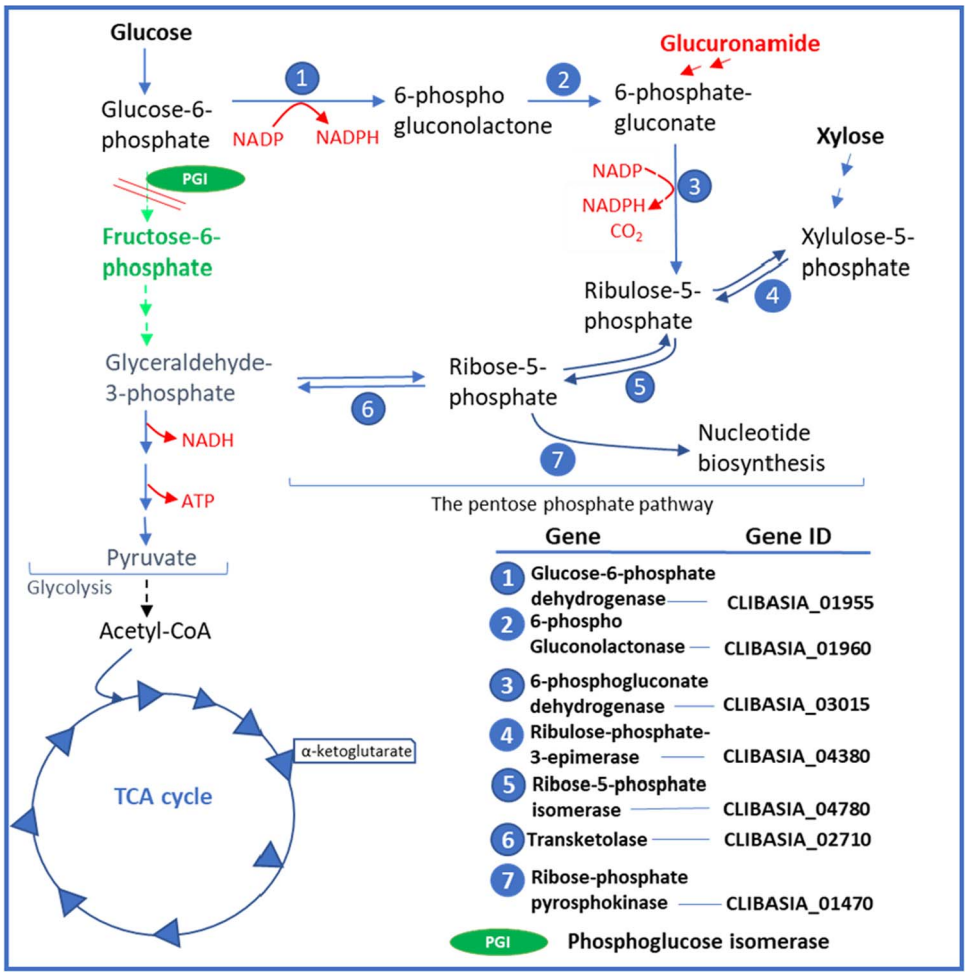


Figure 12. Scheme of glycolysis and pentose-phosphate pathway in Las during growth *in vitro*.

We have experimentally provided evidence for the first time that Las is the causative agent of HLB in citrus. We cannot rule out the formal possibility that HLB requires an additional bacterial agent, although that agent must have also been present in the co-culture. The natural infection cycle of Las involves both plant host (citrus) and insect vector (psyllid). To infect the host with cultivated Las, we followed the same infection pathway as Las does in nature through the vector. First, we fed psyllid nymphs with co-cultivated Las and then reared the fed nymphs on healthy citrus seedlings. After being ingested in and propagated within the psyllids, Las was transmitted to citrus plants and multiplied within the citrus phloem, eventually causing HLB in the citrus plant. It is important to note that the co-cultivated Las did cause HLB-like symptoms with most of the plants (88.9%), even though the Las titers remained low (Ct > 33.0). Further, these low titer infections had not developed high titer for 3–5 years after inoculation in greenhouse settings. Some of these plants only showed HLB symptoms in one of the original inoculated branches and did not develop systemic disease throughout the infected plant. The long-lasting low titer infections with differing symptoms warrant further study to elucidate the causal agents in the HLB complex. In conclusion, for the first time we have fulfilled more than one of Koch's postulates and laid additional groundwork towards obtaining an axenic Las culture.

