Adaptive Strategies of *Bacillus thuringiensis* Isolated from Acid Mine Drainage Site in Sabah, Malaysia

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Abstract The adaptive process in bacteria is driven by specific genetic elements which regulate phenotypic characteristics such as tolerance to high metal ion concentrations and the secretion of protective biofilms. Extreme environments such as those associated with heavy metal pollution and extremes of acidity offer opportunities to study the adaptive mechanisms of microorganisms. This study focused on the genome analysis of *Bacillus thuringiensis* (Bt MCMY1), a gram positive rod shaped bacterium isolated from an acid mine drainage site in Sabah, Malaysia by using a combination of Single Molecule Real Time DNA Sequencing, Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The genome size of Bt MCMY1 was determined to be 5,458,152 bases which was encoded on a single chromosome. Analysis of the genome revealed genes associated with resistance to Copper, Mercury, Arsenic, Cobalt, Zinc, Cadmium and Aluminum. Evidence from SEM and FTIR indicated that the bacterial colonies form distinct films which bear the signature of polyhydroxyalkanoates (PHA) and this finding was supported by the genome data indicating the presence of a genetic pathway associated with the biosynthesis of PHAs. This is the first report of a *Bacillus* sp. isolated from an acid mine drainage site in Sabah, Malaysia and the genome sequence will provide insights into the manner in which *B. thuringiensis* adapts to acid mine drainage.

Keywords *Bacillus thuringiensis* · Acid mine drainage · Polyhydroxyalkanoates · Metal tolerance · Single molecule real time (SMRT) sequencing

Introduction

Microorganisms from the domain of Bacteria and Archaea with special adaptations to survive and propagate in extreme environments are known as the Extremophiles [1]. Among them, microbes can be further classified according to their adaptations to specific ecological niches, for instance, bacteria which thrive at extremely low values of pH value are termed Acidophiles [2, 3]. Acidophiles are generally associated with heavy metal pollutions, and they have adapted to survive in this environment by virtue of their metal resistance gene clusters which are involved in the maintenance of cytoplasmic conditions via various mechanisms [4–6]. Acidophiles have also been reported to acquire traits via horizontal gene transfer as part of their adaptive process [7]. Bacteria of the genus *Bacillus* have been reported to thrive in acidic environments, for instance *Bacillus cereus*, *B. licheniformis*, *B. thuringiensis* and *B. subtilis* have all been employed as bio-sorbents for wastewater treatment to sequester heavy metal pollutants [8, 9]. *B. thuringiensis* (Bt) is a member of the genus *Bacillus* which expresses toxic crystalline proteins that have been reported to be effective in targeting a wide range of pests which include insects and nematodes [10–13].

Bacillus thuringiensis, *B. cereus* and *B. anthracis* have been classified as the *Bacillus cereus sensu lato* group. Previous investigations have revealed that they exhibit a low degree of morphological and genetic diversity as evinced by 16S rDNA sequence data, gene content and multi-locus genotyping [14, 15]. This investigation was

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directed towards the characterization of Bt isolated from an abandoned acid mine drainage in Sabah, Malaysia with the primary objective of identifying the genomic signatures of adaptation by employing the PacBio SMRT long-read sequencing platform which has been proven to be effective for the characterization of bacteria genomes by virtue of its ability to sequence long reads of DNA in excess of 10,000 bases [16].

Mamut Copper Mine, the only copper mine in Malaysia, was discovered by the a United Nations Exploration Team in the mid 60's and the subsequent exploratory works by the Geological Survey Malaysia in 1966 confirmed the presence of a disseminated porphyry copper deposition [17]. Acid mine Drainage (AMD) was recorded at the mining site after the operations ceased in 1999 [18, 19]. Analysis of the seepage of water at the mine pit area were indicative of low pH value (2.90–3.75), high and variable total acidity (176–1697 mg CaCO_3/L), high TDS (302–2673 mg/L), high sulfate (292–2808 mg/L) and elevated concentrations of dissolved metals (Al, Mn, Fe, Cu and Zn) [20]. There has been no previous study pertaining to the assessment of AMD on microbial communities at this location. This study was driven by the need to assess the impact of AMD on the microbial communities with special reference to Bt MCMY1.

Materials and Methods

Sample Collection and Determination of Metal Element Concentration

Soil samples were collected at MCM's sampling site (6°1.801'N 116°39.496'E) at a depth of 10 cm below the surface by employing sterile augers, stored in sterile collection tubes and shipped to the laboratory for processing. Soil samples were analyzed for elemental composition with the assistance of an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) on an Optima 5300 DV (Perkin-Elmer). The Aqua digestion method using *Aqua Regia* was employed on all the soil samples. All samples were heated, filtered and analyzed by using ICP-OES in triplicates.

