**Original Article**

# Use of Rich BHI Medium Instead of Synthetic TMH Medium for Gene Regulation Study in *Yersinia pestis*[*]

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**Abstract**

**Objective** This study is to verify the use of rich BHI medium to substitute synthetic media for gene regulation studies in *Yersinia pestis*.

**Methods** The transcriptional regulation of *rovA* by PhoP or via temperature upshift, and that of *pla* by CRP were investigated when *Y. pestis* was cultured in BHI. After cultivation under 26 °C, and with temperature shifting from 26 to 37 °C, the wild-type (WT) strain or its *phoP* or *crp* null mutant (*ΔphoP* or *Δcrp*, respectively) was subject to RNA isolation, and then the promoter activity of *rovA* or *pla* in the above strains was detected by the primer extension assay. The *rovA* promoter-proximal region was cloned into the pRW50 containing a promoterless *lacZ* gene. The recombinant LacZ reporter plasmid was transformed into WT and *ΔphoP* to measure the promoter activity of *rovA* in these two strains with the β-Galactosidase enzyme assay system.

**Results** When *Y. pestis* was cultured in BHI, the transcription of *rovA* was inhibited by PhoP and upon temperature upshift while that of *pla* was stimulated by CRP.

**Conclusion** The rich BHI medium without the need for modification to be introduced into the relevant stimulating conditions (which are essential to triggering relevant gene regulatory cascades), can be used in lieu of synthetic TMH media to cultivate *Y. pestis* for gene regulation studies.

**Key words:** *Yersinia pestis*; BHI; TMH; Gene regulation

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## INTRODUCTION

Plague is a fatal infectious disease caused by *Yersinia pestis*. *Y. pestis* is cycled among flea vectors and rodent reservoirs in natural plague foci, and it also infects humans by bite of infected flea, contact with infected tissues, or inhalation of respiratory droplets or aerosols, during which the expression of virulence/transmission-required genes is tightly regulated[1-3]. PhoP is the

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response regulator of the two-component regulatory system PhoP-PhoQ, and its activity is activated by low $Mg^{2+}$ signals through the PhoQ sensor[4]. In *Y. pesits*, PhoP directly stimulates a set of stress-responsive genes[5-6], which promotes the intracellular growth of *Y. pestis* at early stages of systemic infection[5-9]. The *rovA* gene in *Y. pesits* encodes the virulence regulator RovA that is required for the expression of the pH6 antigen[10] and for the secretion of YOP effectors of the Type III secretion system (T3SS)[11]. Two promoters P2 and P1 are transcribed for *rovA* in *Y. pestis*[12]. The temperature shift from 26 to 37 ℃ down-regulates both of P2 and P1 at the stationary growth phase, whereas the down-regulatory effect occurs only for P2 at the middle-exponential phase[12]. PhoP directly represses the transcription of *rovA* in *Y. pestis* when it grows under low magnesium conditions[12]. cAMP receptor protein (CRP) binds to its sole cofactor cAMP to form the CRP-cAMP complex, and this complex can bind to the target promoters to regulate a large set of target genes in bacteria[13]. CRP is also a virulence-required regulator of *Y. pestis*[14], and it directly stimulates the virulence gene *pla* encoding the plasminogen activator[14-15]. CRP still represses the *sycO-ypkA-yopJ* operon encoding the components of T3SS[16].

TMH[17] is a chemically defined medium containing carbohydrates, amino acids, vitamins, salts, and others, and it provides balanced nutrients for the growth of *Y. pestis*. Since one or more constituents can be conveniently removed from or added into TMH, different modified TMH media were used for *Y. pestis* cultivation in our previous gene regulation studies[1,5,7,11-12,14,16,18-25], which included those describing the above-mentioned temperature/PhoP/CRP-dependent regulatory circuits. However, the preparation of TMH in laboratory conditions is extremely laborious due to its very complex composition of ingredients. In addition, the environmental perturbations (e.g. low magnesium conditions) introduced for stimulating the function of relevant regulators (e.g. PhoP) are often suboptimal for the normal bacterial growth[12]. The present work verified that the rich Brain Heart Infusion (BHI) medium could be used in lieu of TMH to cultivate bacteria for gene regulation study in *Y. pestis*, since the nature of PhoP/temperature-dependent transcription of *rovA* and CRP-dependent transcription of *pla,* remained identical no matter whether BHI or TMH was used for bacterial cultivation.

