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# Characterization of a novel cryptic plasmid, pHLHK26, in *Laribacter hongkongensis*

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**SUMMARY**

We report the complete nucleotide sequence and characterization of a cryptic plasmid, pHLHK26, recovered from a strain of *Laribacter hongkongensis* isolated from a patient with community acquired gastroenteritis. pHLHK26 consists of 8700 bp, with G + C content 51.3%. The copy number (mean±SD) is 0.57±0.07 and it is stable after four passages (about 240 generations) in the absence of selection. There is a predicted origin of replication that consists of a DnaA box and five 22-bp direct repeats. pHLHK26 has four ORFs with two genes encoded in the sense direction and the other two in antisense direction. These four ORFs encode a putative plasmid partitioning protein of the ParA family, a putative protein that contains putative ADP-ribose 1"-phosphatase activity belonging to the Appr-1-p processing enzyme family, a putative recombinase (TniR) of the resolvase/invertase family, and a putative replication protein, respectively. We speculate that pHLHK26 is a theta, possibly Class A, replicative plasmid, as it contains an origin of replication with AT-rich region, a number of iterons and a DnaA box and a gene that encodes a replicative protein most homologous to those of other theta replicative plasmids and it shares eight of the nine positions of the consensus sequence TTAT(C/A)CA(C/A)A (TTTTCCACA in pHLHK26) in the DnaA boxes observed in other classical examples of Class A plasmids of this group.

*KEY WORDS: Laribacter hongkongensis*, plasmid

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## INTRODUCTION

*Laribacter hongkongensis*, a novel genus and species, was first discovered in Hong Kong in 2001 from the blood and empyema pus of a 54-year-old Chinese man with alcoholic cirrhosis and bacteremic empyema thoracis (Yuen *et al.*, 2001). Phenotypically, it is a facultative anaerobic, motile, non-sporulating, urease-positive, Gram-negative, S-shaped bacillus. Genotypically, by phylogenetic analysis using 16S rRNA gene sequences, *L. hongkongensis* belongs to the *Neisseriaceae* family of the β-subclass of *Proteobacteria*. In a recent multi-centered prospective study using cefoperazone MacConkey agar as the selective medium (Lau *et al.*, 2003), we confirmed that *L. hongkongensis* is associated with community-acquired gastroenteritis and traveler's diarrhea (Woo *et al.*, 2004). Furthermore, freshwater fish was also confirmed to be a reservoir of *L. hongkongensis* (Teng *et al.*, 2005, Woo *et al.*, 2004).

By comparing the pulsed-field gel electrophoresis patterns of fish and patient isolates, it was observed that most patient isolates were clustered together, suggesting that some clones could be more virulent than others (Teng *et al.*, 2005). The isolation of *L. hongkongensis* from patients who

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resided in or have recent travel histories to Asia, Europe, America, and Africa implied that the bacterium is likely to be of global importance (Woo *et al.*, 2003, Woo *et al.*, 2005a). Recently, a class C beta-lactamase gene of *L. hongkongenis*, responsible for its resistance to multiple beta-lactam antibiotics, was cloned and characterized (Lau *et al.*, 2005).

Characterization of plasmid vectors recovered from *L. hongkongensis* may contribute to the understanding of the mechanism of pathogenesis of *L. hongkongensis*. Recently, we reported the complete sequence of the first plasmid isolated from *L. hongkongensis* and the construction of a shuttle vector for *L. hongkongensis* (Woo *et al.*, 2005b). In this article, we report the characterization of another cryptic plasmid recovered from a strain of *L. hongkongensis* isolated from a patient with community acquired gastroenteritis.

## MATERIALS AND METHODS

### Bacterial strain

The *L. hongkongensis* strain HLHK26 used in this study was isolated from a patient with community-acquired gastroenteritis.

### Sequencing and in silico analysis of pHLHK26

pHLHK26, the 8700-bp plasmid of *L. hongkongensis* strain HLHK26, was digested with *Pst*I and the fragments were cloned into pBSKII(-). Both strands of the plasmid were sequenced twice with an ABI 3700 DNA analyzer according to the manufacturer's instructions (Applied Biosystems), using primers T7 and T3 and additional primers designed from the first and second rounds of the sequencing reactions.

