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Research article

Genome sequence of *Acidomonas methanolica* CPK24 isolated from sugarcane with plant growth-promoting traits

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Abstract

Importance of the work: Endophytic bacteria have been recognized as powerful plant growth promoters. The beneficial strains isolated from sugarcane, which produce phytohormones, raise nutrient bioavailability and act as biocontrol agents, might encourage the production of important agricultural crops.

Objectives: To evaluate the plant growth-promoting (PGP) activities of an endophytic bacterium isolated from the root of sugarcane and to characterize the genes related to its PGP mechanisms.

Materials & Methods: The strain CPK24 was characterized using combined data from phenotypic tests, phylogenetic analysis based on 16S rRNA gene and genome features. The PGP activities of the bacterial strain were determined. Genome analysis was used to examine the genes responsible for PGP traits.

Results: The endophytic acetic acid bacterium *Acidomonas methanolica* strain CPK24 (=TBRC 14896) was isolated from the root of sugarcane in Nakhon Ratchasima province, Thailand. The strain CPK24 was able to produce indole-3-acetic acid (49.75 µg/mL), solubilize zinc oxide and zinc phosphate with solubilization index values of 3.47 and 6.44, respectively. In addition, this strain inhibited the growth of the fungal pathogen, *Fusarium moniliforme* AIT01 (19.65%). The draft genome consisted of 3,708,126 bp with an average GC content of 64.8%. Genome annotations predicted 3,405 proteins coding genes, including 46 tRNAs and 3 rRNAs. The strain contained the PGP genes involving phytohormone production and mineral solubilization. In addition, some genes were identified that responded to produce volatile organic compounds, acetoin and 2,3-butanediol that exhibited antagonistic activity.

Main finding: The strain CPK24 had the potential to stimulate plant growth-promoting activities. The genome information of *A. methanolica* CPK24 should provide a valuable overview of the genetics of its plant growth-promoting functions.

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Introduction

A facultative methylotrophic acetic acid bacterium, *Acidomonas methanolica* (formerly *Acetobacter methanolicus*) is the only single species in the genus *Acidomonas*, with the type strain, *A. methanolica* MB58^T being isolated from a septic methanol-processing mixture in Germany (Uhlig et al., 1986). Later strains were found from a patient with chronic granulomatous disease exacerbated with cervical lymphadenopathy, indicating the emerging pathogenicity of these bacteria (Chase et al., 2012; Falcone et al., 2016). There is still no report on the strains isolated from plant samples. Several genera and species of the family *Acetobacteraceae*, such as *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae*, *Glucoacetobacter azotocaptans*, *Swaminathania salitolerans*, *Acetobacter peroxydans*, *Acetobacter nitrogenifigens* and *Acetobacter sacchari*, were observed to be associated with different plant species colonizing within tissues and roots (Pedraza, 2016; Vu et al., 2019). Various species have been reported for the ability to promote plant growth with direct and indirect mechanisms, such as nitrogen fixation, phytohormone production, mineral solubilization and antagonistic activity against phytopathogens (Reis and Teixeira, 2015; Pedraza, 2016). The current isolated an endophytic bacterium, strain CPK24 from the root of sugarcane collected in Nakhon Ratchasima province, Thailand. The draft genome sequence of this strain was investigated to obtain information providing insights into its identification and genes associated with its plant growth-promoting (PGP) activities.

Materials and Methods

Isolation of bacterial endophyte

The roots of sugarcane were cut into small pieces and surface sterilized in 70% ethanol for 3 min, 2.5% fresh sodium hypochlorite solution for 5 min and 70% ethanol for 30 s, successively, and finally rinsed five times with sterile distilled water (Tam and Diep, 2014). The surface-sterilized roots were mashed with a sterile mortar and pestle; then, they were inoculated in LGI broth (weight per volume, w/v; 10% sucrose, 0.06% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4$, 0.002% $CaCl_2$, 0.001% $FeCl_3$ and 0.0002% Na_2MoO_4). After incubation at 30 °C for 4–6 d, the culture was streaked and purified on LGI agar (Vu et al., 2013). To validate the disinfection process, aliquots of the sterile water used in the final rinse were inoculated on tryptic soy agar and incubated at 30 °C for 3 d, after which the plates were examined for any microbial growth.