## MATERIALS AND METHODS

### *Bacterial Strains and Growth*

The wild-type (WT) *Y. pestis* strain 201 (biovar *Microtus*), which was avirulent to humans but highly virulent to mice, was isolated from *Microtus brandti* in Inner Mongolia, China[26]. The base pairs 41 to 631 of *phoP* (672 in total) or the entire region of *crp* were replaced with the kanamycin resistant cassette by using the one-step inactivation method based on the lambda red phage recombination system with the helper plasmid pKD46[27]. This generated the *phoP* and *crp* mutants of *Y. pestis*, designated as *ΔphoP*[5,7,12] and *Δcrp*[14,16,18-19], respectively. Chromosomal integration of the mutagenic cassette was confirmed by PCR and sequencing with oligonucleotides external to the integrated cassette. The elimination of pKD46 in the mutants was verified by PCR. All primers used in this study are listed in Table 1.

The BHI broth contains 3.7% Bacto™ Brain Heart Infusion (BD Biosciences). Overnight cell culture with an optical density ($OD_{620}$) of about 1.0 in BHI was diluted at 1:20 into 18 mL of fresh BHI, and the bacteria were allowed to grow at 26 °C with shaking at 230 rpm. For the determination of bacterial growth curves, the $OD_{620}$ values were monitored for each culture with a 2 h interval. For the primer extension or LacZ fusion assay of the PhoP or CRP regulation, cells were harvested at the middle-exponential phase. To determine the effect of temperature upshift on gene transcription, cell cultures were divided equally at about 3h before entering the middle-exponential phase; and then, half of the cell cultures were incubated at 37 °C for 3 h, and the remaining half were allowed to grow continuously at 26 °C for 3 h; the above cultures undergoing different treatments were designated as “shift from 26 to 37 °C” and “maintaining 26 °C continuously”. Cells were cultivated at least in triplicate (biological replicates) for each strain under different conditions.

### *Primer Extension Assay*

Total bacterial RNAs were extracted with the TRIzol Reagent (Invitrogen)[5,14]. Immediately before harvesting, bacterial cultures were mixed with two pieces of the RNAprotect Reagent (Qiagen) to minimize RNA degradation. RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was determined by spectrophotometry. For

the primer extension assay[5,14], the oligonucleotide primer(s) complementary to the RNA transcript of each target gene was employed to synthesize cDNAs from the RNA templates. About 10 μg of the total RNA from each strain under different conditions was annealed with 1 pmol of [γ-$^{32}$P] end-labeled reverse primer and by using a Primer Extension System (Promega) according to the manufacturer's instructions. The same labeled primer was also used for sequencing with the fmol® DNA Cycle Sequencing System (Promega). The primer extension products and sequencing materials were concentrated and analyzed in a 6% polyacrylamide/8 (mol/L) urea gel. The result was detected by autoradiography (Kodak film).

**Table 1.** Oligonucleotide Primers Used in This Study

| Target | Primers (forward/reverse, 5'-3') |
|---|---|
| | **Gene mutation** |
| *phoP* | ATGCGGGTTCTGGTTGTGGAAGATAACGCG |
| | TTGTTGCGTCAGTTGTGTCTCAAAATCTCTG/ |
| | CTAGTTGACGTCAAAACGATATCCCTGACCA |
| *crp* | CGAATAGTCGAAAGCCGCCGTCCCGTCAAG |
| | TGTTAAGTTAGGCAGCGATAACAACAGAGG |
| | ATAACAGCGAAGATTGCAGCATTACACG/ |
| | CGCTAATAGCTTCAGCTTTAACGCCGGTTT |
| | TTAGAGGGAATGTAACGCACTGAGAAGC |
| | **Primer extension** |
| P1 of *rovA* | /GTATCCTCATTACCCAGCATCG |
| P2 of *rovA* | /GTGCTAGATCAGATCCTAATGTCG |
| *pla* | /ATGATGCTGCATTAGCACTCC |
| | **LacZ fusion** |
| *rovA* | GCGGGATCCCGTTCGTTACTCTGCCCATC |
| | /GCGAAGCTTTTGTGATTGCTCTGGTGGTAAAC |

### *LacZ Fusion and β-Galactosidase Assay*

The promoter-proximal DNA region of each target gene was obtained by PCR using the ExTaq™ DNA polymerase (Takara) and with *Y. pestis* 201 genome DNA as the template. PCR fragments were then directly cloned into the *Eco*RI and *Bam*HI sites of the low-copy-number plasmid pRW50 that harbored a tetracycline resistant gene and a promoterless *lacZ* reporter gene[28]. The recombinant plasmids were verified by DNA sequencing, and then transformed into *Y. pestis* strains, and an empty pRW50 plasmid was also introduced into each strain to serve as negative control. The *Y. pestis* strains transformed with the recombinant plasmids and the empty pRW50 were cultivated to measure the β-galactosidase activity in the cellular extracts with the β-Galactosidase Enzyme Assay System (Promega)[14].