The nucleotide and deduced amino acid sequences of the open reading frames of pHLHK26 were compared with sequences in the GenBank. Protein family analysis was performed using PFAM and InterProScan (Bateman *et al.*, 2004). Direct and inverted repeats were determined using dotmatcher (EMBOSS-GUI). Phylogenetic tree construction was performed by using the neighbor-joining method with GrowTree software (Genetics Computer Group, Madison, WI).

### Determination of copy number of pHLHK26

The copy number of pHLHK26 was determined according to a published protocol (Smith and Bidochka, 1998). Overnight culture of HLHK26 was inoculated into brain heart infusion (BHI) broth. When the culture reached a turbidity of 0.6-1.0 at $OD_{600}$, 1 ml of the culture was used for plasmid DNA extraction using the High Pure Plasmid Isolation kit (Roche Applied Science), and the number of bacteria was determined by back titration. The concentration of plasmid DNA was calculated by measuring the absorbance of the plasmid DNA solution at 260 nm. A plasmid of known copy number (pBR322 in *E. coli* DH5α) was used as the control. The experiment was performed three times and the copy number of pHLHK19 was calculated using the following formula:

$$\frac{\text{No. of plasmids}}{\text{Bacterium}} = \frac{6.02\times10^{23} \text{ plasmids}}{\text{mass of 1 plasmid}} \times \frac{\text{mass of plasmid DNA in 1 ml bacterial culture}}{\text{No. of bacteria in 1 ml of bacterial culture}}$$

### Segregational plasmid stability studies

The plasmid stability of pHLHK26 was determined according to a published protocol (Inui *et al.*, 2000). Single colony of HLHK26 containing pHLHK26 was inoculated into BHI broth. Cells in the late exponential growth phase (12 h after inoculation) were diluted 1000-fold and the procedure was repeated four times. After these subcultures, bacterial cells were plated on BHI agar plates. Ten colonies were picked and the presence of pHLHK26 was determined by plasmid DNA extraction.

### Accession numbers

The nucleotide sequence of pHLHK26 has been lodged within the GenBank sequence database under accession no. DQ341277.

## RESULTS AND DISCUSSION

### Sequencing and characterization of pHLHK26

The plasmid pHLHK26 extracted from *L. hongkongensis* HLHK26 consists of 8700 bp. The

TABLE 1 - *Open reading frames on the L. hongkongensis plasmid pHLHK26 having significant database matches*

| *ORFs* | *Characteristics of ORFs* | | | | *Best match to known sequences in public databases* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Start-end (base)* | *No. of bases* | *Number of amino acids* | *Frame* | *Organism* | *Description* | *GenBank accession no.* | *E-value* |
| ORF 1 | 1601-2251 | 651 | 216 | +2 | *Sodalis glossinidius* | Partition protein A | YP_257082 | $4e^{-80}$ |
| ORF 2 | 3311-4387 | 1077 | 358 | -3 | *Gloeobacter violaceus* | Appr-1-p processing enzyme family | BAC91312 | $8e^{-98}$ |
| ORF 3 | 5052-5675 | 624 | 207 | -2 | *Sinorhizobium meliloti* | TniR protein (Plasmid pSB102) | CAC79209 | $1e^{-104}$ |
| ORF 4 | 6257-7180 | 924 | 307 | +2 | *Laribacter hongkongensis* HLHK8 | Replication protein | AAZ91682 | $1e^{-122}$ |

overall G + C content is 51.3%. There is a predicted origin of replication that consists of a DnaA box and five 22-bp direct repeats. The copy number (mean±SD) of pHLHK8 is 0.57±0.07. The plasmid is stable after four passages (about 240 generations) in the absence of selection.