Isolation of Microbes

1.0 g of soil sample collected was suspended in sterile water, mixed by vortex and incubated in oven pre-warmed to 30 °C. The sample was then serially diluted to 10^{-5} and spread on Nutrient Agar plates by using a sterile inoculating loop and cultured in an incubator at 15 °C incubator until visible colonies were observed. Selection of potential *Bacillus* species for Whole Genome Sequencing (WGS)

was done on the basis of their morphology as observed using Scanning Electron Microscope (SEM).

Total DNA Extraction and Quality Control

Selected strains were cultured in Nutrient Broth (HI-MEDIA) on a rotary shaker at 180 rpm and a temperature of 15 °C. Bacterial cells were collected by pelleting down 5 ml of culture media on a centrifuge set at $13,000 \times g$ for a 1 min. Total DNA of the selected culture was extracted by using Qiagen DNeasy Kit based on the pretreatment protocol for Gram-positive bacteria as described in [21]. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit based on the protocols for plasmid DNA purification [22]. The quantity and quality of the DNA extracted were evaluated by using NanoDrop DNA spectrophotometer (GE Healthcare) and by gel electrophoresis.

Whole Genome Sequencing

Whole Genome Sequencing was conducted using Single Molecule Real-Time Sequencing Platform (Pacific Bioscience) in accordance with the manufacturers' protocols. The library size was set at 20,000 base pairs. The libraries were processed using a Pacific Biosciences RS 2 sequencing platform (Biotechnology Research Institute, UMS). The raw sequencing data were submitted to NCBI's Sequence Read Archive (SRA) and assigned an accession number PRJNA374601.

Assembly and Annotation

The raw sequence data were imported into Pacific Bioscience SMRT Portal. Hierarchical Genome Assembly Process (HGAP) was conducted to preassemble and assemble the raw sequencing data by using Celera® Assembler with the genome size estimated to be 5,000,000 bp. Consensus polishing was completed using Quiver. Annotation was done using Rapid Annotation using Subsystem Technology (RAST) where FIG's "Subsystem Approach" for High-Throughput Comparative Analysis was practiced to rapidly call and annotate the genes. The whole genome sequence was used for nucleotide blast using BLAST+ 2.6.0.

Comparative Genomics

The genome of *Bacillus thuringiensis* strain IS5056 (Accession No.: CP004123) was used as a reference genome on the basis of its similarity to the genome of Bt MCMY1. BLAST Ring Image Generator (BRIG) was used to compare the genomes of Bt MCMY1 and the reference genome and to obtain a graphical representation based on similarity.

Culture of Bt MCMY1 Under Simulated Copper Stress

1 µl of the solution that contained Bt MCMY1 spores was spotted onto a series of nutrient agar plates that contained Copper sulfate with concentrations of 0, 25, 50, 75 and 100 µM. The control contained no Copper ions. The cultivation was carried out at 15 °C for 7 days in an incubator (Olympus). The growth of the bacteria was observed and documented using a stereo light microscope. The nutrient agar plates with different concentrations of copper sulfate were prepared in order to determine the minimum inhibitory concentration up to two decimal places. 5 replicates of nutrient agars of each copper sulfate concentration were prepared and the final minimum inhibitory concentration was calculated by taking the average of the last concentration of copper sulfate nutrient agar which permitted the growth of Bt MCMY1.

Scanning Electron Microscopy

Bt MCMY1 was cultured in nutrient broth, incubated in an orbital shaker set at 25 °C at a speed of 200 rpm. The bacteria colonies were harvested in a vial after 3 days of incubation and centrifuged at 11,000×g for 15 min. 5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) was used to fix the sample which was incubated at 4 °C overnight. Phosphate buffer was used to wash the sample three times with a duration of 10 min per wash. The sample was dehydrated using ethanol with different concentration (35, 50, 75 and 95%) for 5 min per wash and the final dehydration was done twice with absolute ethanol for 5 min per wash. The sample was air dried by placing the vial in a desiccator at room temperature for 16 h. The dried cells were mounted onto an SEM specimen stub with double sided sticky carbon tape and coated with gold. Scanning Electron Microscope (Hitachi S-3400N) was used to view the sample.

