

Separation and Detection of Phosphorylated and Nonphosphorylated BvgA, a *Bordetella pertussis* Response Regulator, *in vivo* and *in vitro*

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[Abstract] Protein phosphorylation plays a central role in signal transduction in bacteria. However, separation and detection of the phosphorylated protein from its nonphosphorylated form remain challenging. Here we describe a method to detect phosphorylation of the *Bordetella pertussis* response regulator BvgA, which is phosphorylated at an aspartate residue (Boulanger *et al.*, 2013). This method is based on the proprietary adduct, Phos-tagTM, a dinuclear metal complex, which together with Zn²⁺ or Mn²⁺, forms a complex with a phosphomonoesterdianion, such as the phosphorylated aspartate of a response regulator (Barbieri and Stock, 2008; Kinoshita and Kinoshita-Kikuta, 2011). For *in vivo* detection, *B. pertussis* cells are lysed in mild formic acid at 4 °C to minimize the disruption of the phospho-aspartate bond, and the phosphorylated BvgA is separated from its nonphosphorylated form by electrophoresis (SDS-PAGE) containing Phos-tagTM. Both forms of BvgA are subsequently detected by Western Blot analysis. Quantification of the level of phosphorylated BvgA formed after treatment with acetyl phosphate *in vitro* is also easily accomplished. Thus, this technique allows one to readily assess the levels of BvgA phosphorylation in *B. pertussis* and in *E. coli* under different laboratory conditions *in vivo* or after phosphorylation under varying reaction conditions *in vitro* (this research was supported in part by the Intramural Research Program of the NIH, NIDDK).

Materials and Reagents

1. *Bordetella pertussis* strain BP536 is the *tet*-resistant derivative of the clinical isolate Tohama I strain (laboratory inventory)
2. Formic acid (> 95%) (Sigma-Aldrich, catalog number: F0507)
3. NaOH (Thermo Fisher Scientific, catalog number: SS255-1)
4. Tris base (MP Biomedicals, catalog number: 819620)
5. Bromophenol blue (Sigma-Aldrich, catalog number: B8026)
6. Glycerol (Invitrogen, catalog number: 15514-011)
7. Butanol (Sigma-Aldrich, catalog number: 15467-9)
8. 30% 29:1 Acrylamide/bis-acrylamide (Sigma-Aldrich, catalog number: A3574)

9. 10% SDS (Hoefer, catalog number: GR155-1)
10. Phos-tag™ acrylamide (Wako Pure Chemical Industries, catalog number: AAL-107)
11. Zn(NO₃)₂ (Sigma-Aldrich, catalog number: 228737)
12. Ammonium persulfate (Sigma-Aldrich, catalog number: 3678)
13. N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, catalog number: T9281)
14. 0.5 M EDTA, pH 8.0 (ResGen, catalog number: 750009)
15. MOPS (Fluka, catalog number: 69947)
16. Sodium metabisulfite (Na₂S₂O₅) (Sigma-Aldrich, catalog number: S9000)
17. Glycine (Sigma-Aldrich, catalog number: G7126-1KG)
18. Methanol (EMD Millipore, catalog number: MX0485-7)
19. PBS buffer (Gibco, catalog number: 2014-10)
20. Bacto™ proteose peptone (Becton, Dickinson and Company, catalog number: 211684)
21. Difco™ Bordet Gengou agar base (Becton, Dickinson and Company, catalog number: 248200)
22. Defibrinated sheep blood (LAMPIRE® Biological Labs, catalog number: 7239001)
23. Lithium potassium acetyl phosphate (Sigma-Aldrich, catalog number: A0262-500MG)
24. Colloidal Blue staining kit (Life Technologies, catalog number: LC6025)
25. Tween-20 (Bio-Rad Laboratories, catalog number: M3524)
26. Non-fat dry milk (Giant Food Store, catalog number: 688267078330)
27. Monoclonal anti-BvgA antibody (laboratory inventory)
28. HRP-conjugated Secondary Antibody (Santa Cruz, catalog number: SC2005)
29. Amersham ECL Primer Western Blotting Detection System (General Electric Company, catalog number: RPN2232)
30. PVDF filter (Invitrogen, catalog number: LC6025)
31. BSA (Albumin from bovine serum, Sigma-Aldrich, catalog number: A2153)
32. 1 M formic acid (see Recipes)
33. 5 N NaOH (see Recipes)
34. 1 M Tris-Cl solutions (see Recipes)
35. 1% Bromophenol blue (see Recipes)
36. 5x Loading Solution (see Recipes)
37. Water-saturated butanol (see Recipes)
38. 5 mM Phos-tag™ acrylamide (see Recipes)
39. 10 mM Zn(NO₃)₂ (see Recipes)
40. 10% APS (see Recipes)
41. 1x MOPS Running Buffer, pH 7.8 (see Recipes)
42. Transfer Buffer (see Recipes)
43. BG agar plates (see Recipes)

44. 200 mM acetyl phosphate (see Recipes)
45. 1% or 5% BSA in PBS (see Recipes)
46. 0.05% Tween-20 in PBS (see Recipes)
47. 1% Non-fat milk in PBS (see Recipes)

Equipment

1. Polyester-tipped applicator (Puritan Medical, catalog number: 25-806 1PD)
2. Spectrophotometer
3. Mini gel cassettes (1.0 mm) (Invitrogen, catalog number: NC2010)
4. 10, 12 or 15-well combs (1.0 mm) (Invitrogen, catalog number: NC3015)
5. XCell SureLock[®] Mini-Cell (Invitrogen, catalog number: EI0001)
6. Platform shaker
7. Eppendorf microfuge
8. Bio-Rad Mini-Protean II (Bio-Rad Laboratories)

Procedures

A. Whole cell lysate preparation for *in vivo* detection of BvgA phosphorylation

Note: This protocol has been successfully used with another gram-negative bacteria, E. coli.