**Open reading frames of pHLHK26**

The plasmid has four ORFs with two genes encoded in the sense direction and the other two in antisense direction (Table 1 and Figure 1). ORF1 (bases 1601-2251) encodes a putative plasmid partitioning protein of the ParA family

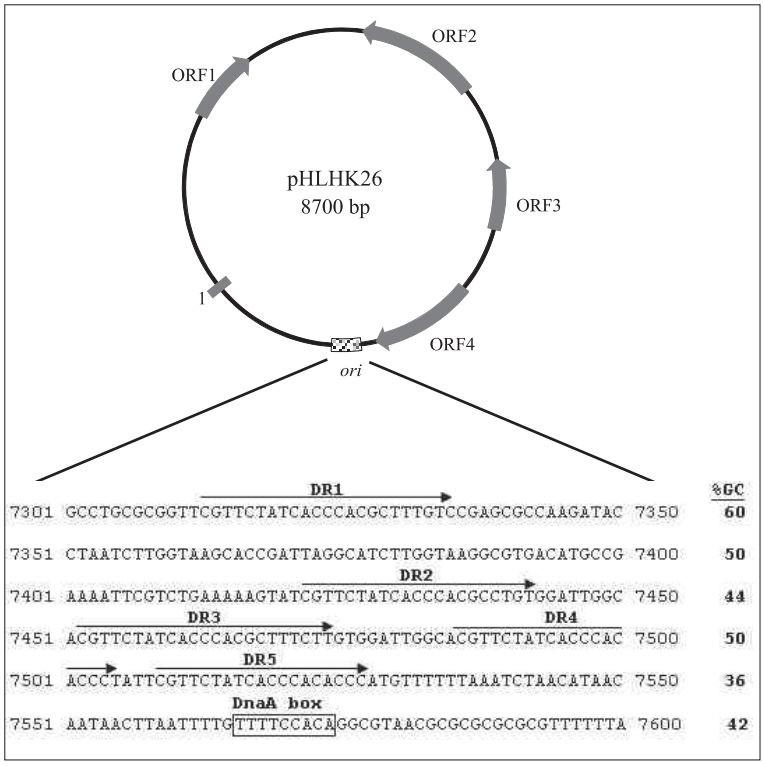



FIGURE 1 - *Circular map of pHLHK26. The %GC content of each line in the putative origin of replication is shown at the right. The arrows indicate the five 22-bp imperfect direct repeats (DR) of the putative origin of replication.*

(PFAM accession no. PF01656) with 216 amino acids. Phylogenetic analysis showed that it is most closely related (70% amino acid identity) to the partition protein of a recently published plasmid in *Sodalis glossinidius*, a g-proteobacteria and symbiont of tsetse flies (Figure 2) (Darby *et al.*, 2005). Multiple alignments with the amino acid sequences of closely related partition proteins revealed four conserved motifs that are characteristically present in partition proteins of the ParA family of ATPases (Figure 3). Two of the four motifs (motif I and III) showed high homology to an ATP-binding motif, whereas the other two motifs (motif II and IV) are thought to be involved in protein-protein interactions or interaction with the cell membrane. This partition pro-

