Extraction and detection of guanosine 5'-diphosphate-3'-diphosphate in *Clavibacter michiganensis* cells

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Abstract

Guanosine 5'-diphosphate-3'-diphosphate (ppGpp) is a small molecule nucleotide alarmone that can accumulate under the amino acid starvation state and trigger the stringent response. This study reported the extraction of ppGpp from the Gram-positive bacteria *Clavibacter michiganensis* (Cm) through methods using formic acid, lysozyme, or methanol. Following extraction, ppGpp was detected through ultra-high-performance liquid chromatography (UHPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The methanol method showed the highest extraction efficiency for ppGpp among the three methods tested. Amino acid starvation was induced in *C. michiganensis* cells during logarithmic phase using serine hydroxamate (SHX). ppGpp was extracted and detected at 0 min, 15 min, 30 min and 1 h. When using the methanol extraction method, the results show that ppGpp concentrations in SHX-treated samples were 15.645 nM, 17.656 nM, 20.372 nM and 19.280 nM at 0 min, 15 min, 30 min and 1 h, respectively, when detected using LC-MS/MS. This is the first report on ppGpp extraction and detection in *Clavibacter* providing a new idea and approach for nucleotide detection and extraction in bacteria.

Key Points

Methanol was the optimum extraction method for ppGpp when compared to formic acid and lysozyme

UHPLC and LC-MS/MS method were developed for detecting ppGpp

ppGpp could be extracted and detected in *Clavibacter michiganensis* cells under SHX treatment

Introduction

Guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp) (collectively referred to as (p)ppGpp) is a small molecule nucleotide alarmone first reported in *Escherichia coli* in 1969 (Cashel and Gallant 1969). It can accumulate under various environmental stresses and trigger the stringent response (Lu et al. 2012; Potrykus and Cashel 2008). (p)ppGpp influences transcription, translation, replication, and metabolism in bacteria (Gupta et al. 2016; Hallez et al. 2017; Liu et al. 2015; Magnusson et al. 2005; Ross et al. 2013; Srivatsan and Wang 2008). Enzymes related to (p)ppGpp metabolism can be divided into three major groups: long RelA/SpoT homologue proteins (RSH), small alarmone synthetases (SAS) and small alarmone hydrolases (SAH) (Atkinson et al. 2011). RSH contains both synthetase and hydrolase domains. SAS and SAH only harbor the synthetase domain or the hydrolase domains, respectively (Atkinson et al. 2011). RSH and SAS can catalyze ppGpp/pppGpp synthesis from phosphate group of ATP to 3' hydroxide radical of GDP/ GTP (Xiao et al. 1991).

pppGpp can be converted to ppGpp by pppGpp phosphatase (GppA) and other GTPases (Hamel and Cashel 1973; Hauriuk et al. 2015). It was reported that ppGpp is more potent than pppGpp with respect to regulation of growth rate and stress responses in *Escherichia coli* (Mechold et al. 2013). Many studies

have demonstrated that ppGpp can be extracted from bacterial cells using formic acid (Nanamiya et al. 2007; Ochi 1986; Ochi et al. 1981; Washio et al. 2010). However, this extraction method resulted in ppGpp degradation to ppGp and GDP *in vitro* (Lagosky and Chang 1978). Other extraction methods for ppGpp have used lysozyme or methanol (Chatnaparat et al. 2015a; Chatnaparat et al. 2015b; Lagosky and Chang 1978). These methods show a significant in ppGpp degradation when extraction conditions have neutral pH.

