The effect of simulated copper stress on genes associated with the metabolism of polyphosphates and polyhydroxyalkanoates in *Bacillus thuringiensis* Y1

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ABSTRACT

Aims: *Bacillus thuringiensis* Y1 is a copper-tolerant bacterium that can serve as a model for the elucidation of the mechanism of energy metabolism under simulated copper stress. This study aimed to elucidate the effects of simulated copper stress on the genes associated with the biosynthesis of polyhydroxyalkanoates (PAH) and the metabolism of polyphosphates (PP).

Methodology and results: The gene expression study involved the growth of the bacterium in nutrient broth supplemented with two concentrations of copper sulphate (0.4 mM and 0.8 mM), followed by RNA extraction and quantification of four and 11 genes associated with the metabolism of polyphosphate (PP) and polyhydroxyalkanoates (PAHs) respectively, using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Three genes associated with polyphosphate metabolism, which are polyphosphate kinase (*ppk*), exopolyphosphatase (*ppx*) and NAD kinase (*ppnk*), were all shown to be upregulated by both 0.4 mM and 0.8 mM copper, except for the 5'-nucleotidase (*surE*) gene that was downregulated under the second treatment. Among the 11 genes associated with the metabolism of polyhydroxyalkanoates, only the 3-ketoacyl-CoA-thiolase (*phaA*) gene was upregulated in both treatments and highly expressed in the second treatment; the majority were downregulated and repressed.

Conclusion, significance and impact of study: The study demonstrated that copper induces the metabolism of polyphosphates in *B. thuringiensis* Y1 that serve as an alternative source of energy under copper stress. This model can be extended to the study of other species of *Bacillus* under environmental stress.

Keywords: Bacillus thuringiensis, copper stress, gene expression, polyhydroxyalkanoates, polyphosphates

INTRODUCTION

Bacteria isolated from sites contaminated with copper have been reported to be tolerant to a range of metal ions, however, tolerance will vary within species (Santo *et al.*, 2010; Altimira *et al.*, 2012). Concentrations of metal ions that exceed the limits of tolerance, overload the cellular medium and disrupt homeostasis (Argüello *et al.*, 2013), limit growth, damage nucleic acid structure and impair cellular membranes because of protein and lipid instability (Dupont *et al.*, 2011; Ladomersky and Petris, 2015; May *et al.*, 2019). To maintain cellular processes because of the stress induced by copper, energy management via gene regulation in bacteria plays a critical role. The storage, conversion and utilisation of energy metabolic compounds are important to support the active transportation of copper ions between the internal and external of cellular medium. Polyphosphate, glycogen, polyhydroxyalkanoates, triacylglycerol and wax ester are five types of energy storage chemical compounds identified in bacteria (Wang *et al.*, 2019). In addition to facilitating the production of energy, their metabolic pathways are involved in regulatory signalling, intracellular persistence, pathogenicity and environmental long-term survival of bacteria (Wang and Wise, 2011). The model of energy storage compounds in bacteria was originally proposed by (Wilkinson, 1959) and confirmed by (Wang *et al.*, 2017). This model is based on the premise that the cycling of energy storage compounds is driven by abiotic and biotic factors such as the introduction of heavy metals, changes in temperature, oxidative stress and nutrient limitation.

The five classes of the energy storage compounds, that comprise polyphosphate, polyhydroxyalkanoates, glycogen, wax ester and triacylglycerol, are recycled via their own respective metabolic pathways that are regulated by enzymes encoded by specific gene families (Murphy and Saltikov, 2009; Ball *et al.*, 2011; Chandra *et al.*, 2011; Orell *et al.*, 2012). The metabolic pathway of polyphosphate involves the enzymes encoded by the

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genes polyphosphate kinase (*ppk*), polyphosphate (*pp*): AMP phosphotransferase (*pap*), 5'-nucleotidase (*surE*), NAD kinase (*ppnk*) and polyphosphate glucokinase (*ppgk*) (Wang *et al.*, 2018). The pathway that is responsible for the metabolism of polyhydroxyalkanoates comprise the genes 3-ketoacyl-CoA thiolase (*phaA*), Acetoacetyl-CoA reductase (*phaB*), Acetyl-CoA acetyltransferase (*atoB*), Acetoacetyl-CoA synthetase (*aas*), PHA synthase subunit C (*phaC*), D-beta-hydroxybutyrate dehydrogenase (*bhbd*), 3-hydroxybutyryl-CoA dehydratase (*hbh*), Polyhydroxyalkanoate synththesis repressor (*phaR*), Enoyl-CoA hydratase (*eh*), D-beta-hydroxybutyrate permease (*hbp*) and 3-hydroxybutyryl-CoA dehydrogenase (*bdhA*). *B. thuringiensis* has been previously isolated from different extreme environments, such as abandoned mine sites, wastewater effluent and heavy metal contaminated soils (Hassen *et al.*, 1998; Marandi, 2012; Oves *et al.*, 2013; Kumar *et al.*, 2015). However, there is a paucity of information pertaining to the genetic response to changes in environmental conditions. The current study was directed towards elucidating the gene expression profile of the metabolic pathways associated with metabolism of polyphosphate and polyhydroxyalkanoates in *B. thuringiensis* Y1.