## RESULTS

### *Growth of WT, ΔphoP, and Δcrp*

When they grew in BHI (Figure 1), *ΔphoP* and WT showed almost the same rates of growth, indicating that BHI promoted sound growth of both *ΔphoP* and WT and that the *phoP* mutation did not affect the bacterial growth in BHI. *Δcrp* grew much more slowly than WT in BHI, suggesting that the *crp* mutation attenuated the bacterial growth in rich media.

### *Negative Regulation of rovA by PhoP*

The primer extension experiments (Figure 2a) were conducted to determine the yields of primer extension product of *rovA* (i.e., the relative *rovA* promoter activities) in WT or *ΔphoP* at the middle-exponential phase in BHI. This assay detected two transcriptional start sites located at 343 and 78 bp upstream of *rovA*, and thus the two promoters (P1 and P2) were transcribed for *rovA*, respectively. PhoP negatively regulated the P1 activity, but had no effect on P2.

The *rovA-lacZ* fusion vector was transformed into WT or *ΔphoP* to elucidate the effect of the *phoP* mutation on the *rovA* promoter activity (Figure 2b), and it was confirmed that the transcription of *rovA* was significantly enhanced the *ΔphoP* as compared to WT.

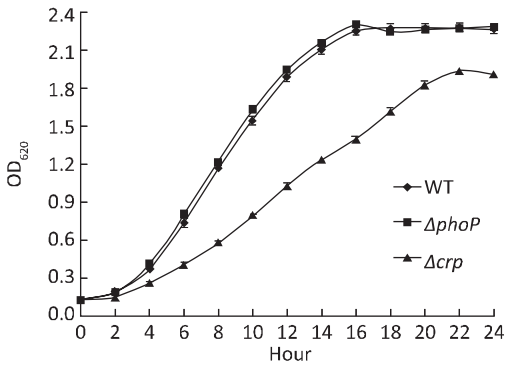


**Figure 1.** Bacterial growth curves in BHI medium. Cell cultures with an $OD_{620}$ value of about 1.0 in BHI were diluted 1:20 into 18 mL of fresh BHI. Bacteria were then cultivated at 26 °C with shaking at 230 rpm, and the $OD_{620}$ values were monitored for each culture with a 2 h interval.

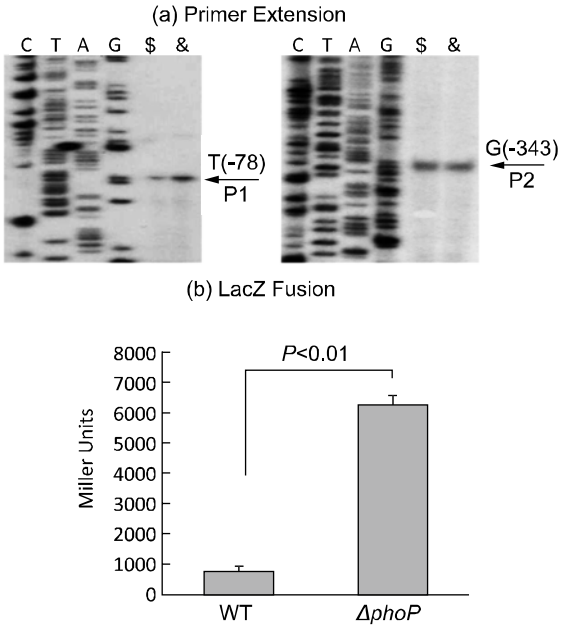


**Figure 2.** Negative regulation of *rovA* by PhoP. WT was denoted with $, while & indicated *ΔphoP*. **a) Primer extension**. The primer extension products were analyzed with 8 M urea-6% acrylamide sequencing gel. Lanes C, T, A and G represented the Sanger sequencing reactions. What were shown on the right side were the two transcriptional start sites T (-78) and G (-343) for both *ΔphoP* and WT, and accordingly two promoters P1 and P2 were identified. The minus numbers in the brackets indicated the nucleotide sites upstream of the translation start (+1). **b) LacZ fusion**. The LacZ fusion reporter vector of *rovA* was transformed into WT or *ΔphoP* to determine the β-Galactosidae activity in cellular extracts. What were shown were the *rovA* promoter activities (Miller units) in *ΔphoP* and WT.

### *Positive Regulation of pla by CRP*

The yield of primer extension product of *pla* in WT or *Δcrp* at the middle-exponential phase in BHI was determined by the primer extension assay (Figure 3). The result showed that a heavy primer extension product was detected for WT but not for *Δcrp*, indicating the positive regulation of *pla* by CRP. Accordingly, a single transcription start of *pla* at A (-77) was located, and thus a single promoter was transcribed for *pla*.