Screening for plant growth promoting activities

The isolate was determined for PGP traits, such as indole acetic acid biosynthesis, ammonia and siderophore production and zinc and phosphate solubilization, following the standard protocols of Glickmann and Dessauk (1995), Cappuccino and Sherman (2001), Schwyn and Neilands (1987), Pande et al. (2017) and Gandhi and Muralidharan (2016), respectively.

Indole acetic acid production was determined using the colorimetric method in the presence and absence of tryptophan as substrate. The supernatant reacting with Salkowski's reagent was quantified at 530 nm spectrophotometrically compared to a standard indole-3-acetic acid solution. The Pikovskaya medium supplemented with tri-calcium phosphate and Tris-mineral salts medium supplemented with insoluble zinc compounds were used to determine the potential of phosphate and zinc solubilization. The strain CPK24 was initially grown in tryptic soy broth and incubated at 30 °C for 24 h. Then the cultivated bacterial suspension was dropped on a plate and incubated at 30 °C for 7 d, with a clear, hollow zone around the bacterial colony indicating solubilizing capacity. The solubilization index (SI) was defined as: Colony diameter / (Colony diameter + Halo zone diameter). Siderophore production was tested on the chrome azurol S medium and the development of an orange halo zone confirmed siderophore production. The ammonia production was examined by cultivating the bacterium in peptone water. Development of a yellow-to-brown color in the supernatant reacting with Nessler's reagent was recorded as a positive test for ammonia production.

The strain was tested for its antagonistic effect against *Fusarium moniliforme* AIT01. A 5 mm mycelial mat of the phytopathogen, *F. moniliforme* AIT01 was placed on one side of potato dextrose agar; then, an endophytic bacterial strain was streaked on the other side of the medium. After incubation at 30 °C for 7–14 d, the %inhibition was calculated according to the formula (Saechow et al., 2018): Percentage inhibition of radial growth = R1 - R2 / R1 × 100, where R1 and R2 are the radial lengths of *F. moniliforme* in the control and antagonist-tested plates, respectively.

Strain identification

Phenotypic characteristics

Colony morphology, cultural, physiological and biochemical tests were performed, consisting of Gram staining, catalase and oxidase activity, methyl red (MR)-Voges-Proskauer (VP) test, indole-3-acetic acid production, utilization of citrate, nitrate reduction, acetate and lactate oxidation, lysine and ornithine decarboxylation and hydrolysis of L-arginine and gelatin (Asai et al., 1964; Barrow and Feltham, 1993). Acid production from carbohydrates was done as described by Tanasupawat et al. (1998).

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene was amplified with two universal primers, 27F and 1525R (Seearunruangchai et al., 2004). The polymerase chain reaction (PCR) product was purified using a GeneJet PCR purification Kit (ThermoFisher; USA) and sequenced using primers 27F, 585F and 1525R. The 16 rRNA gene sequence of strain CPK24 was available under the accession number MT994883 and was used for comparison with those of all type strains in the EzBioCloud database (Yoon et al., 2017). Phylogenetic trees of the 16S rRNA gene sequences were constructed using neighbor-joining methods (Saitou and Nei, 1987) with the MEGA X program (Kumar et al., 2018).