Materials and methods

Biolog phenotype microarray analysis

Biolog plates 1–4, 9, and 10 (Biolog Inc., Hayward, CA) were inoculated with both *L. crescens* BT-1 and inoculum derived from Las-infected periwinkle plants. The *L. crescens* inoculum was prepared by pelleting 3 ml of a 5-day-old culture via centrifugation at 2.3 × 1000 rcf for 2 minutes. The pellet was initially resuspended in 5 ml of the IF M1 inoculating fluid (Biolog Inc., Hayward, CA). Optical density (OD) measurements were taken at 600 nm, and the culture was diluted with IF M1 until the OD600 = 0.01, and at least 10 ml of inoculum was obtained. Then, 100 μl of Biolog Redox Dye Mix D (Biolog Inc.) was added to 10 ml of inoculum, mixed, and 100 μl was dispensed into each well of the Biolog PM plates 1, 2, and 10. The Las inoculum was prepared by soaking 12–14 leaves from a Las-infected periwinkle plant in 70% ethanol for 5 minutes to eliminate surface contamination. The leaves were then rinsed in sterile water for 5 minutes. Midribs were removed from the leaves aseptically and chopped into smaller fragments. The chopped samples were then placed into a 2 ml tube containing 900 μl of water, 100 μl of polyvinylpyrrolidone-40 (50% w/v) (Sigma, St. Louis, MO), and glass beads. Samples were processed via a Fast Prep-24 homogenizer (MP Biomedical, Solon, OH, USA) at speed of 6 m/s for 20 s. Large particulates were removed via centrifugation for 2 minutes at 200 rcf. Then, 0.89 ml of inoculum was removed and placed into a tube containing 10 ml

IF-M1 and 110 μl of Biolog Redox Dye Mix D. After mixing via inversion, 100 μl was added to each well of the Biolog PM plates 1, 2, and 10. All plates were incubated at 25°C for 24 hours and scanned using the OmniLog ID system Model 71000 (TORCON Instruments Inc., Torrance, CA). Biolog PM plates 3, 4, and 9 were processed with both inoculums as stated above, with the addition of 10 mM glucuronamide as a carbon source.

Preparation of Las inoculum from Las-infected psyllids

Las-infected citrus psyllids (*D. citri*) were obtained from artificial rearing colonies housed at the USDA-ARS-USHRL, Fort Pierce, Florida and sterilized in 70% ethanol for 5 minutes. After washing in sterile water once, they were placed into 5% bleach for 5 minutes and then washed with sterile water three times. Psyllids were ground in 2.0 ml Eppendorf tubes with NT solution (1.5% arabinose and 0.85% sodium chloride) using a mini pestle (~100 psyllids in 2.5 ml NT solution). The extraction was conducted twice with the first extraction of 1.5 ml and second extraction of 1.0 ml of NT solution. The extracts were centrifuged at 7000 rpm for 1 minute. The supernatants of the two extractions were combined into Falcon™ 15-ml tube. The supernatants were then used as the Las inoculum from Las-infected psyllids.

Preparation of Las inoculum from Las-infected periwinkle or citrus

Infected leaves were collected from plants kept in the USHRL greenhouse, and midribs of the leaves were excised. The midribs were then surface sterilized by placing them in 70% ethanol for 5 minutes, followed by one wash in sterile water. Then, the midribs were placed in 10% bleach for 5 minutes and washed in sterile water three times. After being dried with sterilized paper towels, the midribs were cut into small pieces and ground with a sterilized mortar and pestle in NT solution at a ratio of ~1.0 g tissue to 2 ml NT solution. The extracts were centrifuged at 5000 rpm for 1 minute, and the supernatants were collected as the Las inoculum from the plant materials.

Medium preparation

The medium was designated as Las growth (LG) medium and is composed of: 10% of stock solution A (2.0 g α-ketoglutaric acid, 1.5 g K_2HPO_4, and 1.0 g KH_2PO_4, with the pH adjusted to 8.2 with KOH, and deionized water added to a final volume of 100 ml before autoclaving), 10% of stock solution B (10 g glucuronamide dissolved in the final volume of 100 ml deionized water, autoclaved), 30% of stock solution C (Difco™ Nutrient Broth, autoclaved) and 50% of sterilized water. The final pH in the LG medium was 7.0 ± 0.1. For Petri dish plate cultures, 15 g of Difco™ agar was added to 1.0 L LG liquid medium. Replacement of each major component of LG was conducted and the preferred pH in LG medium for Las growth was also tested.