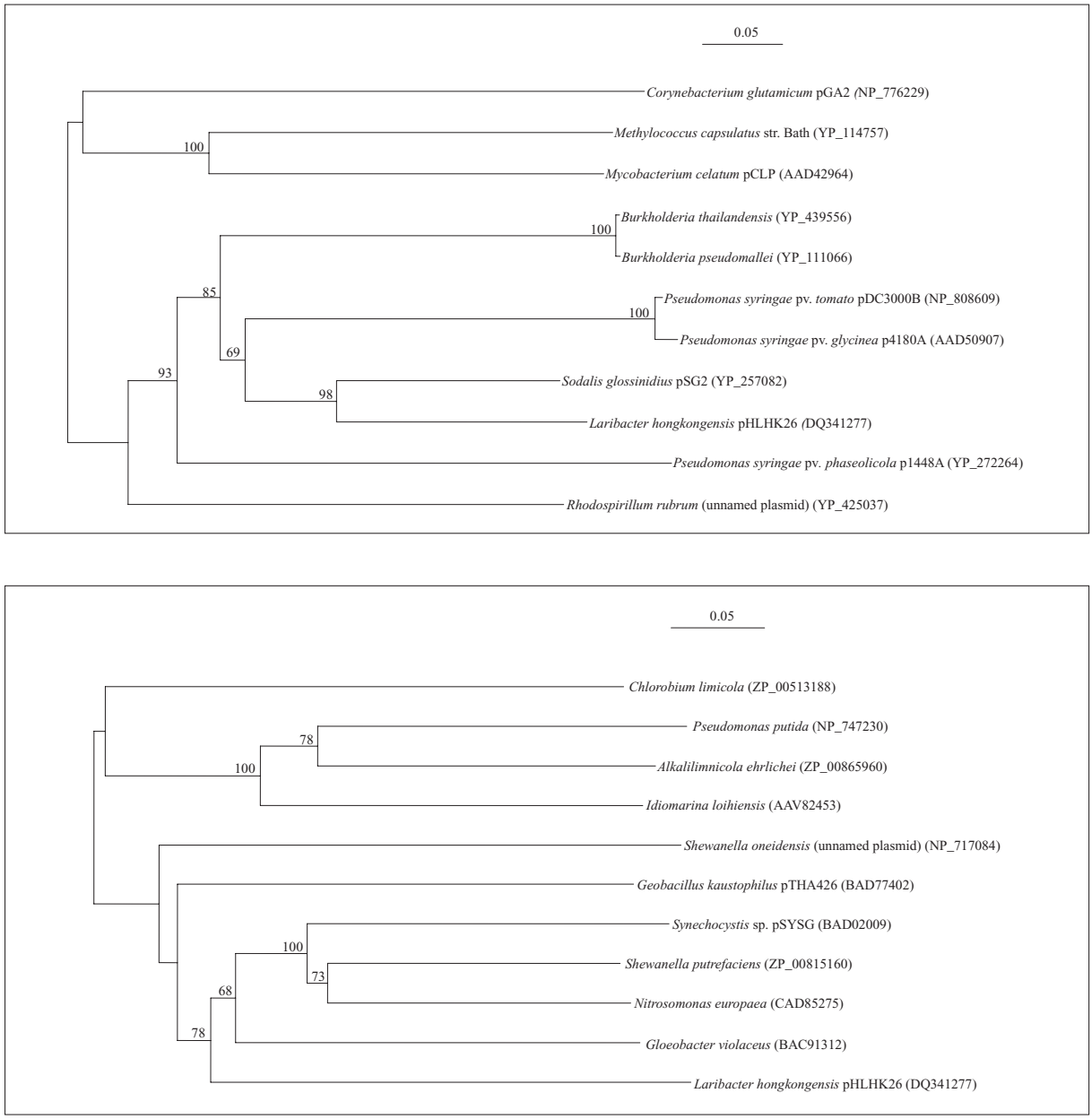
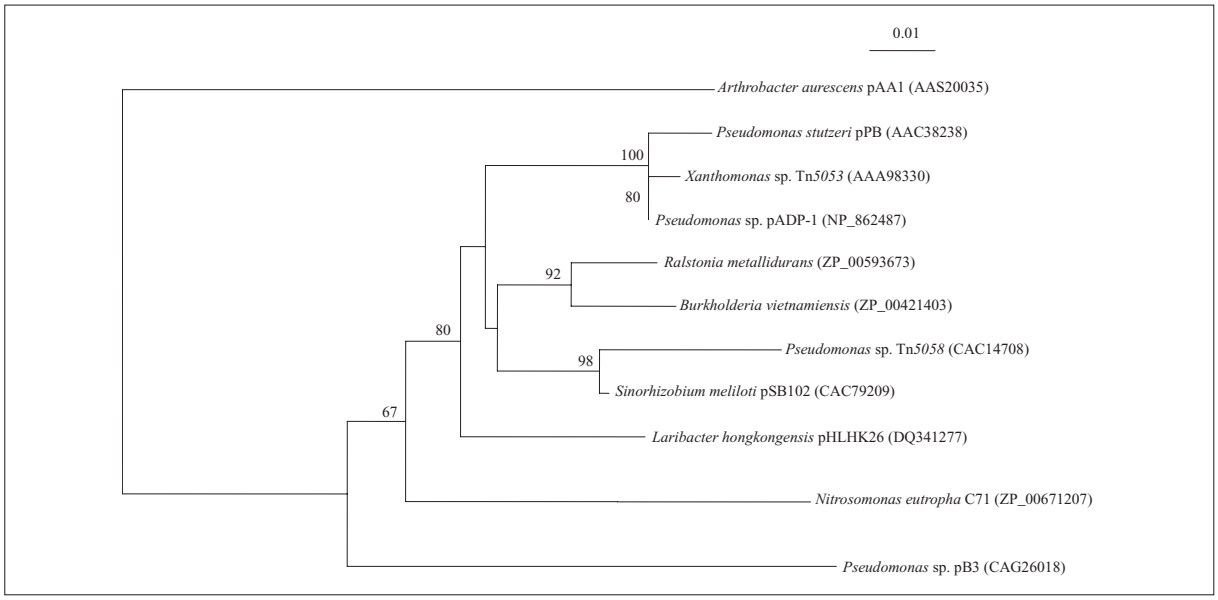