DNA extraction, library construction and genome sequencing

Genomic DNA was extracted from strain CPK24 cultivated aerobically in YPGD broth (weight per volume, w/v; 0.5% yeast extract, 0.5% peptone, 0.5% glycerol, 0.5% glucose) at 30 °C using a GF-1 Bacterial DNA extraction kit (Vivantis; Malaysia). The gDNA (100 ng) was subjected to DNA sequencing library preparation using QIAGEN FX kit (Qiagen; USA). Briefly, gDNA was fragmented using enzymatic reaction and cleaned with magnetic beads. An adaptor index was ligated to the fragmented DNA. The quality and quantity of the indexed libraries were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies; CA, USA) and a Denovix fluorometer (Denovix; DE, USA) and pooled in equimolar quantity. Cluster generation and paired end 2×150 nucleotide read sequencing were performed on an Illumina HiSeq X ten sequencer (Illumina; CA, USA). The DNA fragment library was constructed according to the manufacturer's instructions and sequenced on the HiSeq X ten sequencer (Illumina; CA, USA).

Genome assembly and annotation

High-quality bases were used to assemble the genome by the Unicycler v0.4.4 software (Wick et al., 2017). The whole genome sequence of strain CPK24 was deposited in GenBank under the WGS accession number JAFJZX000000000. A phylogenetic tree of strain CPK24 and its closely related type strains was constructed using the Type (Strain) Genome Server (TYGS) web server (Meier-Kolthoff and Göker, 2019). The genome annotation and metabolic pathways identification was carried out by RASTtk annotation service in PATRIC (Brettin et al., 2015) and functions of the gene products were compared with similar proteins detected by BLAST (Altschul et al., 1997). The web platform OrthoVenn2 was used to identify orthologous gene clusters (Xu et al., 2019).

Genome based taxonomy and phylogenomics

For the identification of *Acidomonas* strains at the species level, digital DNA-DNA hybridization (dDDH) was performed using the GGDC2.1 software (Meier-Kolthoff et al., 2013). The degree of pairwise genome-based relatedness was estimated based on average nucleotide identity (ANI) calculation (Edgar, 2010; Yoon et al., 2017) and average amino acid identity (AAI) values calculated by the orthologous ANI algorithm (Rodriguez-R and Konstantinidis, 2014). The genome sequence data were uploaded to TYGS, a free bioinformatics platform available at https://tygs.dsmz.de, for a whole genome-based taxonomic analysis (Meier-Kolthoff and Göker, 2019).

Results and Discussion

Isolation and identification of strain

A strain CPK24 isolated from the root of sugarcane in Nakhon Ratchasima province, Thailand, was a Gram-negative rod. The colonies cultivated on GYPG agar, were beige to pink, smooth, raised and entire. Based on the physiological and biochemical analysis (Table 1), CPK24 oxidized acetate and lactate. Catalase and oxidase were positive. Urease was negative. Hydrolysis of gelatin and aesculin were not observed. It produced acid from lactose and maltose. Like the type strain *A. methanolica* MB58^T, CPK24 utilized methanol as the sole carbon source for energy and growth (Urakami et al., 1989). Nitrate reduction, the Voges-Proskauer test and indole production were negative. CPK24 grew weakly in 3% (w/v) NaCl. β-hemolysis was not observed in CPK24.

Based on the 16S rRNA gene sequence and phylogenetic analysis (Fig. 1), the strain CPK24 was closely related to *Acidomonas methanolica* MB58^T with 99.4% sequence similarity. Regarding the sequence of 16S rRNA gene extracted from the genomic data, strain CPK24 exhibited 100% similarity with *A. methanolica* MB58^T. Analysis based on genome-based taxonomy, showed that CPK24 had the highest similarity with *A. methanolica* MB58^T based on ANIb (95.29%) and ANIm (96.48%) values (Table 2); this level was above the ANI cut-off value (95–96%) used to delineate bacterial species (Richter and Rosselló-Móra, 2009). Strain CPK24 had a dDDH value of 70.2% (formula d_6) to *A. methanolica* MB58^T. The AAI value between strains MB58^T and CPK24 was 95.1% that was above the AAI cut-off value (85–90%) used to delineate bacterial species. In a phylogenetic tree analysis based on the whole genome, strain CPK24 shared the same node and was closest with *A. methanolica* MB58^T (Fig. 2).