MATERIALS AND METHODS

Genome analysis

The genome of the *Bacillus thuringiensis* Y1 was downloaded from the NCBI GenBank (PRJNA374601) and uploaded to the Rapid Annotations using Subsystems Technology (RAST) version 2.0 for annotation (Brettin *et al.*, 2015). The summary of all annotations was viewed in the SEED viewer of RAST (Overbeek *et al.*, 2014). All genes annotated in the subsystems comprising metabolism of polyphosphates and polyhydroxyalkanoates were retrieved from the database.

Primer design

Primers were designed to target the genes involved in the biosynthesis of PAH and PP using the Primer3Plus web interface (Untergasser *et al.*, 2007) and have been listed in Table 1.

Bacterial culture

The copper sulphate concentrations of 0.4 mM (medium) and 0.8 mM (high) were selected and denoted as treatment 1 and 2 during the simulation test, respectively. Approximately 1% v/v of bacteria culture was inoculated into a sterile 250 mL conical flask that contained 100 mL aseptic nutrient broth without supplementation (control) and with supplementation of copper sulphate in 0.4 mM (treatment 1), 0.8 mM (treatment 2) concentrations. Each condition was prepared as four biological replicates and

the cultivation was conducted in an orbital shaker at 37 °C set at 180 oscillations per minute until the exponential phase (OD600: 0.5-1.5) was attained. The biological replicates represented four individual bacterial cultures prepared for each control, treatment 1 and treatment 2 incubated under the same conditions.

RNA extraction and quality control

RNA was extracted using the RNeasy Mini Kit (Qiagen) followed by DNase treatment using DNase I, RNase-free (Thermo Scientific), based on the manufacturer's protocols. All RNA samples were subject for quality and quantity assessments by gel electrophoresis, NanoDrop™ 2000 Spectrophotometer (Thermo Fisher) and 2100 Bioanalyzer Instrument (Agilent, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) reaction

The RT-qPCR reactions were performed on a Bio-Rad Real Time PCR System. The Brilliant III Ultra-Fast SYBR® Green RT-qPCR master mix (Agilent) was used as the reagent for the qPCR analysis. The reaction mixture was prepared according to the manufacturer instructions which was consisted of 1 µL of RNA sample (diluted 1:100), 10 µL of 2× SYBR Green RT-qPCR master mix, 1 µL of each forward and reverse primers (0.5 µM), 0.2 µL of 100 mM DTT, 1 µL of RT/RNase block and nuclease free PCR grade water to make up the volume to precisely 20 µL. The thermal cycling protocol comprised 10 min of reverse transcription at 50 °C, 3 min of polymerase activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 5 sec and 10 sec of annealing at 60 °C. Negative controls were applied to test for non-specific amplification. A non-reverse transcription control (nRT-control) was included in each RT-qPCR run to monitor the presence of gDNA contamination by replacing the RT/RNase block with nuclease free PCR grade water. Each treatment and control sample were amplified in three technical replicates and three biological replicates. The three biological replicates represented three individual RNA samples and the three technical replicates representing the same RNA samples were used for three separate experimental reactions. The expression levels were retrieved as the individual quantification cycles (Cq) value from the 60 °C annealing and extension step based on the instructions from the manufacturer. The gene expression was determined by the absolute quantification method using a standard curve constructed based on C(t) values of qPCR amplification using 1:100 diluted RNA samples. Relative quantification was calculated by normalisation against a combination of two reference genes as suggested by GeNorm software (Reiter *et al.*, 2011) with Livak's formula (Two Delta Delta Ct method) and formula for calculating the average C_T value of the reference genes (Schmittgen and Livak, 2008; Riedel *et al.*, 2014).