Analysis of Polyhydroxyalkanoates (PHA) Secreted by the Sample Strain

Extraction of the bacteria exopolymer was done by using the dispersion method of sodium hypochlorite and chloroform as described [23, 24] with modifications. Bt MCMY1 was cultured in nutrient broth, incubated in an orbital shaker at 25 °C and 200 rpm setting. The bacteria colonies were harvested into a vial after 3 days of incubation and centrifuged at 11,000×g for 15 min. The supernatant was discarded. The remaining bacterial pellet was suspended in 2.5 µl of 4% sodium hypochlorite and 2.5 µl of chloroform. The mixture was incubated at 37 °C for 1 h. The dispersion was then centrifuged at 8000×g for 10 min at room temperature resulting in a three phase solution with the bottom phase of chloroform containing

the PHA. The chloroform phase solution was transferred into another sterile vial. An equal volume of a mixture of ethanol and acetone (1:1 v/v) was added to the chloroform solution for precipitation. The mixture was centrifuged at 13,000×g for 10 min at room temperature and the precipitate was allowed to evaporate to dryness at 30 °C.

The residue in the vial was subjected to Fourier Transform Infrared Spectroscopy FTIR analysis (Agilent Cary 630). The residue was dissolved in isotonic saline solution (30 kg/m³) and 10 µl of the solution was deposited onto the diamond crystal stage of the spectrometer and the spectrum of the sample was recorded.

Results

Elemental Concentration of the Soil Sample

The elemental concentration of the soil samples was as follows: Iron (Fe) was the element with highest concentration of 645.61 mg/kg, followed by Aluminum (Al) 629.99 mg/kg, Magnesium (Mg) 227.97 mg/kg and Copper (Cu) was recorded at 96.88 mg/kg which exceeded the maximum permissible concentration of Potential Toxic Elements (PTE) in soil (80 mg/kg) [25]. Potential hazardous heavy metal such as Arsenic, As (1.10 mg/kg), Nickel, Ni (0.41 mg/kg), Chromium, Cr (0.84 mg/kg), Manganese, Mn (12.62 mg/kg) were also detected in the soil sample.

Genome Similarity Using Basic Local Alignment Search Tool Nucleotide (blastn)

The raw genome sequence was assembled by using Pacific Bioscience SMRT Portal. Hierarchical Genome Assembly Process (HGAP) was conducted to preassemble and assemble the raw sequencing data by using Celera[®] Assembler with the genome size estimated to be 5,000,000 bp. The assembled sequence was evaluated for similarity with other nucleotide whole genome sequences by using BLAST+ suite 2.6.0. Table 1 shows the top three sequences which exhibited significant similarity with Bt MCMY1. These were the *Bacillus thuringiensis* serovar *thuringiensis* str. IS5056 (CP004123), *Bacillus thuringiensis* serovar *chinensis* CT-43 (CP001907) and *Bacillus thuringiensis* Bt407 (CP003889) (Fig. 1).

Annotation with RAST

The general features of the annotated genome revealed that Bt MCMY1 consisted of a single circular chromosome of 5,458,152 bp in length, with a GC content of 35.60%, encoding 8686 sequences and 123 RNAs which comprised of 85 tRNA genes and 38 rRNA operons (Fig. 2).

Table 1 Top three sequences producing significant alignments based on blastn

Genome	Max. score	Total score	Query cover (%)	E value	ID (%)	Accession number
<i>Bacillus thuringiensis</i> serovar <i>thuringiensis</i> str. IS5056, complete genome	4.542e+05	1.099e+07	99	0.0	99	CP004123
<i>Bacillus thuringiensis</i> serovar <i>chinensis</i> CT-43, complete genome	3.300e+05	1.096e+07	99	0.0	97	CP001907
<i>Bacillus thuringiensis</i> Bt407, complete genome	3.196e+05	1.096e+07	99	0.0	97	CP003889

Fig. 1 Rapid Annotation using Subsystem Technology results for *Bt* MCMY1

Minimum Inhibitory Concentration of Copper

The minimum inhibitory concentration (MIC) is the lowest concentration of metal that inhibits the growth of a microbe. The physical observation of colony morphology revealed a reduction of aerial mycelia and the dimensions of the colony as the concentration of copper ions in the culture medium increased as depicted in Fig. 3. The MIC of copper ions was determined to be 1.7 mM, which was higher than that reported for *Sulfolobus solfataricus* (1 mM) [26] but much more lower than of *Acidothiobacillus caldus* DSM8584 (24 mM) [27].