Table 1 Phenotypic characteristics of strain CPK24 and type strain

Characteristic	CPK24	MB58^T [a]
Growth at 37 °C	+	+
at 42 °C	w	-
Growth in 3% NaCl	w	-
Catalase	+	+
Oxidase	+	+
Urease	-	+
Voges-Proskauer test	-	-
Indole test	-	-
Lactate oxidation	+	-[b]
Acetate oxidation	+	+[b]
Nitrate reduction	-	-
Aesculin hydrolysis	-	-
Gelatin hydrolysis	-	-
Acid production from		
D-Mannitol	-	-
D-Maltose	+	-
D-Sorbitol	-	-
Glycerol	-	-
Ethanol	-	+
Sucrose	-	-
Lactose	+	-

+ = positive; w = weakly positive; - = negative.
Data from [a]Urakami et al. (1989), and [b]Vu et al. (2013)

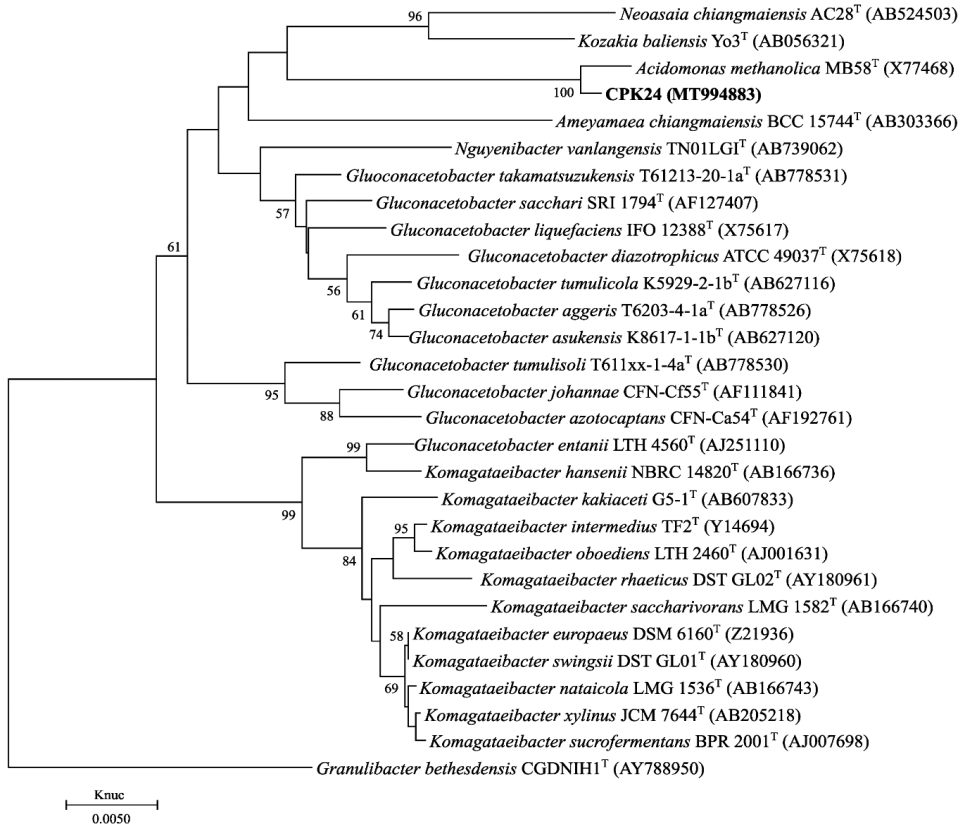

Fig. 1 Neighbor joining tree based on alignment of nucleotide sequences of 16S rRNA gene of strain CPK24 and related species, indicating bootstrap values greater than 50% and scale bar represents number of substitutions per site

Table 2 ANIb (upper triangle in bold) and ANIm (lower triangle) values among draft genomes of strain CPK24 and all closely related type strains