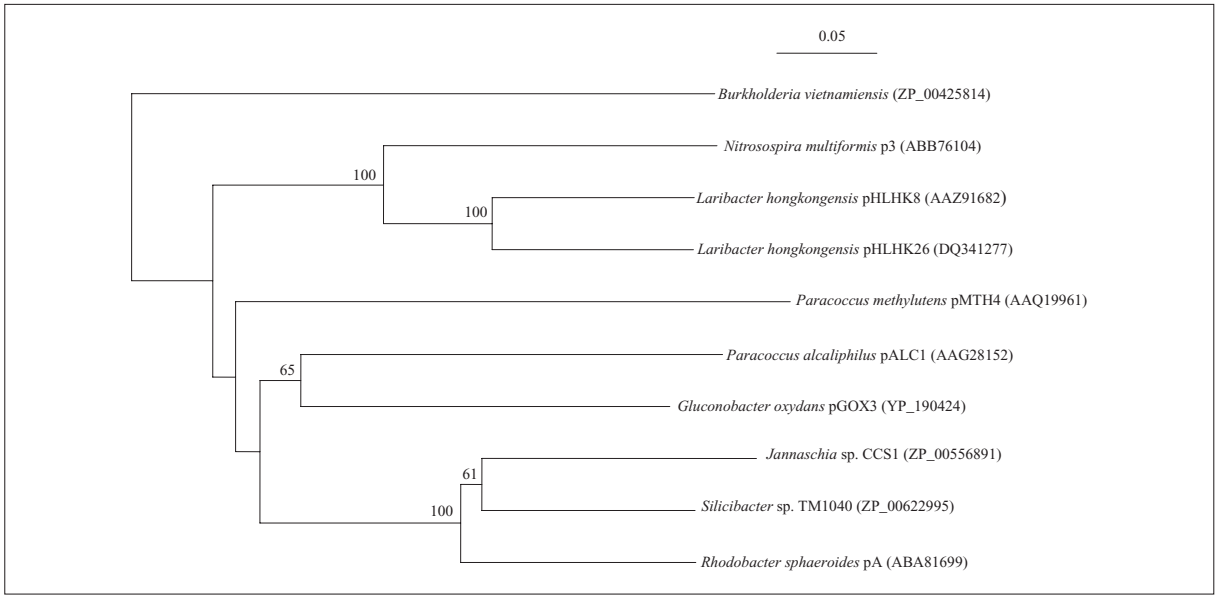