ppGpp was first noticed when thin layer chromatography was used to detect the change in nucleotide concentrations when *E. coli* cells were starved for amino acids (TLC) (Cashel and Gallant 1969) and was detected by the isotope [³²P] in a radioimmunoassay. High performance liquid chromatography (HPLC) was widely used to detect ppGpp by UV absorption starting since 1980s (Ochi et al. 1981). Extracted ppGpp from bacterial cells was eluted by a gradient buffer of low to high ionic strength. The loading of 50–250 µL of sample resulted in a large amount of sample waste in previous studies using HPLC for detection of ppGpp (Fischer et al. 1982; Nanamiya et al. 2007; Ochi 1986; Ochi et al. 1981; Traxler et al. 2008; Washio et al. 2010). Another drawback of detecting ppGpp using HPLC is difficulty in distinguishing the target peak due to background absorption. As a result, LC-MS/MS was accepted to determine ppGpp according to the characteristic ion peak (m/z 604→ m/z 150) and detection limit of 2 nmol/mL at OD₆₀₀ (Chatnaparat et al. 2015a; Chatnaparat et al. 2015b; Kriel et al. 2012). The concentration of ppGpp was very low in the bacterial cells used in these studies, around tens or hundreds of pmoles/mL/OD (Lagosky and Chang 1978; Ochi et al. 1981; Traxler et al. 2008; Zhang et al. 2013). Therefore, sensitive extraction and detection methods of ppGpp are required for its functional analysis in bacteria.

Clavibacter michiganensis (Cm) is the causal agent of bacterial canker in tomato. As a typical seed-borne bacteria, it has caused substantial losses in tomato and seed production (Eichenlaub and Gartemann 2011). When bacteria are experiencing stress conditions, they can be induced into sporulation, dormancy, persistence, and viable but non-culturable (VBNC) state (Pinto et al. 2013). The bacterial cells can be recovered or resuscitated from harsh stress by suitable environmental conditions, allowing the resuscitated cells become culturable. It has been reported that Cm could be induced into VBNC state under stress conditions of low pH and Cu²⁺, and then resuscitated by addition of nutrient or inoculation *in planta* (Jiang et al. 2016). It is well known that ppGpp can accumulate and act as an alarmone molecule in the regulation of growth and survival in bacteria when adverse conditions are encountered. However, there is no report on ppGpp production and its functional analysis in Cm. As a representative of Gram-positive phytopathogenic bacteria, it is necessary to study the function of ppGpp in Cm under stress conditions, especially under the starvation state.

In this study, we established a method to detect the level of ppGpp by UHPLC and LC-MS/MS and developed a suitable method for extracting ppGpp from Cm cells. All these methods were used to analyze the ppGpp level of Cm under amino acid starvation during log-phase. To our knowledge, this is the first study on extraction and quantification of ppGpp in Cm, as well as in Gram-positive phytopathogenic bacteria.

Materials And Methods

Strain and culture conditions

Clavibacter michiganensis (Cm) strain BT0505 (CICC 24944) was isolated from an infected tomato plant in Inner Mongolia, China in 2005 and kept at -80°C at the China Agricultural University. Cm was grown in LB medium at 28°C for 72 h. ppGpp standard was purchased from TriLink Biosciences (Sigma, N6001-T1KX01A). Serine hydroxamate was purchased from Sigma (Sigma, S4503), and used to induce amino acid starvation in Cm cells.

Development of standard curve for detecting of ppGpp by UHPLC

The sample was separated by ion pair reverse phase UHPLC (UHPLC 1290 Infinity II, Agilent) using a 150 mm×3 mm Agilent Zorbax Eclipse Plus C18 column (1.8 µm particle size). Mobile phase A consisted of 5% methanol and 95% K₂HPO₄-KH₂PO₄ buffer (0.1 mol/L, pH 7.0) with 10 mmol/L tetrabutyl ammonium bromide (Washio et al. 2010). Each 20 µL sample was injected at a flow rate of 0.2 mL/min. Signals were detected by UV absorbance at 254 nm. ppGpp standard stock solution was diluted to 0.5 µM, 5 µM, 10 µM, 20 µM, 40 µM and 80 µM to establish the standard curves. The standard curve was made by plotting the peak area against its corresponding concentration.

Development of standard curve for detecting of ppGpp by LC-MS/MS

An Agilent 1290 (Agilent, USA) liquid chromatography equipped with a QQQ (Agilent 6460, USA) tandem mass spectrometer system was used in ppGpp detection. Mass spectra was acquired under positive electrospray ionization. Characteristic ion peak of ppGpp (m/z 604.1- m/z 152.0) was detected (Chatnaparat et al. 2015a; Chatnaparat et al. 2015b).