Strain	1	2	3	4	5
1		**100**	**95.29**	**71.21**	**73.89**	**73.32**
	100	96.48	87.34	84.54	85.66	
2		**94.9**	**100**	**71.5**	**73.94**	**73.23**
	96.49	100	87.92	84.37	84.42	
3		**70.32**	**70.36**	**100**	**69.82**	**73.47**
	87.34	87.92	100	86.19	84.01	
4		**73.7**	**73.71**	**69.75**	**100**	**72.04**
	84.54	84.37	86.19	100	84.23	
5		**72.62**	**72.07**	**73.12**	**71.26**	**100**
	85.69	84.4	84.01	84.19	100	

Strains: 1 = CPK24; 2 = *Acidomonas methanolica* MB58^{T}; 3 = *Komagataeibacter hansenii* ATCC 23769^{T}; 4 = *Neoasaia chiangmaiensis* NBRC 101099^{T}; 5 = *Nguyenibacter vanlangensis* LMG 31431^{T}

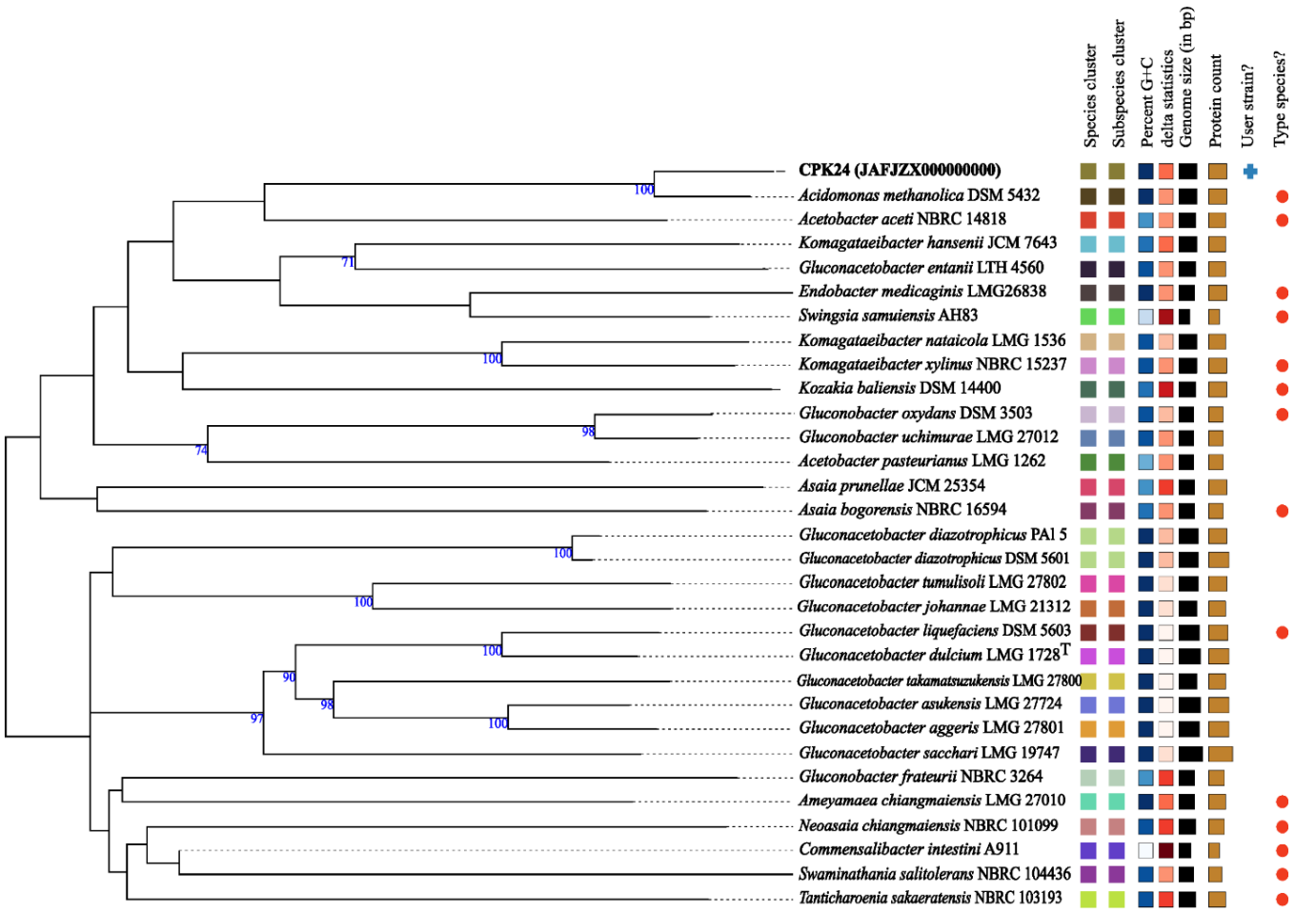

Fig. 2 Genomic blast distance phylogeny (GBDP) of strain CPK24 and related genera based on whole genome, where tree is inferred with FastME 2.1.6.1 from GBDP distances calculated from either genome sequences (Lefort et al., 2015), the branch lengths are scaled in terms of GBDP distance formula d_5, numbers above branches are GBDP pseudo-bootstrap support values from 100 replications, with an average branch support of 52.5% and tree was rooted at the mid-point (Farris, 1972)

Plant growth promoting activities

The strain CPK24 possessed some PGP activities. It produced indole-3-acetic acid (IAA, 49.75 µg/mL) in GYPG medium supplemented with tryptophan. This strain also produced IAA (13.25 µg/mL) in the absence of tryptophan. In addition, the strain solubilized insoluble ZnO and $Zn_3(PO_4)_2$ medium with SI values of 3.47 and 6.44, respectively, but not $ZnCO_3$ and $Ca_3(PO_4)_2$. Siderophore and ammonia production were not observed in this strain. The