Table 1: Primers designed to amplify the reference genes and target gene transcripts associated with the PP and PAH metabolic pathways indicating the acronym for the gene, the encoded protein, the primer sequences for the forward and reverse primers and the expected size of the amplicons.

| Gene code	Protein	Primer sequences	Product size (bp)
pspA	Reference Gene 1: Phage shock protein A	5′-TGCTTCATAATACGAGCTAATTCAT-3′ 5′-GCTGGTTTAAGCCGTATGGA-3′	158
rpsU	Reference Gene 2: 30S ribosomal protein S21	5′-AAGATCGGTTTCTAAAACTGGTACA-3′ 5′-TTTCTTGCCGCTTCAGATTT-3′	102
aas	Acetoacetyl-CoA synthetase	5′-TGAGGCATTACCGCCAGGAG-3′ 5′-TCACCTGTATGGAGCCAGCC-3′	126
atoB	Acetyl-CoA acetyltransferase	5′-AAACGAGGCGGGGTAGAACC-3′ 5′-CGCTAATGCAGCCGTTCTCG-3′	102
bhbd	D-beta-hydroxybutyrate dehydrogenase	5′-AACAGGTGCTGCGAGTGGTA-3′ 5′-GCTCAGCAGCTTCTTTCGCA-3′	113
bdhA	3-hydroxybutyryl-CoA dehydrogenase	5′-AATTGCTCCAGAACACGCGA-3′ 5′-TTTCCGGGCGCTTCGTTAC-3′	86
eh	Enoyl-CoA hydratase	5′-CGTTTCTTCTCAGCGGGAGC-3′ 5′-AGTGCCGCTCCATGAATTGC-3	152
hbh	3-hydroxybutyryl-CoA dehydratase	5′-ACCAACTGCTGGCCTTGGAT-3′ 5′-GAAGGAAGAAATGGCCGCCG-3′	131
hbp	D-beta-hydroxybutyrate permease	5′-CGATGGATGCGCTACCTGGA-3′ 5′-TCCAGCTGCTTTCGCCTTCT-3′	170
phaA	3-ketoacyl-CoA thiolase	5′-ATTGCGCTCGGTCATCCGTA-3′ 5′-AGTGCAAGACCTATCCCGCC-3′	131
phaB	Acetoacetyl-CoA reductase	5′-GACAAGCGCGGTTCTTCCAT-3′ 5′-TGGCCAAATCCACCTGCTTG-3′	93
phaC	PHA synthase subunit C	5′-CGCTTCGTCGAAAGCTGGAG-3′ 5′-CCGCGAATAACGAGTTCACCC-3′	137
phaR	Polyhydroxyalkanoate synththesis repressor	5′-ATTCTGCTTGGATGGGCAGC-3′ 5′-AAGCGTAGCCACTCTAGCGA-3′	131
ppk	Polyphosphate kinase	5′-CGGCTCTTATTCCGCAACCAC-3′ 5′-TACATTCGGATCGTCCGCCG-3′	152
ppx	Exopolyphosphatase	5′-TGAGGAAGCGCGTTACGGAT-3′ 5′-ACTTCCGTACTTCCCCCACC-3′	93
surE	5'-nucleotidase	5′-CCATGAAGGCATGGAGCCTG-3′ 5′-GCTGGGCATCAGCAACCAAA-3′	170
ppnk	NAD kinase	5′-CGCCACTCGTATTGCCGAAG-3′ 5′-AACGGGCGGAAACGAACAAA-3′	163

Statistical analysis

As stated in the formula above, the fold change values were calculated by converting the $2^{-\Delta\Delta Ct}$ values into logarithm to the base of 2. In order to define the level of expression for the genes, the fold change values were categorised into three different percentiles. In the 75th percentile, the genes were categorised as highly expressed, in the 50th percentile, the genes were categorised as moderately expressed and in the 25th percentile, the genes were categorised as repressed. These thresholds were set relatively based on the current dataset. In gene normalisation, there is no definite benchmark to indicate the level of expression for each comparative study. However, the setting of percentile or quantile to categorise the level of expression were widely implemented in gene expression studies. As for the differential gene expression results, the means of the ΔΔ Ct values at two different treatment levels were tested for significant differences. The statistical significance was determined by a student t test for testing the mean of the differences between the treatments using IBM SPSS software version 23 with significance level of $p < 0.05$.