Cultivation of Las *in vitro*

In liquid culture, 50 to 100 μl of inoculum from psyllids or 200 to 500 μl of inoculum from plant material was added to 10 ml of media in a 50 ml Falcon tube and incubated at 28°C while shaking at 100 rpm. Each culture had three replicate tubes. Cultures with larger volumes (50, 75, or 100 ml) were carried out in appropriate flasks at 28°C while shaking at 100 rpm.

For co-culture of the helper *S. maltophilia* FLMAT-1 with Las inoculum, single colonies of the helper were picked from an NBA (agar) plate and inoculated in 5 ml NB medium and incubated at 28°C while shaking at 100 rpm overnight or until the OD_{600} was ~1.20. On Day 0, 10 μl of the overnight culture of the helper was added into a 10 ml Las culture and incubated at 28°C while shaking at 100 rpm.

Monitoring Las growth in liquid cultures

The growth of Las in liquid medium was monitored using qPCR with HLBas/HLBr/HLBp primers and TaqMan probe as described by Li et al. [37]. The dynamics of the helper bacterium, *S. maltophilia* FLMAT-1 was also monitored by qPCR using FLMAT-1 strain-specific primers, Stp-F, 5′-TCGCCATCAGCAACATCTC-3′ and Stp-R, 5′-CCTTGATGACCACGCTGAA-3′. After the inoculum was added to the medium and mixed well, 500 μl of the culture mixture was taken immediately from each tube as day-0 samples. Then, 500 μl individual samples were taken at different time intervals (usually once a week). DNA extraction for qPCR was performed using a simple method called osmotic shock, as described by Masson et al. [38]. The samples (500 μl culture mixture) were centrifuged at 15000 rpm for 5 minutes with a table centrifuge. The supernatants were discarded, and the pellets were re-suspended in 25 μl DNA-free water and heated at 100°C for 5 minutes. The suspensions were spun down, and the supernatants were used as DNA sources for qPCR. 2 μl of the supernatant was used in each qPCR reaction. The individual qPCR reactions containing 0.05 μl of each specific primer (100 pmol/μl), 0.025 μl of probe (100 pmol/μl), 7.5 μl of 2X TaqMan Reaction Mixture (Thermo Fisher Scientific, MA) and water to make a final volume of 15 μl. The following qPCR program was used: 95°C for 3 minutes followed by 40 cycles of 95°C for 30 seconds and 62°C for 30 seconds.

The Ct value of qPCR with Li primers and probe was used to estimate the bacterial titer of Las in liquid culture, presented as the number of cells per milliliter (cells/ml) of liquid cultures. The Ct value was converted to Las copy number first based on a standard curve generated in our laboratory [Log Copy Number = 11.5–0.3X (Ct)], and then calculated as cell number after taking into consideration three copies r of 16S rDNA genes per genome [3] and the volume of DNA used in the individual qPCR reaction.

Detection of antibiotic sensitivity of *S. Maltophilia* FLMAT-1

A pure culture of the helper bacteria was used for determining the sensitivity to antibiotics. l ml helper cell suspension of cultures that had grown overnight was added to 5 ml top agar (0.7% agar in water) and poured on LB agar plates. Paper discs were placed on the top agar in the LBA plate and 5 μl of antibiotics was added on the paper discs. Incubation of the plates was performed at 28°C. The inhibition zones were measured after overnight incubation. Twelve antibiotics were used in the assays, including neomycin, ampicillin, amikacin, cefalexin, colistin methanesulfonate, gentamicin, kanamycin, penicillin, polymyxin B (PMB), spectinomycin, streptomycin, and tetracycline. The concentration of each antibiotic used in the assays was the same as that used for treating HLB-affected citrus [39].

Scanning electron microscope (SEM) observation

The petioles of infected periwinkle and Las cultures were used as samples for SEM examination. The periwinkle samples were prepared as described by Sivager et al. [40]. 1.5 ml of a liquid culture was centrifuged at 10000 × *g* for 5 minutes. The pellet was washed with 1X phosphate-buffered saline (PBS) and then fixed in 500 μl

fixative solution (0.1 M PBS supplemented with 2% paraformaldehyde) for 2 hours. The fixed samples were centrifuged at 10 000 × *g* for 5 minutes and the pellets were resuspended in 0.1 M PBS. The samples were processed and sputter-coated with gold for SEM observation as described by Ha et al. [10].