FIGURE 2 - *Phylogenetic analysis of the putative plasmid partitioning protein, ADP-ribose 1"-phosphatase, recombinase and replication protein encoded by ORF1, 2, 3 and 4 of pHLHK26. The trees were constructed by neighbor joining method using Jukes-Cantor correction and bootstrap values calculated from 1000 trees. 213, 331, 204*

tein is putatively involved in active partitioning of the plasmid.

ORF2 (bases 3311-4387) encodes a putative protein with 358 amino acids containing a domain that contains putative ADP-ribose 1"-phosphatase (ADRP) activity belonging to the Appr-1-p processing enzyme family (PFAM accession no. PF01661). Phylogenetic analysis showed that it is most closely related (50-51% amino acid identity) to similar proteins in *Gloeobacter violaceus*, a cyanobacterium isolated from calcareous rock in Switzerland; *Shewanella putrefaciens*; and *Nitrosomonas europaea* (Figure 2). This domain is found in proteins present in all three domains of life, Bacteria, Archeae and Eukarya and viruses. This ubiquity suggests that




*and 237 amino acid positions in the four putative proteins respectively were included in the analysis. The scale bar indicates the estimated number of substitutions per 1 or 5 amino acids as indicated. Names and accession numbers are given as cited in the GenBank database.*

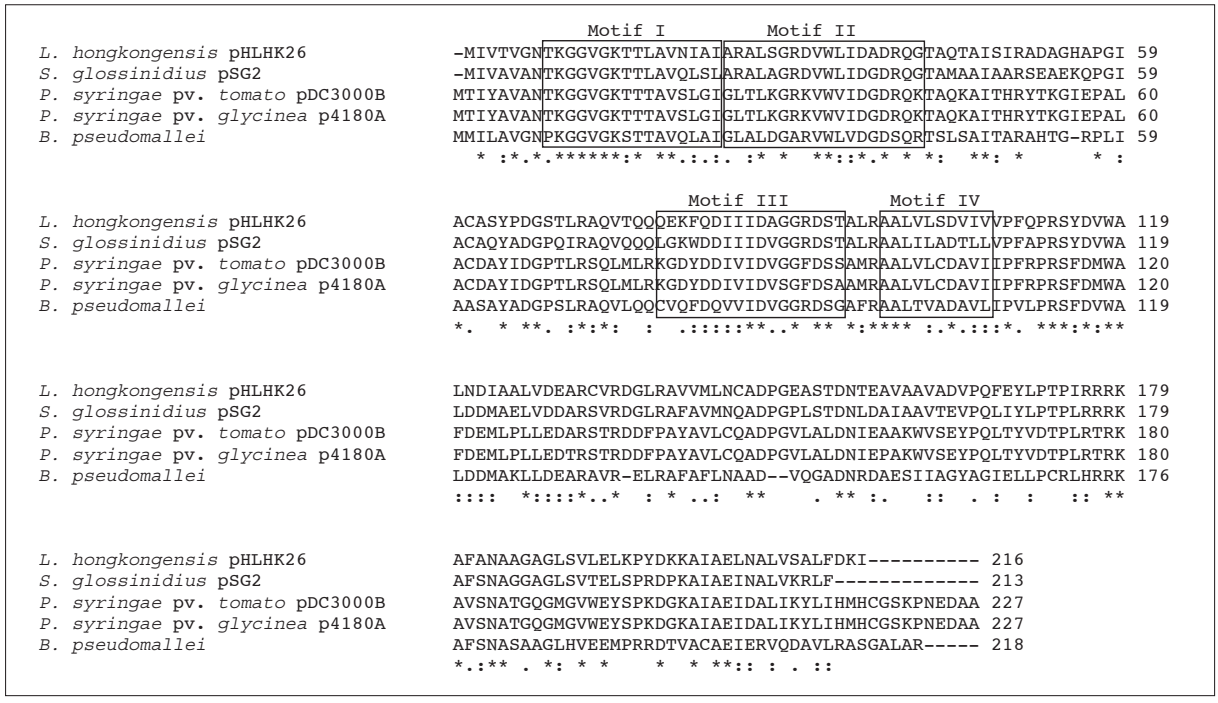



FIGURE 3 - *Comparison of the deduced amino acid sequence of* parA *of pHLHK26 with a number of homologous proteins. (1) Putative plasmid partitioning protein of* L. hongkongensis*, accession no. DQ341277. (2) ParA of* Sodalis glossinidius*, accession no. YP_257082. (3) Hypothetical protein of* Pseudomonas syringae *pv.* tomato *NP_808609. (4) Putative partition protein of* Pseudomonas syringae *pv.* glycinea*, accession no. AAD50907. (5) Putative partition protein of* Burkholderia pseudomallei*, accession no. YP_111066. The four conserved motifs are framed and the consensus amino acids are shaded in gray.*

it is involved in an important cellular process. In other microorganisms, such as *Saccharomyces cerevisiae* and other eukaryotes, ADRP and its functionally related enzyme cyclic nucleotide phosphodiesterase (CPDase) were important for tRNA processing (Nasr and Filipowicz, 2000). ADP-ribose 1",2"-cyclic phosphate (Appr>p) is produced as a result of tRNA splicing. Appr>p is converted to ADP-ribose 1"-phosphate (Appr-1"p) by CPDase. Appr-1"p is then further processed by ADRP.