antagonistic effect was tested *in vitro* against *Fusarium moniliforme* based on dual plate techniques. The strain CPK24 inhibited growth of the fungal pathogen (19.65%). Three genera in the family *Acetobacteraceae* (*Gluconacetobacter, Acetobacter* and *Swaminathania*) consist of nitrogen-fixing species (Pedraza, 2016). *Gluconacetobacter diazotrophicus* has been reported to possess plant growth promoting activities such as biological nitrogen fixation, mineral nutrient solubilization and phytohormone production, mainly auxins (Eskin et al., 2014; Rodrigues et al., 2016).

Genomic features

The assembled genome of CPK24 comprised 113 contigs with a total of 3,708,126 bp and 64.8% of GC content (Table 3). The *A. methanolica* CPK24 genome contained 3,469 total genes, 3,405 protein-coding genes, 46 copies of tRNA genes and 3 copies of rRNA gene. Comparative analysis of the presence and absence of genes between *A. methanolica* MB58^T and CPK24 revealed that the core shared by the two strains accounted for 2,638 core genome orthologs. There were 60 and 42 unique orthogroups in the strains MB58^T and CPK24, respectively. The orthologous groups specific to CPK24 were related to siderophore transport, lipid and organic metabolic process, biological regulation and cellular process. The CPK24 genome contains genes that code for alcohol dehydrogenase, aldehyde dehydrogenase, glucose dehydrogenase and methanol dehydrogenase that have been reported in the type strain MB58^T (Higashiura et al., 2014). These enzymes belong to the group of quinoproteins that consists of the cofactor PQQ, except aldehyde dehydrogenase.