An ACQUITY UPLC® HSS T3 column (2.1 mm × 50 mm, particle size 1.8 µm) was used for LC separation, using gradient elution with methanol was solvent A, and 10 mM ammonium acetate and 0.02% ammonium hydroxide in water as solvent B. The gradient program was as follows: 0–3 min 0% A, 3–5 min 0% A to 90% A, 5–6 min 90% A, 6–6.1 min 90% A to 0% A, 6.1–10 min 0% A. The flow rate was set at 0.2 mL/min, and the injection volume was 10 µL. The total run time was 10 min for each sample.

The positive ion mode was used for the detection of ppGpp by LC-MS/MS. The mass spectrometer was operated in both electrospray ionization mode and in multiple-reaction monitoring mode using the following main working parameters: the nebulizer was 45 psi, the capillary was 4000V, and high-purity nitrogen served as the nebulizing dry gas and was held at a temperature of 350°C at a flow of L/min.

The method of establishing the standard curve was same as UHPLC. The ppGpp standard stock solution was diluted to 1 nM, 5 nM, 10 nM, 50 nM, 100 nM and 200 nM to establish the standard curves, and the standard curve was made by plotting the peak area against its corresponding concentration.

Recovery rate of ppGpp standard sample by three extraction methods

ppGpp standard at 10 μ M was “extracted” using formic acid, lysozyme, or methanol. The concentration of ppGpp “extracted” using each method was determined by UHPLC. Ten micromoles per liter of ppGpp standard was added as a control for estimating the recovery rate.

The formic acid method was used as previously described in Ochi 1986. One milliliter of 10 μ M ppGpp standard sample was immediately transferred into a new tube containing 0.1 mL of 0.5 M formic acid. The liquid was then vigorously mixed and incubated on ice for 30 min. The samples were centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was then filtered through 0.22 μ m filters and stored at -20°C until quantification.

For the lysozyme (Lysozyme from chicken egg white, Sigma) method, 1 mL of 10 μ M ppGpp standard sample with 50 mg/mL lysozyme was incubated at 28°C for 30min. The supernatant was then filtered through 0.22 μ m filters and stored at -20°C until quantification (Lagosky and Chang 1978).

As for the methanol method (Chatnaparat et al. 2015b), the 200 μ L of 10 μ M ppGpp standard sample was transferred into a new tube containing 2 mL 100% cold methanol. The sample was vortexed for 50 s. Then the suspension was frozen in liquid nitrogen and thawed on ice. The bacterial suspension was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and stored on ice. Then the pellet was resuspended in 2 mL 100% cold methanol. The freeze-thaw cycle was repeated. Supernatants extracted by methanol were combined and filtered through 0.22 μ m filters. The filtrate was dried by centrifugation or N₂. The sample was resuspended in 200 μ L distilled water and stored at -20°C until UHPLC quantification.

Recovery rate of ppGpp standard from Cm bacterial suspension

ppGpp standard was added to Cm bacterial suspensions and extracted using formic acid method, lysozyme method, and methanol method. Then concentration of ppGpp extracted using each method was determined by UHPLC. Twenty micromoles per liter of ppGpp standard sample was added as a control for estimating the recovery rate.

Cm cells were grown on LB medium for 72 h. A single colony was transferred into 10 mL LB broth for 20 h incubation to log-phase at 28°C while shaking (120 rpm). The ppGpp standard was added into Cm suspension at a final concentration of 20 μ M and extracted from 1 mL Cm bacterial suspension using formic acid or lysozyme. For the methanol extraction, ppGpp was extracted from 1 mL Cm bacterial suspension. 200 μ L of 20 μ M ppGpp standard sample was added, and the extracted ppGpp samples were resuspended in 200 μ L distilled water and stored at -20°C until UHPLC analysis. All details of extraction methods were similar to above.