Fluorescent *in situ* hybridization (FISH)

The FISH assay followed the protocol described by Parsley et. al with minor modifications [41]. Briefly, 2 ml bacterial suspensions were centrifuged at 15 000 rpm for 5 minutes. The samples were fixed by re-suspending the cell pellets in 1 ml fixative solution (4% formaldehyde in 1X phosphate buffered saline (PBS), pH 7.4) per tube and incubated at room temperature for 3 hours. The cell mixture was centrifuged at 15 000 rpm for 5 minutes, and the cell pellet was washed by re-suspending the pellet in 50% ethanol, incubated at room temperature for 5 minutes, and centrifuging at 15 000 rpm for 2 minutes. The washing was repeated with 80% and 95% ethanol, respectively. The cell pellet was then dried in a speed vacuum dryer for 10 minutes.

For the hybridization assay, fixed cells were re-suspended in 500 μl hybridization solution (20 mM Tris-Cl, pH 8.0; 0.9 M sodium chloride, 0.01% sodium dodecyl sulfate, 40% formamide) and incubated at 37°C for 30 minutes. A fluorescently labeled Las-specific oligonucleotide probe LSS [42] (LSS: 5'-Alexa-555-CCC ACC ATC TAG GTA AAA ACC TAA ACT TGA-3') was added to the prehybridization solution to a final concentration of 5 ng/μl. The hybridization mixture was incubated at 42°C for 3 hours in the dark and then centrifuged at 15 000 rpm for 5 minutes. The cell pellet was washed by re-suspending it in 40 μl of 0.1× SSC and incubating at 37°C for 15 minutes in the dark. The mixture was centrifuged at 15 000 rpm for 5 minutes. The washing was repeated twice. The cell pellet was re-suspended in 300 μl of 4,6-diamidino-2-phenylindole (DAPI) solution (300 nM of DAPI in 1X PBS) and incubated at room temperature for 5 minutes. The solution was centrifuged at 15 000 rpm for 5 minutes and the cell pellet was washed twice by re-suspending in 500 μl of PBS and centrifuging at 15 000 rpm for 5 minutes. The final pellet was re-suspended in 20 μl of 0.1 x SSC. The cell-suspension (12 μl) was loaded onto a microscope slide and 6 μl of SlowFade reagent was added. A coverslip was placed over the mixture, and the slide was dried using a slider dryer. The sample was viewed under a fluorescence microscope, and the excitation light emission was kept at the minimal level (low-photobleaching mode). The filters included the BZ-X filter GFP (OP-87763, Keyence), BZ-X filter Cy5 (OP-87766, Keyence), BZ-X filter TRITC (OP-87764, Keyence), Keyence Corp, of America, Tampa, FL 33607).

Reverse transcriptional quantity polymerase chain reaction (RT-qPCR)

RNA isolation from Las culture and psyllids were conducted using an RNA Clean & Concentrator kit (Zymo Research Corp) following the Manufacture's instruction.

First-strand cDNA was synthesized at 42°C from total RNA using M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The individual qPCR reactions contained 1 μl of cDNA, 0.05 μl of each specific primer (100 pmol/μl), 7.5 μl of 2X SYBR Green Reaction Mixture (Promega), and water to a final volume of 15 μl. The following qPCR program was used: 95°C for 3 minutes, followed by 40 cycles of 95°C for 30s, and 60°C for 30s. The primers used in the RT-qPCR were listed in Table-S1.

Analysis of antibiotic effects on Las growth in culture *in vitro*

Eleven antibiotics were used in the assays: ampicillin, amikacin, cefalexin, colistin methanesulfonate, gentamicin, kanamycin, penicillin, polymyxin B (PMB), spectinomycin, streptomycin, and tetracycline. The concentration of each antibiotic used in the assays was the same as that used for treating citrus in a previous study [39]. They were listed as follows: ampicillin (100 μg/ml), amikacin (100 μg/ml), celalexin (100 μg/ml), colistinmethane sulfonate (40 μg/ml), gentamycin (100 μg/ml), kanamycin (100 μg/ml), neomycin (100 μg/ml), penicillin (100 μg/ml), polymyxin B (300 μg/ml), −spectinomycin (100 μg/ml), streptomycin (100 μg/ml), tetracycline (100 μg/ml). A 10 ml culture was prepared using LG medium, and after measuring the Ct value of Las, the culture was aliquoted into a 96-well cell growth plate. Individual antibiotics were added and mixed with 100 μl of liquid culture in a well. Each treatment had three replicate wells. The plate was incubated at 28°C and shaken at 100 rpm for 5 days. The cultures from each well were harvested and centrifuged. The pellets were suspended in 15 μl water and heated at 100°C for 5 minutes. The heated cell suspension was centrifuged for 1 minute and 2 μl of supernatant was used to detect Las in a qPCR reaction with 16S primers and their TaqMan probe as published [37]. Similar assays were also performed with a larger volume (10 ml). The same set of eleven antibiotics mentioned above, as well as neomycin (Neom), were used to test the sensitivity of the helper *S. maltophilia* FLMAT-1on agar plates with pure cultures.