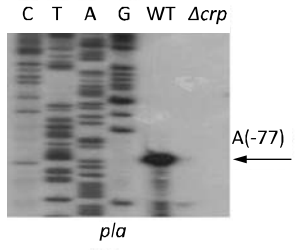


**Figure 3.** Positive regulation of *pla* by CRP. Electrophoresis of the primer extension products was performed with a 6% polyacrylamide/8 (mol/L) urea gel. Lanes C, T, A, and G represented the Sanger sequencing reactions. The transcriptional start site was underlined. The minus numbers in the brackets indicated the nucleotide sites upstream of the translation start (+1).

### *Repression of rovA Transcription under Temperature Upshift*

The primer extension experiments (Figure 4) were performed to determine the relative activities of promoters P1 and P2 of *rovA* in WT at the middle-exponential phase in BHI, when the temperature was upshifted from 26 to 37 °C. The two promoters P1 and P2 were detected again for *rovA*. The activity of P2 promoter showed no obvious

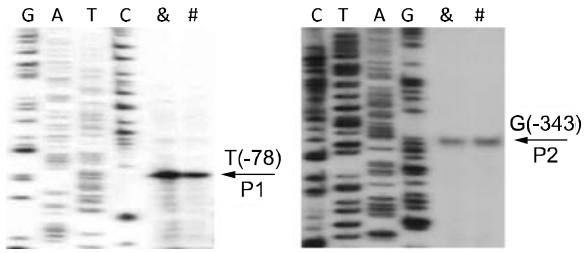


**Figure 4.** Repression of *rovA* transcription under temperature upshift. Electrophoresis of the primer extension products was performed with a 6% polyacrylamide/8 (mol/L) urea gel. Lanes C, T, A, and G represented the Sanger sequencing reactions. What were detected were the two transcriptional start sites T (-78) and G (-343) that corresponded to the two promoters P1 and P2, respectively. &: 26 °C continuously; #: shift from 26 to 37 °C. The minus numbers in the brackets indicated the nucleotide sites upstream of the translation start (+1).

change under the temperature upshift, while the activity of P1 promoter decreased significantly. Thus, the temperature shift triggered the negative regulation of P2 rather than that of P1 at the middle-exponential growth phase.

## DISCUSSION

When being cultivated in the original TMH medium (containing 20 mmol/L $Mg^{2+}$), WT grew well while *ΔphoP* grew at a rate much lower than WT; a similar growth restriction effect was also observed for *ΔphoP* relative to WT cultivated in the TMH containing 10 μmol/L $Mg^{2+}$[12]. In addition, bacterial growth of either *ΔphoP* or WT was impeded seriously under this suboptimal 10 μmol/L $Mg^{2+}$ TMH relative to the original TMH[12], since the magnesium cation is one of the essential elements for bacterial cell growth due to its function as a cofactor of enzymes. In the present study, the rich BHI medium was used instead of TMH for *Y. pestis* cultivation. *ΔphoP* and WT exhibited almost the same good rates of growth when cultivated in the rich BHI medium, and there was no growth restriction for *ΔphoP* relative to WT (Figure 1). Thus, the BHI medium promoted the good growth of both WT and *ΔphoP*. However, as CRP controlled the expression of a large set of energy metabolism genes[14], the *crp* mutation attenuated the bacterial growth *in vitro* in both synthetic medium[14] and rich medium in this study.

Data presented herein confirmed that PhoP-PhoQ and CRP-cAMP were able to sense unknown signals in BHI to regulate their known target genes *rovA* and *pla* respectively, and that the known temperature-dependent *rovA* gene was also repressed when the temperature was upshifted from 26 to 37 °C in BHI; these observations were consistent with those when TMH or its modifications was used previously[12,14]. Notably, the introduction of low magnesium conditions or the addition of cAMP into the medium was needed for triggering the PhoP-or CRP-dependent regulatory circuits, when the TMH media were used. However, the relevant introduction/addition processes could be omitted when BHI was employed instead. The above results verified that the rich BHI medium without the need for modification to be introduced into relevant stimulating conditions, can be employed instead of the synthetic TMH media for *Y. pestis* gene regulation study.

The commercial BHI is a powdered mixture of beef heart and calf brain infusions, peptone, sodium chloride, and disodium phosphate, and it is very simple to prepare into broth or agar for laboratory use. However, compared to TMH, BHI is much less convenient to be modified. TMH will have to be used when relevant ingredient modifications (e.g. the introduction of low magnesium or low iron conditions) are necessary.

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