Genes involved in plant growth-promoting activities

Functional genes related to PGP activities were identified in this strain (Table 4). Various genes are putatively involved in the production of IAA, such as the tryptophan biosynthesis gene cluster (*trp*ABCDEFGS). This gene cluster is related to tryptophan synthase (alpha and beta chains), phosphoribosylanthranilate isomerase, phosphoribosyltranferase, anthranilate synthase and indole-3-glycerol phosphate synthase (Singh et al., 2017). Rodrigues et al. (2016) reported that *Gluconacetobacter diazotrophicus* produced IAA via the indole-3-pyruvate (IPyA) pathway in cultures supplemented with tryptophan. It had the *pdc* gene encoding a pyruvate decarboxylase (PDC) that catalyzed the decarboxylation of IPyA to an indole-3-acetaldyhyde (IAAld) intermediate. Then, IAAld was further oxidized to IAA. The *pdc* gene was also observed in the genome of CPH24 and the type strain MB58^T. For mineral solubilization, the mechanisms related to organic acid production, proton extrusion and phosphatase enzyme (Swetha and Padmavathi, 2016). Consequently, the genes involved in organic acid production and phosphatase, such as lactate dehydrogenase, citrate synthase, exopolyphosphatase and inorganic pyrophosphatase, were found in the genome of the strains CPK24 and MB58^T. The genes responsible for phosphate metabolism include the ABC transporter complex, PstABCS related to inorganic phosphate uptake under starvation conditions and the Pho regulon (PhoB-PhoR, PhoU) that were observed in the strains CPK24 and MB58^T. These genes are co-regulated by extracellular phosphate and are involved in phosphorus assimilation (Kaur et al., 2016). The direct periplasmic oxidation of glucose to

Table 3 Genome features of strain CPK24 and type strain

Genome feature	CPK24	M58^T
GenBank assembly accession	GCA_024539955.1	GCA_000617865.1
Genome size (bp)	3,708,126	3,690,031
G+C content (%)	64.8	65.4
Number of contigs	113	546
Total genes	3,469	3,525
Protein-coding genes	3,405	3,270
tRNA	46	44
rRNA	3	3

Table 4 Potential plant growth-promoting genes in genome of strain CPK24

Gene name	Locus tag	Gene annotation
IAA-related gene		
trpA	JZX93_00755	Tryptophan synthase subunit alpha
trpB	JZX93_00750	Tryptophan synthase subunit beta
trpC	JZX93_02205	Indole-3-glycerol phosphate synthase
trpD	JZX93_02200	Anthranilate phosphoribosyltransferase
trpE	JZX93_02190	Anthranilate synthase component I
trpF	JZX93_11975	N-(5'-phosphoribosyl) anthranilate isomerase
trpG	JZX93_02195	Anthranilate synthase component II
trpS	JZX93_04805	Tryptophan-tRNA ligase
pdc	JZX93_06830	Pyruvate decarboxylase
Phosphate solubilization and transport gene		
gcd	JZX93_05005	PQQ-glucose dehydrogenase
dld	JZX93_08460	Quinone-dependent D-lactate dehydrogenase
aarA	JZX93_13680	Citrate synthase
ppx	JZX93_03690	Exopolyphosphatase
ppa	JZX93_05450	Inorganic pyrophosphatase
ppk	JZX93_12865	Polyphosphate kinase
ppc	JZX93_03510	Phosphoenolpyruvate carboxylase
phoB	JZX93_08450	Phosphate regulon transcriptional regulatory protein
phoR	JZX93_08455	Phosphate regulon sensor protein
phoU	JZX93_08445	Phosphate-specific transport system accessory protein
pstA	JZX93_08435	Phosphate transport system permease protein
pstB	JZX93_08440	Phosphate import ATP-binding protein
pstC	JZX93_08430	Phosphate transport system permease protein
pstS	JZX93_07790	Phosphate-binding protein
Siderophore gene		
entS	JZX93_01595	Enterobactin exporter
Nitrogen fixation gene		
fixK	JZX93_15120	Nitrogen fixation regulation protein
fixL	JZX93_16540	Putative oxygen sensor protein
Acetoin-related gene		
ilvC	JZX93_06040	Ketol-acid reductoisomerase
ilvH	JZX93_06035	Acetolactate synthase isozyme 3 small subunit
ilvI	JZX93_12220	Acetolactate synthase isozyme 3 large subunit
ilvX	JZX93_06030	Putative acetolactate synthase large subunit
bdhA	JZX93_13245	2,3-Butanediol dehydrogenase

