Genome sequence of an uncharted halophilic bacterium *Robertkochia marina* with deciphering its phosphate-solubilizing ability

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Abstract

The wide use of whole-genome sequencing approach in the modern genomic era has opened a great opportunity to reveal the prospective applications of halophilic bacteria. *Robertkochia marina* CC-AMO-30D^T is one of the halophilic bacteria that was previously taxonomically identified without any inspection on its biotechnological potential from a genomic aspect. In this study, we present the whole-genome sequence of *R. marina* and demonstrated the ability of this bacterium in solubilizing phosphate by producing phosphatase. The genome of *R. marina* has 3.57 Mbp and contains 3107 predicted genes, from which 3044 are protein coding, 52 are non-coding RNAs, and 11 are pseudogenes. Several phosphatases such as alkaline phosphatases and pyrophosphatases were mined from the genome. Further genomic study (phylogenetics, sequence analysis, and functional mechanism) and experimental data suggested that the alkaline phosphatase produced by *R. marina* could potentially be utilized in promoting plant growth, particularly for plants on saline-based agricultural land.

Keywords *Robertkochia* · Genome · Phosphate solubilization · Halophiles · Phosphatase

Halophiles are highly specialized organisms that thrive in hypersaline environments [1, 2]. They possess the ability to produce enzymes with high biotechnological potential including proteases, amylases, cellulases, lipases, and phosphatases for industrial applications [3–11]. For instance, halophilic bacteria could enhance phosphate uptake by plants through solubilizing the insoluble phosphate into soluble forms by producing alkaline phosphatase [12]. Thus, the phosphate-solubilizing bacteria could be used as biofertilizer to promote plant growth

[13] that could be applied on saline-based agricultural land [14, 15].

An increasing number of halophilic bacteria has been taxonomically identified in recent years; nonetheless, majority of these halophiles remained under-explored and are potential for biotechnological applications [16]. *Robertkochia marina* CC-AMO-30D^T is one of such bacteria in which its potential has not been revealed yet until today. This halophilic bacterium was originally isolated from seawater at the harbor of Taiwan and is one of the two species within the *Robertkochia* genus aside from *Robertkochia solimangrovi* [17, 18]. With the advancement in next-generation sequencing technology, whole genomes of halophiles have been sequenced and studied. This resulted in a plethora of genomic information that was mined for potential industrial applications [19, 20]. Therefore, in this study, we employ the whole-genome sequencing approach to disclose the biotechnological prospective of *Robertkochia marina* CC-AMO-30D^T.

The genomic DNA of *Robertkochia marina* CC-AMO-30D^T was extracted and purified by using the DNeasy Blood and Tissue kit (Qiagen) and DNA Clean & ConcentratorTM-25 (Zymo Research) respectively following the protocol from the

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manufacturers. The quality and quantity of purified DNA were verified by using the Qubit 3.0 fluorometer and NanoDrop™ 1000 spectrophotometer accordingly. The genomic DNA library was constructed by using the Nextera XT library preparation kit. Paired-end genome sequencing (2×250 bp) was performed on an Illumina MiSeq (Illumina, San Diego, USA) using MiSeq Reagent Kit v2 (500-cycles). After sequencing, low-quality sequences, adapters, and contaminants were trimmed and filtered by BBDuk (BBTools v. 36). The quality-filtered sequences were de novo assembled using SPAdes v. 3.9 [21] to generate contigs. The genome assembly quality was assessed using QUAST v. 4.0 [22]. The assembled genome was then annotated following NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v. 4.6 [23] and DOE-JGI Microbial Genome Annotation Pipeline (MGAP) v. 4 [24]. The protein-coding genes were functionally grouped according to Pfam v. 32.0 with the aid of the WebMGA server [25]. The predicted genes were also mapped on reference canonical pathways using the KEGG automatic annotation server (KAAS) [26]. The whole-genome shotgun project of *Robertkochia marina* CC-AMO-30D^T has been deposited at DDBJ/EMBL/GenBank under accession [QXMP00000000](#).