RESULTS

The four genes associated with the metabolism of polyphosphates in the genome of *B. thuringiensis Y1* are *ppk*, *ppx*, *surE* and *ppnk* (Table 2), were all upregulated under both treatments with the exception of *surE* gene during treatment 2. The expression of the *ppk* gene was upregulated for a mean value of 2.7-fold at 0.4 mM of induced copper concentration. As the concentration of copper increased to 0.8 mM, the expression increased to 3.2-fold. The improvement of the expression level was insignificant, t (4) = -0.74, $p = 0.25$ (Figure 1). On the other hand, the *ppx* and *ppnk* genes which also showed increment of expression level have had significance at

Polyphosphate Genes Expression
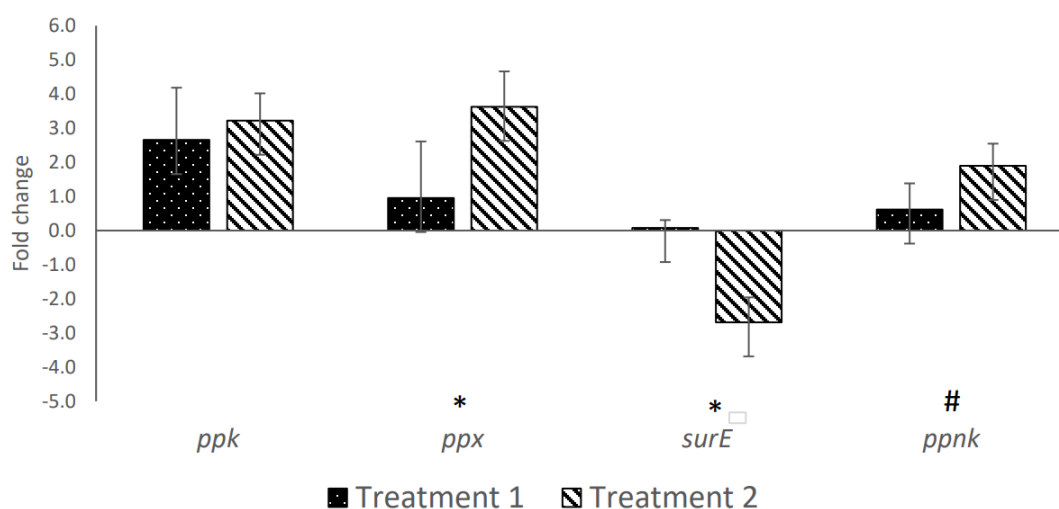
Figure 1: The relative fold change in gene expression of the four genes implicated in polyphosphate metabolism during growth in nutrient agar supplemented with copper sulphate at concentrations of 0.4 mM (Treatment 1) and 0.8 mM (Treatment 2). The significant differences detected at $p<0.05$ with student t-test are indicated with an asterisk '*', slight difference at $p<0.1$ are indicated with a hash '#'. Error bars indicate the standard deviations of the mean.

Table 2: The compilation of the fold change in gene expression as detected from qRT-PCR data normalization.

| System	Gene	Treatment 1		Treatment 2	
		Fold change	SD +/-	Fold change	SD +/-
Polyphosphate	*ppk*	2.7	1.5	3.2	0.8
	ppx	1.0	1.7	3.6 *	1.0
	surE	0.1	0.2	-2.7 *	0.7
	ppnk	0.6	0.8	1.9 #	0.7
Polyhydroxyalkanoates	*phaA*	0.8	1.7	3.5	2.8
	phaB	0.4	0.6	-0.6	1.3
	atoB	-1.6	0.8	-5.3 *	1.8
	aas	-0.9	0.3	-2.2 #	1.9
	phaC	-0.2	1.0	-3.3 *	1.0
	bhbd	-1.7	1.5	-0.9	2.4
	hbh	-1.8	1.1	-5.2 *	0.4
	phaR	-3.4	0.8	-3.3	1.7
	eh	-3.0	0.6	-6.2 *	0.6
	hbp	-1.0	1.1	-0.5	0.4
	bdha	-0.8	1.9	-4.4 #	1.4

The significant differences detected at $p<0.05$ with student t-test are indicated with an asterisk '*', slight difference at $p<0.1$ are indicated with a hash '#'. 'SD' indicates the standard deviations of the mean.
Colour codes: Green: Highly expressed (fold change >1.25 in 75th percentile); Yellow: Moderately expressed (0.1>fold change>1.24 in 50th percentile); Red: Repressed (fold change <0 in 25th percentile).

$p<0.05$ and $p<0.1$ levels, respectively. The *ppx* gene was upregulated for 1-fold in treatment 1 and 3.6-fold for treatment 2, t (4) = -2.26, $p=0.04$. The *ppnk* gene was upregulated by 0.6-fold in treatment 1 and 1.9-fold in treatment 2 with low significance of t (4) = -1.9, $p=0.07$. Gene *surE* showed minimal upregulation at 0.1-fold in treatment 1. It was significantly downregulated by 2.7-fold in treatment 2, t (4) = 5.44, $p=0.00$. The fold changes of the *ppk* gene in treatment 1 and the fold changes for all

polyphosphate metabolism genes in treatment 2 were all highly expressed (75th percentile) except for the *surE* gene.