ORF3 (bases 5052-5675) encodes a putative recombinase (TniR) of the resolvase/invertase family (PFAM accession no. PF00239) with 207 amino acids. Phylogenetic analysis showed that it is highly related (95% amino acid identity) to the recombinase in a mercury resistance plasmid found in *Sinorhizobium meliloti* (formerly *Rhizobium meliloti)*, an a-proteobacteria with a multipartite genome found in soil and forms nitrogen-fixing nodules on the roots of certain genera of leguminous plants, including *Medicago, Melilotus* and *Trigonella* (Schneiker *et al.*, 2001). Multiple alignments with the amino acid sequences of closely related resolvases revealed highly conserved motifs and amino acid residues that are functionally important as determined by X-ray crystallography (Figure 4) (Yang and Steitz, 1995). In the N-terminal catalytic domain, a mixed b sheet is surrounded by a helices. In the C-terminal DNA-binding domain, the last residues form a three-helix bundle (aF, aG and aH) surrounding a compact hydrophobic core, where aG and aH form a helix-turn-helix DNA major groove-binding motif. This protein is putatively involved in replicative transposition. It has been shown in other bacteria, such as *S. meliloti* and *Xanthomonas* species, that the presence of additional genes, including *tniA*, *tniB* and *tniQ*, were essential for the transposition of the corresponding transposons, Tn*5718* and Tn*5053* (Kholodii *et al.*, 1993, Schneiker *et al.*, 2001). Therefore, we speculate that there may be chromosomal genes in the *L. hongkongensis* genome