The genome of *R. marina* consists 58 contigs, with a genome size of 3,571,649 bp and G+C content of 43.67% (Table 1). A total of 3107 genes were predicted, with 3044 protein-coding genes, 52 non-coding RNA genes (42 tRNAs, 4 ncRNAs, and 6 rRNAs), and 11 pseudogenes (Table 1). The protein-coding genes were then functionally annotated and assigned into 22 categories according to Pfam as indicated in Table 2. The genes grouped under cell wall/membrane/envelope biogenesis, translation, ribosomal structure, and biogenesis, and amino acid transport and metabolism were among the top three most abundant, which were 10.81%, 10.49%, and 9.69% respectively. In terms of metabolic pathways, based on KEGG annotation, the presence of glucokinases and phosphoglucumutase suggests that this bacterium can utilize α -D-glucose, β -D-glucose, and α -D-glucose-1-phosphate as substrates for glycolysis. This is due to the presence of encoded glucokinase and phosphoglucumutase in order to convert those substrates into glucose-6-phosphate for further conversion into pyruvate. Nevertheless, this bacterium is unable to utilize extracellular D-glucose as substrate for glycolytic cycle as the phosphotransferase was absent. This enzyme transfers the phosphate group to the D-glucose into glucose-6-phosphate. Apart from that, a total of 104 horizontally transferred genes was predicted. Within these genes, several genes related to defense mechanism such as penicillin amidases from *Glaciecola pallidula* DSM 14239 and *Roseivirga spongicola* UST030701-084 were acquired by *R. marina* for survival in its natural habitat.

To reveal the biotechnological potential of *R. marina*, an effort on functional screening was performed from a genomic

Table 1 Genome attributes of *Robertkochia marina* CC-AMO-30D^T according to Minimum Information about a Genome Sequence (MIGS) specification

Attribute	Description
Genome assembly statistics	
Sequencing platform	Illumina MiSeq
Assembly	SPAdes v. 3.9
Finishing strategy	High-quality draft
Genome coverage	$\times 76.28$
Genome quality	No contamination and no mismatch
Genome metrics	
Genome size (bp)	3,571,649
G+C content (%)	43.67
Number of contigs	58
Longest contig length (bp)	654,345
N ₅₀ value	240,013
L ₅₀ value	4
Total genes	3107
Non-coding RNA genes	52
tRNA	42
ncRNA	4
5S rRNA	4
16S rRNA	1
23S rRNA	1
Pseudogenes	11
Protein-coding genes	3044
With Pfam	2416
With TIGRfam	903
Connected to KEGG pathways	770
NCBI accession	QXMP00000000

aspect, including its phosphate-solubilizing ability. In general, bacteria could enhance phosphate uptake by plants through solubilizing the insoluble phosphate into soluble forms by producing alkaline phosphatase [12]. Thus, the phosphate-solubilizing bacteria could be used as biofertilizer to promote plant growth [13]. Halophilic bacteria were previously demonstrated to possess phosphate-solubilizing ability that could be applied on saline-based agricultural land [14, 15]. By inferring from genomic data, a total of eight putative phosphatase genes were mined from the genome of *R. marina*, as shown in Table 3. Based on the BLASTp search against NCBI database, the phosphatase genes of *R. marina* shared 56.67–94.44% with its closely related members including *R. solimangrovi*, and *Arenibacter* spp. and *Polaribacter* sp. (Table 3). Particularly, all the alkaline phosphatases (TRZ46762, TRZ45488, and TRZ45685) and HAD family phosphatases (TRZ42656 and TRZ40862) of *R. marina* have relatively low similarity (56.67–68.25%) when compared to the sequences found in the NCBI database (Table 3), indicating the novelty of these phosphatases. From the eight of the

Table 2 Functional assignment of protein coding genes of *Robertkochia marina* CC-AMO-30D^T according to Pfam categories

Pfam category	Gene count	Percentage (%)
Cell wall/membrane/envelope biogenesis	135	10.81
Translation, ribosomal structure, and biogenesis	131	10.49
Amino acid transport and metabolism	121	9.69
Function unknown	120	9.61
General function prediction only	102	8.17
Carbohydrate transport and metabolism	90	7.21
Energy production and conversion	83	6.65
Coenzyme transport and metabolism	74	5.92
Posttranslational modification, protein turnover, and chaperones	70	5.60
Inorganic ion transport and metabolism	61	4.88
Replication, recombination, and repair	57	4.56
Transcription	44	3.52
Lipid transport and metabolism	42	3.36
Nucleotide transport and metabolism	36	2.88
Intracellular trafficking, secretion, and vesicular transport	33	2.64
Defense mechanisms	16	1.28
Signal transduction mechanisms	16	1.28
Cell cycle control, cell division, and chromosome partitioning	9	0.72
Secondary metabolites biosynthesis, transport, and catabolism	6	0.48
Cell motility	1	0.08
Chromatin structure and dynamics	1	0.08
RNA processing and modification	1	0.08

putative phosphatases, one alkaline phosphatase (TRZ46762) was predicted to possess signal peptide based on SignalP v. 5.0 [27], which indicated that this enzyme is an extracellular enzyme.