The genome of *B. thuringiensis* Y1 encodes eleven genes associated with the metabolism of polyhydroxyalkanoates. Only the 3-ketoacyl-CoA thiolase (*phaA*) gene upregulated for in both treatments and highly expressed in treatment 2, the majority were downregulated and repressed (25th percentile) (Table 2).
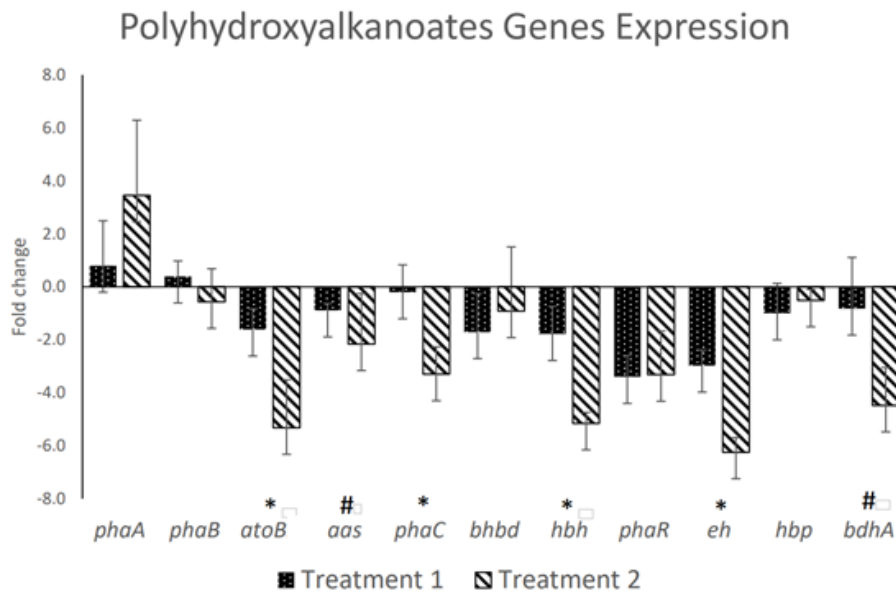
Figure 2: Polyhydroxyalkanoates metabolism genes resulting in fold change during the simulation of copper induced treatment 1 and 2. The significant differences detected at $p<0.05$ with student t-test are indicated with an asterisk '*', slight difference at $p<0.1$ are indicated with a hash '#'. Error bars indicate the standard deviations of the mean.

The *phaA* gene was upregulated at 0.8-fold in treatment 1 and 3.5-fold in treatment 2 but there were no significant differences detected at $p<0.05$ between the means of the two expression levels t (4) = -0.63, $p=0.28$ (Figure 2). The *phaB* gene only showed minor upregulation at 0.4-fold in treatment 1. For treatment 2, the *phaB* gene was downregulated by 0.6-fold. The difference between the two mean fold changes was insignificant, t (4) = 1.42, $p=0.11$. The (*atoB* gene was downregulated by 1.6-fold in treatment 1 and the downregulation increased significantly to 5.3-fold in treatment 2, t (4) = 3.23, $p=0.02$. The genes encoding the enzymes acetoacetyl-CoA synthetase (*aas*) and PHA synthase subunit C (*phaC*) both showed minor downregulation at 0.9-fold and 0.2-fold in treatment 1, respectively. During treatment 2, the *aas* gene was further repressed at 2.2-fold with a slightly significant increment, t (4) = 1.7, $p=0.08$. While *phaC* gene showed a significant increase in downregulation at 3.3-fold in treatment 2, t (4) = 2.97, $p=0.02$. The D-beta-hydroxybutyrate dehydrogenase (*bhbd*) was downregulated during both treatments 1 and 2, with 1.7 and 0.9 average fold change values, respectively. The changes of the mean value of this gene between the two treatments were insignificant, t (4) = 2.13, $p=0.45$. The polyhydroxyalkanoates synthesis repressor, *phaR* as well showed insignificant changes between the two treatments, t (4) = 0.47, $p=0.33$ with downregulation of 3.4-fold and 3.3-fold in treatment 1 and 2, respectively. The 3-hydroxybutyryl-CoA dehydratase gene (*hbh*) on the other hand, was significantly downregulated between the two treatments from 1.8-fold to 5.2-fold, t (4) = 3.81, $p=0.01$. The gene encoding enoyl-CoA hydratase (*EH*) was downregulated in treatment 1 with a repression of 3-

fold and further decline to 6.2-fold of repression in treatment 2. The increment of repression in gene EH was significant at t (4) = 5.68, $p=0.00$. The gene of D-beta-hydroxybutyrate permease, (*hbp*) showed 1-fold of downregulation in treatment 1 and this repression was not statistically significant at 0.5-fold in treatment 2, t (4) = -0.93, $p=0.2$. Finally, the gene for 3-hydroxybutyrylCoA dehydrogenase (*bdha*) was downregulated by 0.8-fold and 4.4-fold in treatment 1 and 2, respectively. The difference between the repression of the two treatment conditions was slightly significant, t (4) = 1.8, $p=0.07$.