Discussions

Genetic Adaptations of *Bacillus thuringiensis*

The genome size of *Bt* MCMY1 was estimated to be 5,458,152 bp, which is almost similar to the average genome size of other *Bt* genomes (5.5 Mb), interestingly

virulence plasmids were absent, unlike those reported in most of the *Bt* genomes [28–31]. The circular plasmid of the *Bt* genome has been reported to contain the *Cry* gene which encodes genes responsible for the production of insecticidal parasporal crystal. The absence of plasmids in the genome sequence as well as in the total DNA, which was confirmed by using GeneJET Plasmid Miniprep Kit, indicates that *Bt* MCMY1 appears to have lost its plasmid and its associated genes.

This situation has led us into the controversial topic of the identification of *Bacillus cereus sensu lato* group. Previously, the presence of this virulence plasmid was relied upon as the criterion to differentiate between *B. thuringiensis* and *B. anthracis* from *B. cereus* due to the absence of differences in phenotypical features and the similarity of the 16S rRNA gene sequence, but [32] have posited that the *Cry* gene plasmids in *B. thuringiensis* cannot be relied upon to confirm the identity of the species. Moreover, [33] also pointed out that different culture media and processes that are adopted in the laboratory cause the *Bt* strain to be cured of its plasmid DNA. Their experiments have also shown that the *Bt* strain

Fig. 2 Comparison results for the Blast Ring Image Generator between the genomes of *Bt* MCMY1 and *Bt* serovar *thuringiensis* str IS5056

devoid of the *Cry* gene plasmid has a higher growth rate which facilitates its dominance in the population as compared to other *Bt* strains that harbor *Cry* encoding plasmids. We posit that, the lack of virulence plasmid in *Bt* MCMY1 can be a possibly adaptation which facilitates rapid growth and survival under unfavorable nutrient limiting conditions of the copper mine.

Adaptations for Survival at Extreme Environment

Microorganisms must be able to achieve an ideal internal cytoplasmic condition unaffected by the harsh external

environment in order to survive in extreme environments. One of the mechanism to counteract with the effects of proton leakage is by maintaining a positive internal membrane potential as a charge barrier [34, 35]. We noted a total of 19 subsystem features in the genome of *Bt* MCMY1 that are responsible for potassium metabolism, these include the genes for ATPase transporting systems for potassium cations. This active transporting system is vital for potassium homeostasis mechanisms which involves in distributing the potassium cations within the cell. According to the findings of [36], putative cation transporters in the genomes of acidophiles are required in

Fig. 3 *Bt* MCMY1 colonies grown in different concentration of copper ions on nutrient agar

order to generate the Donnan potential of positively charged molecules and inhibit the influx of protons through the cellular membrane by relying on a chemiosmotic barrier against the proton gradient. This leads to the production of greater cation influx than the outward flux of protons and the pH of internal cytoplasmic conditions is maintained.

Furthermore, the genome of *Bt* MCMY1 also shows some potential adaptations in cell motility which included 67 subsystem features in the genome that are associated with cell motility and chemotaxis, these included the gene clusters responsible for the biosynthesis of flagella. The formation of flagella indicates that the bacterial cells are capable active movement in order to disperse and seek an optimum ecological niche [37–39]. Cell motility and chemotaxis is an important feature which permits acidophiles to survive and to locate suitable conditions for survival, a phenomenon which has also have been reported in other bacteria such as *Leptospirillum ferrooxidans* and *L. ferriphilum* [40, 41]. The genome of *Bt* MCMY1 encoded 318 subsystem features for DNA metabolism, which are vital for the repair of DNA. It is logical for microorganisms that survive in acidic and heavy metal polluted conditions to harbor an abundance of genes for replication, recombination and repair of DNA in order to deal with the high rate of DNA damage [42]. Metal resistance genes were also encoded in the subsystem features related to virulence, disease and defense, including the resistance genes for Tellurium, Mercury, Copper, Cobalt, Zinc, Cadmium, Arsenic and Aluminum. These metal resistance genes are associated with the proteins involved in the ATPase transporting systems.