The extracellular alkaline phosphatase (TRZ46762) of *R. marina* was then inspected in detail. The sequence of

alkaline phosphatases that was closely related to alkaline phosphatase of *R. marina* was downloaded from NCBI and aligned using Clustal Omega [28] for subsequent analysis. The phylogenetic tree was constructed by using MEGA v 7.0 based on neighbor-joining method and 1000 bootstrap replications. According to the results, alkaline phosphatase of *R. marina* is closely affiliated to that of *R. solimangrovi* (Fig. 1a). While the alkaline phosphatase of *R. marina* has far distant relationship with other phosphatases of related genera such as *Cellulophaga*, *Muricauda*, and *Arenibacter* (Fig. 1a). In terms of sequence analysis based on the InterPro 77.0 server [29], the signal peptide domain and alkaline phosphatase functional domain were located at position 1–19 and position 31–480 respectively (Fig. 1b). Within the alkaline phosphatase domain, the active sites are located at positions D37–Q38, A77–T78, N99, R162, D299, H303, and D346–H347 of the sequence (Fig. S1). Among these active sites, D37, T78, D299, H303, and D346–H347 are catalytic zinc-binding sites that could mediate the zinc by imidazole and carboxyl groups [30]. Furthermore, there is an additional zinc-binding site located at H482 of the alkaline phosphatase sequence (Fig. 1b). Interestingly, all the active sites and zinc-binding sites are also located at similar positions for closely related alkaline phosphatases (Fig. S1).

To investigate the ability of *R. marina* to produce alkaline phosphatase for phosphate solubilization, phosphatase activity was measured. A 10% (v/v) inoculum was added into marine broth 2216 (MB; BD Difco) supplemented with 0.5% (w/v) tricalcium phosphate and was cultured at 30 °C and 150 rpm for phosphatase production. After 24 h incubation, the culture was centrifuged at 4 °C and 8000 rpm for 20 min. The supernatant was used as crude enzyme for phosphatase assay. The alkaline phosphatase activity was assessed by using 5 mM *p*-nitrophenyl phosphate (*p*NPP; HiMedia) as substrate in a reaction tube containing sodium phosphate buffer (pH 8), following a modified method as described [31]. The mixture (1 ml crude enzyme and 1 ml *p*NPP dissolved in buffer) was incubated at 30 °C for 60 min. The reaction was

Table 3 BLASTp search on putative phosphatase genes encoded in the genome of *Robertkochia marina* CC-AMO-30D^T

Gene	NCBI accession	Top hit sequence	Identity
Alkaline phosphatase family protein	TRZ46762	Alkaline phosphatase family protein of <i>Arenibacter</i> (WP031445200)	56.67%
Alkaline phosphatase family protein	TRZ45488	Alkaline phosphatase family protein of <i>Arenibacter</i> sp. (HCO84308)	68.25%
Alkaline phosphatase family protein	TRZ45685	Alkaline phosphatase family protein of <i>Robertkochia solimangrovi</i> (WP143954842)	62.24%
Sodium-translocating pyrophosphatase	TRZ44743	Sodium-translocating pyrophosphatase of <i>Aquimarina pacifica</i> (WP025741192)	81.92%
HAD family phosphatase	TRZ42656	HAD-IA family hydrolase of <i>Robertkochia solimangrovi</i> (WP143956584)	58.42%
HAD family phosphatase	TRZ40862	Cof-type HAD-IIB family hydrolase of <i>Robertkochia solimangrovi</i> (WP143956209)	59.18%
Pyrophosphatase	TRZ41149	Nucleotide pyrophosphohydrolase of <i>Robertkochia solimangrovi</i> (WP143954535)	94.44%
Exopolyphosphatase	TRZ45494	Exopolyphosphatase of <i>Robertkochia solimangrovi</i> (WP143955483)	79.66%