DISCUSSION

We speculated that the conversion of ATP via the metabolism of PP is likely to be involved in the supply of energy to the copper homeostasis ATP-dependent system in *B. thuringiensis* Y1 and designed the experiment to identify the genes associated with the PP pathway that were induced by treatment with copper. PP metabolism in bacteria has been linked to the process of adaptation to extreme environments contaminated by copper which is also supported by other studies (Remonsellez *et al.*, 2006; Voica *et al.*, 2016). Inorganic PP represents a reservoir of phosphate residues held together by high energy bonds and PP metabolism can catalyse the interconversion of ADP and ATP through phosphorylation (Nocek *et al.*, 2008). Four genes related to polyphosphate metabolism *ppk*, *ppx*, *surE* and *ppnk* were found to be encoded in the genome of *B. thuringiensis* Y1. The enzyme PPK catalyses the synthesis of PP, whereas the enzymes PPX, SurE and PPNK are involved in the degradation of PP (Doruk *et al.*,

2013). The *surE* gene that was not induced by 0.4 mM copper and was significantly repressed by 0.8 mM copper implied that it is likely to be regulated by a distinct promoter that is not induced by copper and has been reported in similar studies (Terakawa *et al.*, 2016; Yusupova *et al.*, 2020). PPK, responsible for directly regulating the interconversion between ADP and ATP, showed regulation in both treatment 1 and 2. However, increasing the copper sulfate concentration did not significantly enhance its expression level. The findings were highlighted when the copper concentration was increased from 0.4 mM to 0.8 mM, the expression fold changes were significantly increased at $p<0.05$ and $p<0.1$ levels for the *ppx* and *ppnk* genes respectively implying that an increase in the copper concentration had a direct effect on the degradation of PP. The findings of this study that provide evidence for the mediation of copper tolerance by polyphosphate degradation, as reported in a previous study (Grillo-Puertas *et al.*, 2014), by the enzyme exopolyphosphatase (*ppx*) which triggers the release of inorganic phosphate (PI) followed by the formation of the complex of copper (II)-phosphate that is subsequently scavenged by the efflux protein. This mechanism assists the process of bacterial adaptation to the accumulation of copper ions in the cellular medium. PP degradation in the presence of copper has also had been reported by in the acidophilic strain, *Acidithiobacillus ferrooxidans* is resistant to copper and shows a high increase in PPX enzymatic activity when the cells were exposed to copper ions (Alvarez and Jerez, 2004). The *ppnk* gene that encodes the NAD kinase, PPNK, showed a small difference ($p<0.1$) when the copper concentration was increased. PPNK is a key enzyme that converts nicotinamide adenine dinucleotide (NAD) into nicotinamide adenine dinucleotide phosphate (NADP) via the catalysis of ATP into ADP (Agledal *et al.*, 2010). NADP is subsequently involved in the combat of reactive oxidative stress (ROS) in the cell via the redox reaction (Kawai and Murata, 2008) which is a mechanism that can be reverted to when the concentration of copper is in excess (Yu *et al.*, 2008). The mechanism of the phosphorylation of the NAD and NADP involves the removal of inorganic phosphate (Pi) from ATP or polyphosphate (Li *et al.*, 2018) and the breaking of the chemical bonds releases the energy to for the functioning of the multicopper oxidase (CopA) protein, which removes the excess copper ions from the cytoplasmic medium. The upregulation and significant difference detected in *ppx* and *ppnk* genes implies that they are involved in the copper homeostasis mechanism. The enzymes, PPX and PPNK have both been reported to be involved in the degradation of polyphosphate.

In this study, the *ppx* gene was induced by the introduction of copper, which in turn led to the release of inorganic phosphate, Pi, from a complex of copper (II)-phosphate. This complex enables a stabilisation of copper (II) ions, that are subsequently transported out of the cellular medium by the efflux protein. The enzyme PPNK converts the nicotinamide adenine dinucleotide (NAD) into nicotinamide adenine dinucleotide phosphate (NADP)

by breaking down ATP into ADP. The function of NADP involved the amelioration of ROS condition in the cell via the redox reaction. The mechanism of the phosphorylation of the NAD into NADP involves the removal of inorganic phosphate (Pi) from ATP or polyphosphate and breaking of the energy bonds provides energy to the copper homeostasis ATPases protein for the removal of excessive copper ions from the cytoplasmic medium. This study provides cogent evidence for the genetic basis for the role of polyphosphate in the copper induced stress response and further studies involving radioactive labelling of polyphosphates will be necessary to support this finding.