Extraction of ppGpp from starvation state Cm cells

Serine hydroxamate (SHX) was used to induce amino acid starvation in the Cm cells at a final concentration of 0.8 g/L.

The log-phase Cm cells were prepared as above. Eight hundred milliliters of Cm cells ($OD_{580} = 0.5$) was divided into four aliquots of 200 mL each. All aliquots were centrifuged at 8000 rpm for 15 min. The pellet in each aliquot was then resuspended in 50 mL M9 broth. SHX was added at a final concentration of 0.8 g/L to all aliquots and cultured for 0 min, 15 min, 30 min, and 1 h at 28°C, respectively (Zhang et al. 2013). Following SHX treatment, the Cm cells were harvested and used for ppGpp extraction immediately (Chatnaparat et al. 2015b).

ppGpp was extracted by methanol method as described above. The 50 mL SHX treated bacterial suspension was harvested by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was immediately suspended in 2 mL of 100% cold methanol and vortexed for 50 s. The suspension was then frozen in liquid nitrogen and thawed on ice. This suspension was centrifuged at 4000 rpm for 10 min at 4°C prior to supernatant collection in a new tube and storage on ice. The pellet was resuspended by 2 mL 100% cold methanol. This entire process was repeated, and both supernatants extracted by methanol were combined and filtered through 0.22 µm filters. The filtrate was dried by centrifugation. The sample was resuspended in 200 µL of distilled water and stored at -20°C until LC-MS/MS analysis.

Results

Determination of ppGpp by UHPLC and development of standard curve for quantification

The new method for detection of ppGpp by UHPLC was established. The ppGpp standard sample produced a chromatogram with a single and symmetrical peak, eluting at ≈ 13.1 min (Fig. 1). The retention time was optimal to avoid the influence of early solvent peaks. Standard curves were developed using different concentrations of ppGpp with a detection limit of 0.5 µM (Fig. 2). The correlation coefficient of the standard curve was 0.9997.

Determination of recovery rates of ppGpp by three extraction methods

ppGpp standard (10 µM) was used in formic acid, lysozyme and methanol extraction procedures, then detected by UHPLC for estimating the recovery rate. Twenty microliters of a 10 µM ppGpp standard without any treatment was injected and used as positive control. The recoveries of ppGpp were 41.71%, 62.23%, 83.08% and 86.15% extracted by formic acid, lysozyme, methanol with drying by N₂ and methanol with drying by centrifugation, respectively, according to the peak area (Table 1). The results indicate that the level of ppGpp was highest when extracted with methanol followed by centrifugation.

Furthermore, different extraction procedures were used to extract ppGpp from the bacterial suspension which added the ppGpp standard to achieve the final concentration of 20 µM for formic acid and lysozyme extraction procedures, whereas for methanol extraction procedure which was added 200 µL of 20 µM ppGpp standard. Twenty microliters of 20 µM ppGpp standard was used as positive control. The recoveries were evaluated by calculating peak area and compared with the positive control. The recovery rates were 44.94%, 89.42%, 116.50% and 117.34% extracted by formic acid, lysozyme, methanol with drying by N₂ and methanol with drying by centrifugation, respectively (Table 2). Combined with the above

results, the data indicate that methanol extraction followed by centrifugation is optimal for ppGpp extraction.

Extraction and detection of ppGpp from Cm cells by UHPLC

ppGpp was extracted using the methanol method from Cm cells treated with SHX. Unfortunately, ppGpp concentration has not been determined by UHPLC without the addition of ppGpp standard to the cells. Only other substances were visible in the chromatogram (Fig. 3). However, the ppGpp standard, added as positive control at a final concentration of 20 μ M to the Cm bacterial suspension, was successfully extracted and detected by UHPLC. It is likely that the concentration of ppGpp in Cm cells was too low to be detected by UHPLC. A detection method with higher sensitivity, such as LC-MS/MS, is necessary for further analysis.