Fig. 1 Phylogenetic (a) and sequence analyses (b) of alkaline phosphatase of *Robertkochia marina*. Corresponding GenBank accession numbers are indicated in parentheses and bootstrap values $\geq 50\%$ based on 1000 resampled datasets are shown as percentages at nodes in the phylogenetic tree (a). Domain organization of alkaline phosphatase with SP as signal peptide, the position of the sequence is not drawn according to scale (b)

stopped by adding 4 ml 0.5 M NaOH and 1 ml 0.5 M CaCl₂. Absorbance at 430 nm was measured using a spectrophotometer. One unit of phosphatase activity (U/ml) is defined as the amount of enzyme releasing 1 μ mol of *p*-nitrophenyl per minute under assay conditions. The phosphate that was solubilized by the bacterium in the medium was quantified (mg/l) by employing the method of Murphy and Riley [32]. The effect of time course (24 h interval until 120 h) and tricalcium phosphate concentration (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% (w/v)) on the phosphatase activity were investigated. A negative control was prepared (inactivated supernatant) and all the experiments were performed in biological triplicates. The statistical test (*t*-test) was performed to examine the significant difference between phosphatase activities.

According to Fig. 2a, the alkaline phosphatase activity of *R. marina* was observed throughout 120 h of incubation. This suggests that the extracellular alkaline phosphatase produced by *R. marina* (TRZ46762) is most likely responsible for the observed phosphatase activity. The amount of soluble phosphate that was detected by the end of incubation (120 h) was found to be 4.87 ± 0.01 mg/l. This was due to the alkaline phosphatase that cleaved the phosphoserine intermediate via hydrolysis to

release soluble ionic phosphate (HPO_4^{2-}) [13, 33, 34], which could have an uptake and be utilized by plant for growth.

In a general trend, the bacterial growth is associated with the alkaline phosphatase activity present (Fig. 2a). The alkaline phosphatase activity of *R. marina* demonstrated its optimal activity at 96 h of incubation (Fig. 2a). Unlike *R. marina*, other halophilic isolates such as *Serratia* sp. PSB-37 and *Alcaligenes faecalis* PSB-26 demonstrated their maximal phosphatase activities at 48 h and 144 h of incubation, respectively [35, 36]. This stipulates that the optimal incubation time for maximum phosphatase activity depends on bacterial strains. Subsequently, the effect of different tricalcium phosphate concentrations on phosphatase activity of *R. marina* was investigated. The result showed that *R. marina* was able to solubilize tricalcium phosphate with up to 3%, w/v (Fig. 2b). The optimal alkaline phosphatase activity of *R. marina* was examined at 2.5% (w/v) tricalcium phosphate (Fig. 2b). On the other hand, the optimal phosphatase activities for halophilic *Serratia* sp. PSB-37 and *Alcaligenes faecalis* PSB-26 were observed at 0.25% (w/v) tricalcium phosphate [35, 36], a lower concentration was observed as compared to *R. marina*.

Fig. 2 The alkaline phosphatase activity produced by *Robertkochia marina* throughout 120 h of incubation (**a**) and at different tricalcium phosphate concentration (**b**). Relative activity (%) was calculated by relativity to the case of reaction at which maximum activity was taken as 100% [9]. Mean values ($n = 3$) are reported and standard deviations are indicated as error bars

Collectively, the genome of an uncharted halophilic *R. marina* was studied, and the biotechnological potential of this bacterium was explored. Based on genomic study and experimental data, *R. marina* possesses the ability to solubilize phosphate. This highlights the potential application of this bacterium as a promising beneficial microbe for promoting the plant growth.

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Authors’ contributions Ming Quan Lam designed and performed the experiment, analyzed the data, and wrote the manuscript. Sye Jinn Chen assisted in performing the experiment and

provided the expertise. Kian Mau Goh, Adibah Yahya, Fazilah Abd Manan, and Mohd Shahir Shamsir provided the expertise. Chun Shiong Chong conceived the presented idea, designed the experiment, and provided the expertise. All authors read, edited, and approved the final manuscript.

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Data availability The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession [QXMP000000000](https://www.ncbi.nlm.nih.gov/nuccore/QXMP000000000), with BioProject and BioSample accession [PRJNA489156](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA489156) and [SAMN09949184](https://www.ncbi.nlm.nih.gov/biosample/SAMN09949184), respectively. The version described in this paper is the first version (accession number QXMP01000000). The strain, *Robertkochia marina* CC-AMO-30D^T (=JCM 18552) was obtained from the Japan Collection of Microorganisms.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflicts of interest.

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