gluconic acid by PQQ-glucose dehydrogenase (*gcd*) has been reported as one of the major metabolisms with inorganic mineral solubilization in bacteria (Chakdar et al., 2018; Costerousse et al., 2018). This *gcd* gene also was observed in the CPK24 and MB58^{T}genome. The genes involved in the biosynthesis of the siderophore enterobactin (operon *entCDEBAH*) and in uptake and utilization (*fepE*, *fepG*, *fepD*, *fepC* and *fepB*) (Khan et al., 2018) were absent in the CPK24 genome but not *entS*, whereas the *entS* gene was not observed in the genome of the strain MB58^T according to pairwise genome comparative analysis using OthoVenn2. Paul and Dubey (2015) reported that *entS* mutants produce less enterobactin than the wild type so that the *entS* gene is also involved in enterobactin synthesis. The *nif* and *fix* genes, related to nitrogen fixation, are organized in distinct clusters whose structure and genomic location are species specific (Fischer, 1994). The nitrogen fixation gene clusters (*nif* and *fix*) were not discovered in this bacterial genome. Only the *fixK* and *fixL* genes related to the nitrogen fixation regulatory protein were identified in the genome of CPK 24 and MB58^T. The *fixK* gene was required for activation of the

fixN promoter in *Rhizobium meliloti* (Fischer, 1994). The *fixL* gene was the putative oxygen sensor protein that modulated the activity of FixJ (transcriptional activator of nitrogen fixation *fixK* gene) (Fischer, 1994). Furthermore, acetoin and butanediol have been reported to promote plant growth by induced systematic resistance or by reducing phytotoxic microbial communities (Ping and Boland, 2004; Shemshura et al., 2020). The genome of strains CPK24 and MB58^T contained gene encoding acetolactate synthase (*ilvX*, *ilvI* and *ilvH*), ketol-acid reductoisomerase (*ilvC*) and 2,3-butanediol dehydrogenase (*bdhA*) that were related to acetoin production. A zinc-type alcohol dehydrogenase-like protein that related to the production of acetoin and 2,3-butanediol was observed in CPK24. Yu et al. (2015) characterized the gene encoding (*2R,3R*)-2,3-butanediol dehydrogenase from *Rhodococcus erythropolis* WZ010 belonging to the family of the zinc-containing alcohol dehydrogenase that can reduce diacetyl to acetoin and then 2,3-butanediol. In the context of plant-microbe interactions, nitrilases involve the hormone synthesis, nutrient assimilation and detoxification of exogenous and endogenous nitriles (Howden and Preston, 2009). The *nit* gene encoding nitrilase was observed in the CPK24 genome. One of the orthologous groups specific to CPK24 was the *nodD* gene encoding a nodulation protein. The product of the *nodD* gene has a role as a transcriptional activator protein that functions as a receptor for a flavonoid plant compound (Göttfert, 1993). The *nodD* gene was only observed in the strain CPK24 genome that may indicate its association with plants.

In conclusion, the endophytic bacterium, *A. methanolica* CPK24, isolated from a sugarcane root, had various plant growth-promoting traits, such as IAA production, zinc solubilization and antagonistic activity against *F. moniliforme*. Following a genome analysis, the functional genes for IAA synthesis, zinc mobilization and the generation of volatile organic compounds with antagonistic activity, such as acetoin and 2,3-butanediol, were discovered that were associated with its ability to promote plant growth. These findings could help to clarify the biotechnological use of bacterial endophytes in agricultural fields as they represent a step forward in understanding their function.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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