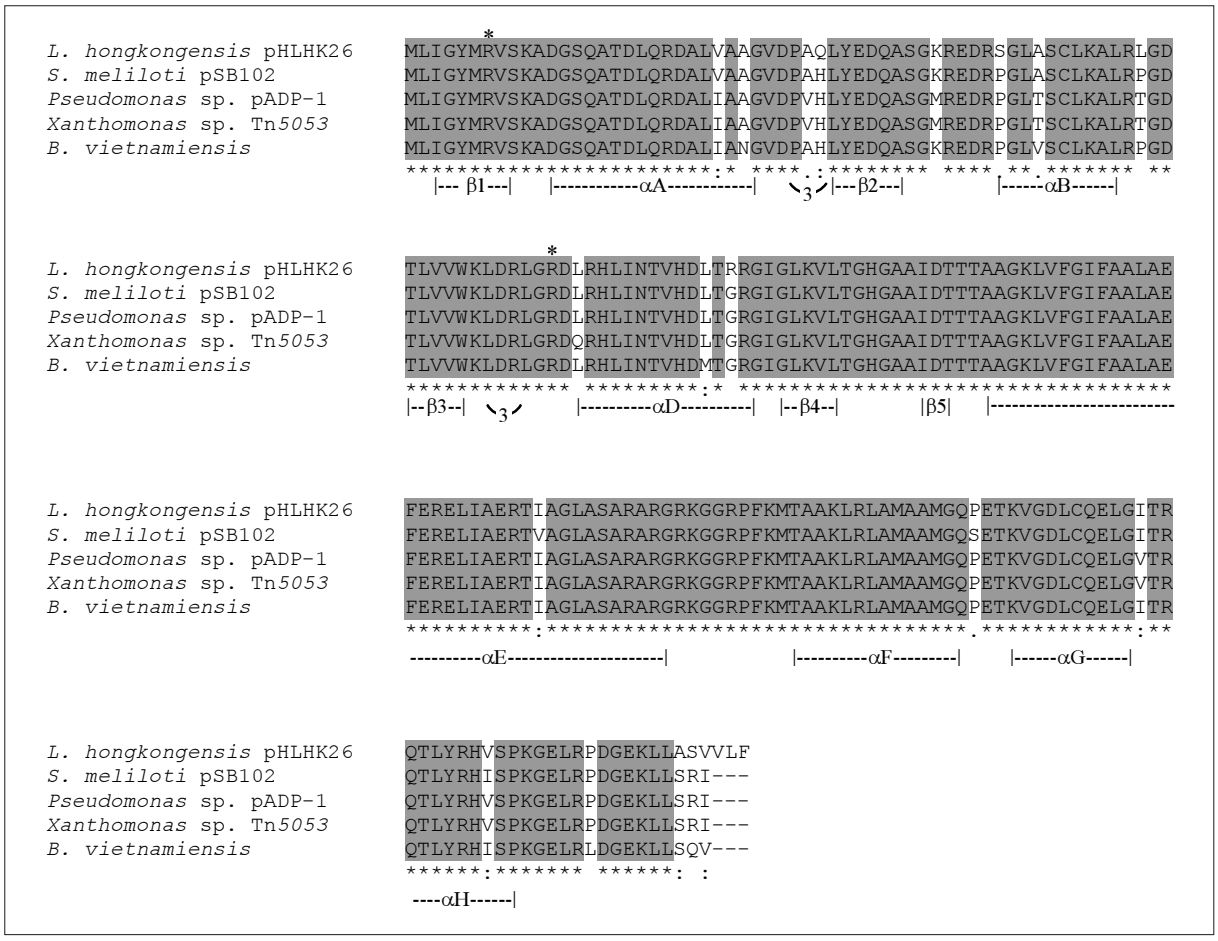



FIGURE 4 - *Comparison of the deduced amino acid sequence of TniR of pHLHK26 with a number of homologous proteins. (1) Putative recombinase of* L. hongkongensis*, accession no. DQ341277. (2) Putative resolvase of* Sinorhizobium meliloti*, accession no. CAC79209. (3) Putative resolvase of* Pseudomonas *sp. ADP, accession no. NP_862487. (4) Resolvase of* Xanthomonas *sp. W17, accession no. AAA98330. (5) Resolvase of* Burkholderia vietnamiensis*, accession no. ZP_00421403. The consensus amino acids are shaded in gray. The putative secondary structures are marked beneath the sequence alignment (*α*, α-helix;* β*, β-strand; 3, 3*$_{10}$ *helix). The two arginine residues shown to be essential for recombination activity by mutagenesis experiments are marked with asterisks.*

that can complement the functions of the genes missing from the plasmid. Moreover, the recombinase may be involved in chromosomal integration of the plasmid itself. Further experiments will determine whether the plasmid can be integrated into the chromosome.

ORF4 (bases 6257-7180) encodes a putative replication protein (PFAM accession no. PF07042) with 307 amino acids. Phylogenetic analysis showed that it is most closely related (77% amino acid identity) to the putative replication protein found in the first plasmid we recently described in another strain of *L. hongkongensis* (Figure 2) (Woo *et al.*, 2005b).

## Putative replicative mechanism of pHLHK26

Two modes of plasmid replicative mechanisms, rolling circle and the theta, are observed in bacterial plasmids. Theta replicative plasmids were classified into six classes, A, B, C, D, E and F. Class A plasmids characteristically consists of an origin of replication with AT-rich region, a number of iterons and a DnaA box and a gene that encodes a replicative protein. We speculate that the present plasmid is a theta, possibly Class A, replicative plasmid, as it contains these characteristics and shares eight of the nine positions of the consensus sequence, TTAT(C/A)CA(C/A)A

(TTTTCCACA in pHLHK26), in the DnaA boxes observed in other classical examples of Class A plasmids of this group, such as plasmid F, pSC101, P1, R1 and R6K (Qin and Hartung, 2001). Furthermore, the putative replicative protein of pHLHK26 is most homologous to those of other theta replicative plasmids, such as pEMT8 and other plasmids in *Paracoccus alcaliphilus*, *Paracoccus methylutens* and *Erwinia stewartii* (Bartosik *et al.*, 2001, Gstalder *et al.*, 2003, Szymanik *et al.*, 2004).

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