Detection of ppGpp from Cm cells by LC-MS/MS

The low concentration of ppGpp extracted from Cm cells could not be evaluated, because of the detection sensitivity of UHPLC. As a result, the LC-MS/MS method was used instead. The standard curve for this method was established using different concentrations of ppGpp. The detection limit of ppGpp was 0.5 nM and the correlation coefficient of the standard curve was 0.9991 (Fig. 4).

In the SHX treated Cm cells, ppGpp was extracted with methanol and detected by the LC-MS/MS method established above. The concentrations of ppGpp were 15.645, 17.656, 20.372 and 19.280 nM at 0 min, 15 min, 30 min and 1 h SHX treated samples, respectively (Fig. 5).

Discussion

Before chromatographic conditions were identified, the influence on peak area with different concentrations of mobile phase, injection volume and flow rate were compared. For UHPLC analysis, mobile phase A consisted of 5% methanol and 95% K_2HPO_4 - KH_2PO_4 buffer (0.1 mol/L, pH 7.0) with 10 mmol/L tetrabutyl ammonium bromide, and each 20 μ L sample was injected at a flow rate of 0.2 mL/min. The data show that these parameters result in greater peak area (data not shown), but also more interference. Therefore, according to the pressure resistance of the instrument and the peak shape parameter, the detection method was optimized as described above. Comparing to previous reports, injection volume (20 μ L) was smaller than former studies (50–250 μ L) (Fischer et al. 1982; Nanamiya et al. 2007; Ochi 1986; Ochi et al. 1981; Traxler et al. 2008; Washio et al. 2010), which means this method is time saving and more efficient. Thus, we have established an optimal method to detect ppGpp by UHPLC. The concentration of ppGpp was very low in the bacterial cells, about 10–100 pmoles/mL/OD (Chatnaparat et al. 2015b; Ihara et al. 2015; Jin et al. 2018; Traxler et al. 2008; Zhang et al. 2013). This low concentration made detection by UHPLC difficult at the detection limit was 0.5 μ M. Although the optimal extraction method was identified by UHPLC, UHPLC was not suitable to detect ppGpp extracted from bacterial suspension due to the extremely low level (Fig. 3A). Furthermore, a component of the bacterial suspension had a similar absorbance to ppGpp, leading to greater interference when detected by

UV at 254 nm. The LC-MS/MS method was more suitable to detect ppGpp from bacterial cells because of its high sensitivity, as the detection limit was 0.5 nM. This detection limit was lower than the method described previously, which indicated that lower concentration of ppGpp could be evaluated from Cm cells. Furthermore, LC-MS/MS was both qualitative and quantitative by detection of characteristic peaks (m/z 150) (Chatnaparat et al. 2015a; Chatnaparat et al. 2015b), resulting in more accurate detection of ppGpp levels.

Most papers described that ppGpp could be extracted by formic acid (Jin et al. 2018; Nanamiya et al. 2007; Ochi 1986; Ochi et al. 1981; Washio et al. 2010). However, it was reported that ppGpp is more likely to degrade into ppGp and GDP *in vitro* when formic acid was present, than it would at neutral pH (lysozyme) (Lagosky and Chang 1978). Here, we also tried to extract ppGpp by using lysozyme which lyse bacteria cells and release metabolite. Indeed, the extraction efficiency of lysozyme was greater than that of formic acid. However, the recovery rate of the lysozyme procedure was smaller than the methanol method. It could be deduced that the concentration of lysozyme was too low to destroy cell wall sufficiently, and was therefore not the ideal way to extract ppGpp from bacterial cells. Comparison of the three extraction methods showed that, the methanol method was optimum for ppGpp extraction (Tables 1 and 2).

Treatment with SHX can induce an amino acid starvation state in bacteria, which can trigger the accumulation of ppGpp (Brockmann-Gretza and Kalinowski 2006). Our data shows ppGpp can be extracted by methanol and be detected by LC-MS/MS in Cm cells at amino acid starvation state (Fig. 5). The concentration of ppGpp was highest at 30 min treated by SHX. The tendency of change in concentration of ppGpp over time was similar to the previous report, which was firstly increased and then decreased (Chang et al. 2002; Traxler et al. 2008; Zhang et al. 2013). As a result, ppGpp likely played a role in adjusting the metabolic balance under adverse conditions in Cm cells, allowing cellular resources to be redirected towards essential activities for bacterial survival.