Metagenomic sequencing of Las co-culture bacterial community and whole genome sequencing of the helper bacterium, *Stenotrophomonas* sp.

Samples were collected from cultures with different inoculum sources and cultivated for different culture times for 16S rRNA gene and whole genome sequencing. The full-length (v1-v9) bacterial 16S rRNA gene was PCR amplified following the protocol of Wagner et al. [43]. PacBio CCS and total DNA sequencing were performed using a PacBio RS II (Pacific Biosciences, Menlo Park, United States) sequencer by BGI Group (Shenzhen, China). Taxonomic classification of the CCS reads was performed using QIIME2 v2022.8 [44] with the SILVA database (Release 138.1) [45]. The metagenome sequencing reads were classified using Kraken 2 [46].

DNA extraction, library construction, and long-read sequencing were performed on the *Stenotrophomonas* sp. FLMAT-1 culture using the PacBio RS II platform (Pacific Biosciences, Menlo Park, USA) by BGI genomics (Shenzhen, China). A total of 7.0 Gb of continuous long reads were obtained, featuring an average length of 10.8 kb. These reads served as the basis for *de novo* genome assembly, which was executed using MECAT2 [47]. Functional annotation of protein-encoding genes, tRNAs, and rRNAs was conducted using the DFAST v1.2.14 pipeline [48]. To identify closely related strains to FLMAT-1, a blastn search against the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) was carried out.

Infection of psyllid and citrus with cultivated Las

Two to three ml of Las culture (500 ml/tube) were centrifuged at 15 000 rpm/min for 5 minutes. The resulting pellets were resuspended in 25 μl water per tube and then the resuspensions were combined into one tube to serve as the inoculum for feeding psyllids. Psyllid infection was performed through topical feeding [24] on fourth or fifth instar nymphs. After feeding, the nymphs

were reared on citrus seedling and maintained in net cages in the lab. At least two to three weeks later, psyllids were collected from the citrus seedings in the net cage, and qPCR was used to detect Las in individual psyllids. The citrus seedlings after insecticide treatment were then moved and maintained in insect-proof greenhouses. After six to eight weeks, the first detection of Las in citrus seedlings was carried out, with second, third, or subsequent detections being performed according to the growth of citrus seedlings.

Statistical analysis

Statistical processing and presentation of the data generated by the project was carried out using Excel and R Statistical software. Prior to statistical analysis, preliminary data exploration was carried out to demonstrate that no further transformation was required to satisfy the assumptions of the proposed statistical comparison methods. All qPCR Ct results displaying no detected level of Las 16S rDNA were arbitrarily assigned the value of 40, indicating zero detection after 40 cycles (Las levels above Ct value = 36.3 are considered negative). For amount of Las titers comparison, Ct values are converted to copy number estimates based on the regression equation, Log CN = 11.5–0.3X (Ct). Boxplot and mean with standard error bars charted across multiple trails were employed to visualize the Las growth dynamics over time after inoculation. These graphical representations allowed us to further illustrate the trends and variations in Las growth. The effect of inoculation source, LG medium, and antibiotics on Las growth across different inoculation period were first analyzed by Analysis of Variance (ANOVA), then pairwise comparisons between groups were determined by subsequent Tukey's honestly significant difference (HSD) tests, with the level of significance set at 0.05. Student t-tests were also used to determine when changes in the number of Las cells were significantly different with p-values being reported.

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Author contributions

DZ, CA, and YD conceptualized this research. DZ, CA, WY, and WH investigated and validated the work. . DZ, CA, BW and WL provided formal analysis and data curation. DZ, CA, CP, DG and YD wrote and modified the manuscript. All authors involved in this study reviewed and edited the manuscript.

Data availability

The data that support the findings are available within the article and supplementary data.

Conflict of interest statement

The authors have no conflict of interest to declare.

Supplementary Data

Supplementary data is available at Horticulture Research online.

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