CONCLUSION

The gene expression analysis of the energy storage mechanisms showed that only the polyphosphate metabolism genes from the degradation pathway had reacted positively during the simulated copper stress condition. These findings imply that the breakdown of polyphosphate has a direct relationship with the copper homeostasis mechanism of *B. thuringiensis Y1*. It is likely to undertake the role as the energy supplier source or its pathway may involve in the formation of copper (II)-phosphate complexes that helps in reducing the accumulation of copper ions in the cellular medium. Future studies can focus on the interaction between the metabolism of polyphosphates and copper homeostasis mechanism to determine how it can help with the adaptation of bacteria when exposed to copper induced stress. The elucidation of the genes associated with the metabolism of polyphosphate in *Bacillus* facilitate the exploitation of this pathway for genetic engineering of bacteria with enhanced tolerance to copper stress in the environment. Potential areas of application include bioremediation of copper mine tailings using genetically modified bacteria.

ACKNOWLEDGEMENTS

REFERENCES

Agledal, L., Niere, M. and Ziegler, M. (2010). The phosphate makes a difference: Cellular functions of NADP. *Redox Report* **15(1), 2-10.**

Altimira, F., Yáñez, C., Bravo, G., González, M., Rojas, L. A. and Seeger, M. (2012). Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. *BMC Microbiology* **12, 193.**

Alvarez, S. and Jerez, C. A. (2004). Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*. *Applied and Environmental Microbiology* **70(9), 5177-5182.**

Argüello, J. M., Raimunda, D. and Padilla-Benavides, T. (2013). Mechanisms of copper homeostasis in bacteria. *Frontiers in Cellular and Infection Microbiology* 3, 73.

Ball, S., Colleoni, C., Cenci, U., Raj, J. N. and Tirtiaux, C. (2011). The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. *Journal of Experimental Botany* 62(6), 1775-1801.

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J. *et al.* **(2015).** *RAST*tk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific Reports* 5(1), 8365.

Chandra, G., Chater, K. F. and Bornemann, S. (2011). Unexpected and widespread connections between bacterial glycogen and trehalose metabolism. *Microbiology* 157(6), 1565-1572.

Doruk, T., Avican, U., Camci, I. Y. and Gedik, S. T. (2013). Overexpression of polyphosphate kinase gene (*ppk*) increases bioinsecticide production by *Bacillus thuringiensis*. *Microbiological Research* 168, 199-203.

Dupont, C. L., Grass, G. and Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance – New sights and applications. *Metallomics* 3(11), 1109-1118.

Grillo-Puertas, M., Schurig-Briccio, L. A., Rodríguez-Montelongo, L., Rintoul, M. R. and Rapisarda, V. A. (2014). Copper tolerance mediated by polyphosphate degradation and low-affinity inorganic phosphate transport system in *Escherichia coli*. *BMC Microbiology* 14, 72.

Hassen, A., Saidi, N., Cherif, M. and Boudabous, A. (1998). Effects of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. *Bioresource Technology* 65(1-2), 73-82.

Kawai, S. and Murata, K. (2008). Structure and function of NAD kinase and NADP phosphatase: Key enzymes that regulate the intracellular balance of NAD(H) and NADP(H). *Bioscience, Biotechnology and Biochemistry* 72(4), 919-930.

Kumar, V., Singh, S., Kashyap, N., Singla, S., Bhadrecha, P., Kaur, P. *et al.* **(2015).** Bioremediation of heavy metals by employing resistant microbial isolates from agricultural soil irrigated with industrial waste water. *Oriental Journal of Chemistry* 31(1), 357-361.

Ladomersky, E. and Petris, M. J. (2015). Copper tolerance and virulence in bacteria. *Metallomics* 7(6), 957-964.

Li, B. B., Wang, X., Tai, L., Ma, T. T., Shalmani, A., Liu, W. T. *et al.* **(2018).** NAD kinases: Metabolic targets controlling redox co-enzymes and reducing power partitioning in plant stress and development. *Frontiers in Plant Science* 9, 379.

Marandi, R. (2012). Bioextraction of Cu (II) ions from acid mine drainage by *Bacillus Thuringiensis*. *International Journal of Biological Engineering* 1(1), 11-17.