Presence of Gene Clusters for Exopolysaccharides

The genome of *Bt* MCMY1 encoded genes involved in the biosynthesis of exopolysaccharides. These included the genes for exopolysaccharide biosynthesis glycosyltransferase, polysaccharide deacetylase and glycosyl transferase

family protein. Microorganisms that secrete exopolysaccharides can contribute to the aggregation of other microbes together with the mixture of protein, carbohydrates, lipids and humic acid substances as they can form a complex structure termed as a biofilm. Each biofilm has its own special physiological function reported to be distinct from the others [43]. The exopolysaccharides produced by microorganisms are capable of forming distinct biofilms that carry out metal chelation and they represent potential agents which can be employed in the bioremediation process to sequester deleterious heavy metal pollutants [44, 45]. The experiments done by [8] have shown that *B. thuringiensis* can carry out bio-sorption of heavy metal rapidly, but the mechanisms of metal chelation by exopolysaccharides produced by *Bt* remains unknown. Observations of *Bt* MCMY1 using SEM (Fig. 4) revealed that bacterial cells were sheathed by multiple layers of bio-product which exhibited the characteristics of a dense biofilm. This biofilm produced by strain grown in media that contained copper sulfate was significantly thicker as compared to the control, thus we posit that the production is associated with the presence of metal stress but further investigation will be required to validate this hypothesis.

Polyhydroxyalkanoates (PHA) Production by *Bacillus thuringiensis*

Gene cluster related to the synthesis of polyhydroxyalkanoate (PHA) were classified by RAST under the subsystem category of fatty acid, lipids and isoprenoids, PHA is a class of thermoplastic or elastomeric polyesters which can be produced by bacteria in forms of intracellular carbon and energy storage compounds [46]. Bacteria that produce PHA generally exist under stressful conditions and a nutrient limiting environment in the presence of excess carbon [47]. *Bt* and some other members from the *Bacillus* genus have been reported to produce PHA in a previous study [48]. A common example of PHA is polyhydroxybutyrate (PHB) which is a type of biodegradable polymer

Fig. 4 SEM image of *Bt* MCMY1 covered in a dense biofilm composed of exopolysaccharides

that can be used as a replacement for the conventional plastics in multiple sectors [49]. We discovered 32 PHA related gene features in the subcategory of PHB metabolism, including the polyhydroxyalkanoate synthesis repressor (PhaR), polyhydroxyalkanoic acid synthase, Acetyl-CoA acetyltransferase, D-beta-hydroxybutyrate dehydrogenase, Acetoacetyl-CoA reductase, D-beta-hydroxybutyrate permease, Enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase and 3-hydroxybutyryl-CoA dehydrogenase.

The PHA produced by *Bt* MCMY1 were extracted using the dispersion method of sodium hypochlorite and chloroform and analyzed using the Fourier Transform Infrared

Spectroscopy (FT-IR). The resulted spectrum is depicted in Fig. 5. Observation on the wavelength of 2900–3700 nm revealed an obvious peak around the wavelength 3300 nm, which represents the hydroxyl (-OH) group. This peak was also observed in PHA produced from other bacteria [50]. Further, the presence of ester carbonyl (C=O) stretching groups was confirmed by the peak at around wavelength 1630 nm. [51] reported C=O peak in PHA standard spectrum consistently appeared in this wavelength. Other small peaks were present between 1000 and 1150 nm which can possibly be the C–O–C stretching of esters and –CH stretching. The small peaks at approximately 1420–1500 probably represent the –CH₃ vibration. Interestingly, the peak that may correspond to β-glycosidic linkage (about 870 nm) is not observed in the study. The presence of this peak in PHA may refer to a covalent bond between the sugar monomers in PHA as reported in [52]. The bond is expected to be present in the PHA produced by *Bt* MCMY1, however the peak was possibly suppressed due to some local structural or environmental effects indicating the PHA has probably been modified in *Bt* MCMY1.

Conclusions

The circular genome of *Bt* MCMY1 isolated from Mamut Copper Mine has been sequenced by using PacBio SMRT sequencer and the sequencing data are available at NCBI Sequence Read Archive (SRA) with accession number PRJNA374601. The findings suggest that, *Bt* has adapted to survive under nutrient limiting conditions by the loss of

Fig. 5 FTIR analysis of the extracted Polyhydroxyalkanoates (PHA)

its virulence plasmid. The genome features revealed the presence of cations transporting ATPase system genes which possibly associated with the mechanisms of potassium homeostasis to create positively charged molecules within the cell and inhibit the influx of protons through the cellular membrane to maintain its cytoplasmic pH condition. Bt MCMY1 harbors metal resistance genes which confer resistance to several metal, such as Tellurium, Mercury, Copper, Cobalt, Zinc, Cadmium, Arsenic and Aluminum. Genes responsible for exopolysaccharides and polyhydroxyalkanoates (PHA) biosynthesis were observed from the genome and the experiment results suggested that they may be involved in the physiological response to stressful conditions. The data provided by this study can contribute to future experiments directed at investigating the role of *B. thuringiensis*, a natively isolated bacteria strain in acid mine drainage.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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