In this study, we have determined the optimal method for the extraction and detection of ppGpp in *Clavibacter michiganensis*. Further research is under way to evaluate the hypothesis that this extraction and detection method can be applied to other nucleotides generally found in bacteria.

Abbreviations

ppGpp: guanosine 5'-diphosphate-3'-diphosphate; pppGpp: guanosine 5'-triphosphate-3'-diphosphate; Cm: *Clavibacter michiganensis*; UHPLC: ultra-high-performance liquid chromatography; LC-MS/MS: liquid chromatography-tandem mass spectrometry; SHX: serine hydroxamate; VBNC: viable but non-culturable.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval and consent to participate

Not applicable

Consent for publication

All authors agree to publish.

Availability of data and materials

The raw data is without undue reservation. The data and material can be available from the authors.

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Author contributions

LL, JL and NJ conceived and designed the research. KB wrote this manuscript and analyzed the data. LL and QL revised the manuscript. KB and XC conducted the experiments. All authors read and approved the final manuscript.

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Tables

Table 1 Recovery of ppGpp standard with different extraction procedures

Extraction procedure	Recovery rate (%)
0.5 M formic acid	41.71 ^c
50 mg/mL lysozyme	62.23 ^b
Methanol*	83.08 ^a
Methanol**	86.15 ^a

Note: Extraction of ppGpp from 10 μM ppGpp standard, and 10 μM ppGpp standard was used as the positive control. The letters (a-c) indicated significant difference (P< 0.05) by Duncan’s multiple range test. *, Drying method with N₂; **, Drying method with centrifugation.

Table 2 Recovery of ppGpp from Cm bacterial suspension which added standard and extracted with different procedures

Extraction procedure	Recovery (%)
0.5 M formic acid	44.94 ^b
50 mg/mL lysozyme	89.42 ^a
Methanol*	116.50 ^a
Methanol**	117.34 ^a

Note: Extraction of ppGpp from bacteria solution with 20 μM ppGpp standard samples. The letters (a-b) indicated signifcant difference (P< 0.05) by Duncan’s multiple range test. *, Drying method with N₂; **, Drying method with centrifugation.

Figures

Figure 1

The chromatogram of ppGpp standard spiked at the level of 10 μ M by UHPLC. The peak appeared at $\approx$ 13.1 min. a, 10 μ M ppGpp standard. b, distilled water as the blank control.

Figure 2

The standard curve of ppGpp determined by UHPLC. Correlation coefficient of standard curve was 0.9997. The error bars represented the mean of two biological replicates $\pm$ SD. The blue spots represent ppGpp standard sampling points.

Figure 3

Detection of ppGpp from bacterial suspension of *Clavibacter michiganensis* by UHPLC. a, The chromatogram of UHPLC. ppGpp levels were detected from bacteria solution treated with SHX at 0 min (with and without addition of 20 μ M ppGpp standard sample) and 15 min, and from 20 μ M ppGpp standard. The all peaks were overlapped together. The suspected target peak was marked by read box. b, Enlarged view of suspected target peak. Blue line: treated with SHX at 0 min and no ppGpp standard addition; Red line: 20 μ M ppGpp standard; Green line: treated with SHX at 0 min and added 20 μ M ppGpp standard; Purple line: treated with SHX at 15 min and no ppGpp standard addition.

Figure 4

The standard curve of ppGpp detection by LC-MS/MS. The error bars represent the mean of two biological replicates $\pm$ SD. The blue spots represent ppGpp standard sampling points.

Figure 5

Level of ppGpp in *Clavibacter michiganensis* cells detected by LC-MS/MS. ppGpp level was detected from bacterial suspension with SHX induction at different time points (0 min, 15 min, 30 min and 1 h). The final concentration of SHX was 0.8 g/L. The letters (a–c) indicate significant difference ($P < 0.05$) by Duncan's multiple range test.