May, K. L., Lehman, K. M., Mitchell, A. M. and Grabowicz, M. (2019). A stress response monitoring lipoprotein trafficking to the outer membrane. *mBio* 10(3), e00618-19.

Murphy, J. N. and Saltikov, C. W. (2009). The ArsR repressor mediates arsenite-dependent regulation of arsenate respiration and detoxification operons of *Shewanella* sp. strain ANA-3. *Journal of Bacteriology* 191(21), 6722-6731.

Nocek, B., Kochinyan, S., Proudfoot, M., Brown, G., Evdokimova, E., Osipiuk, J. *et al.* **(2008).** Polyphosphate-dependent synthesis of ATP and ADP by the family-2 polyphosphate kinases in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 105(46), 17730-17735.

Orell, A., Navarro, C. A., Rivero, M., Aguilar, J. S. and Jerez, C. A. (2012). Inorganic polyphosphates in extremophiles and their possible functions. *Extremophiles* 16(4), 573-583.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T. *et al.* **(2014).** The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Research* 42(D1), D206-D214.

Oves, M., Khan, M. S. and Zaidi, A. (2013). Biosorption of heavy metals by *Bacillus thuringiensis* strain OSM29 originating from industrial effluent contaminated north Indian soil. *Saudi Journal of Biological Sciences* 20(2), 121-129.

Reiter, L., Kolstø, A. B. and Piehler, A. P. (2011). Reference genes for quantitative, reverse-transcription PCR in *Bacillus cereus* group strains throughout the bacterial life cycle. *Journal of Microbiological Methods* 86(2), 210-217.

Remonsellez, F., Orell, A. and Jerez, C. A. (2006). Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus*: Possible role of polyphosphate metabolism. *Microbiology* 152(1), 59-66.

Riedel, G., Rüdrich, U., Fekete-Drimusz, N., Manns, M. P., Vondran, F. W. R. and Bock, M. (2014). An extended ΔCT-method facilitating normalisation with multiple reference genes suited for quantitative RT-PCR analyses of human hepatocyte-like cells. *PLoS ONE* 9(3), e93031.

Santo, C. E., Morais, P. V. and Grass, G. (2010). Isolation and characterization of bacteria resistant to metallic copper surfaces. *Applied and Environmental Microbiology* 76(5), 1341-1348.

Schmittgen, T. D. and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols* 3(6), 1101-1108.

Terakawa, A., Natsume, A., Okada, A., Nishihata, S., Kuse, J., Tanaka, K. *et al.* **(2016).** *Bacillus subtilis* 5′-nucleotidases with various functions and substrate specificities. *BMC Microbiology* 16, 249.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R. and Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35(Suppl 2), W71-W74.

Voica, D. M., Bartha, L., Banciu, H. L. and Oren, A. (2016). Heavy metal resistance in halophilic bacteria and archaea. *FEMS Microbiology Letters* **363(14), fnw146.**

Wang, L. and Wise, M. J. (2011). Glycogen with short average chain length enhances bacterial durability. *The Science of Nature* **98, 719.**

Wang, L., Liu, Q., Wu, X., Huang, Y., Wise, M. J., Liu, Z. et al. (2019). Bioinformatics analysis of metabolism pathways of archaeal energy reserves. *Scientific Reports* **9, 1034.**

Wang, L., Liu, Z., Dai, S., Yan, J. and Wise, M. J. (2017). The sit-and-wait hypothesis in bacterial pathogens: A theoretical study of durability and virulence. *Frontiers in Microbiology* **8, 2167.**

Wang, L., Yan, J., Wise, M. J., Liu, Q., Asenso, J., Huang, Y. et al. (2018). Distribution patterns of polyphosphate metabolism pathway and its relationships with bacterial durability and virulence. *Frontiers in Microbiology* **9, 782.**

Wilkinson, J. F. (1959). The problem of energy-storage compounds in bacteria. *Experimental Cell Research* **7, 111-130.**

Yu, Z. L., Zhang, J. G., Wang, X. C. and Chen, J. (2008). Excessive copper induces the production of reactive oxygen species, which is mediated by phospholipase D, nicotinamide adenine dinucleotide phosphate oxidase and antioxidant systems. *Journal of Integrative Plant Biology* **50(2), 157-167.**

Yusupova, Y. R., Skripnikova, V. S., Kivero, A. D. and Zakataeva, N. P. (2020). Expression and purification of the 5'-nucelotidase YitU from *Bacillus* species: Its enzymatic properties and possible applications in biotechnology. *Applied Microbiology and Biotechnology* **104, 2957-2972.**