1. To collect bacteria sample, *Bordetella pertussis* strain BP536, grown at 37 °C for 2 days on BG agar plate, is swabbed from the plate with a polyester-tipped applicator and resuspended in 1.5 ml of PBS.
2. An aliquot from step A1 is used to determine the OD₆₀₀ reading.
3. A 0.3 ml aliquot from step A1 is centrifuged at 15,600 x g in Eppendorf microfuge for 1 min at room temperature. The supernatant is removed, and the resulting pellet is frozen in dry ice. It can be used directly or stored at -80 °C.
4. The frozen pellet is treated as follows.

Note: Volumes are based on an OD₆₀₀ reading of 0.5 and a pellet obtained from 0.3 ml, one should adjust the volumes proportionally based on the determined OD₆₀₀.

First, 33 µl of ice-cold 1 M formic acid is added, and the pellet is disrupted by pipetting repeatedly. Immediately, a freshly made ice-cold solution (27 µl) containing 2 µl of 5 N NaOH (to neutralize the acid), 10 µl H₂O, and 15 µl of 5x loading Solution is added.

Note: The color of the solution turns yellow due to the acid, but the bromophenol blue changes back to blue once it enters the gel.

5. 4 µl of the resulting cell lysate are loaded onto the Phos-tag[™] gel for electrophoresis.

Note: Do not boil the treated cell lysate; the lysate should be kept on ice before loading.

B. Phos-tag™ SDS-PAGE gel electrophoresis (Recipe makes one mini gel.)

4% stacking gel	
30% Acrylamide/bis-acrylamide	417.5 µl
1 M Tris pH 6.8	1,093 µl
10% SDS	31.3 µl
10% APS (freshly prepared)	25 µl
TEMED	5 µl
H ₂ O	1,570 µl
10% resolving gel	
30% Acrylamide/bis-acrylamide	2,085 µl
1 M Tris pH 6.8	2,185 µl
10% SDS	62.5 µl
5 mM Phos-tag™	93.8 µl
10 mM Zn(NO ₃) ₂	93.8 µl
10% APS (freshly prepared)	25 µl
TEMED	5 µl
H ₂ O	1,715 µl

1. For resolving gel: Acrylamide, Tris, SDS, Phos-tag™, Zn(NO₃)₂, and H₂O are mixed. 10% APS and TEMED are added, and the solution is immediately poured into the gel cassette. The solution is then overlaid with 1 ml of water-saturated butanol. The resolving gel polymerizes in ~40 min at room temperature.
2. The butanol is poured off of the polymerized resolving gel, and the gel surface is then washed with water. Excess water is removed using absorbent paper.
3. For stacking gel: Acrylamide, Tris, SDS, and H₂O are mixed. 10% APS and TEMED are added, and the solution is immediately poured into the gel cassette. The comb is quickly inserted (avoiding the introduction of air bubbles) and the stacking gel is allowed to polymerize for 20 min at room temperature.
4. After insertion of the gel cassette into the electrophoresis apparatus, the ice-cold 1x MOPS Running Buffer is added. The entire apparatus is then placed into an ice-filled bucket and the bucket is placed on a platform shaker so that it can be gently rotated until the temperature of the Running Buffer in the chamber has cooled to 4 °C. This typically takes at least 30 min.

Note: Place only one gel cassette in one XCell SureLock® Mini-Cell to ensure that the temperature remains cold during electrophoresis.

5. The shaker is turned off and the samples are loaded using a pipetman.
6. After resumption of gentle shaking, electrophoresis is performed with constant 100 V for 30 min, 120 V for 30 min, and then 150 V until the bromophenol blue dye is ~ 1 cm from the bottom of the gel. (Temperature within the inner gel chamber will rise to 10 °C to 15 °C.)
7. For Western blot analysis, the gel is washed with 100 ml Transfer Buffer containing 1 mM EDTA for 10 min to remove Zn²⁺ from the gel, followed by washing again with 100 ml Transfer Buffer for 20 min.
8. The gel is transferred to a PVDF filter, which was previously rinsed with methanol and then with Transfer Buffer, in a Bio-Rad Mini-Protean II apparatus filled with ice-cold Transfer Buffer at 100 constant voltage for 1 h at 4 °C.
9. The antibody detection of BvgA is carried out at room temperature as follows: After the transfer, the PVDF filter is blocked by washing with 5% BSA in PBS for 1 h, and then incubated with monoclonal anti-BvgA antibody (1:5,000) in 1% BSA in PBS for 1 h. Three 10-min washes with 0.05% Tween-20 in PBS are conducted before incubation with HRP-conjugated Secondary Antibody (1:2,000) in 1% non-fat milk in PBS for 1 h. The PVDF filter is washed three times for 10 mins, each, with 0.05% Tween-20 in PBS, and then developed with Amersham ECL Primer Western Blotting Detection System, according to the manufacture's instruction.

C. Separation of *in vitro* phosphorylated purified protein

1. To phosphorylate *in vitro*, 9 µl purified BvgA (from ~1 to 25 pmol) is mixed with 1 µl 200 mM acetyl phosphate on ice and then incubated for the desired amount of time at 37 °C.
2. Reaction is stopped by placing the sample on dry ice.
3. Sample is mixed with 2.5 µl 5x loading solution (final concentration of 1x), separated on PhostagTM acrylamide gel, washed, and detected by Western analysis as described above.
4. Alternately, gel can be stained. In this case, the gel is first washed 3 times in 100 ml of water for 10 min. The gel is then treated with Colloidal Blue following manufacturer's instructions.

Note: For best results, use either Colloidal Blue or classic Coomassie blue staining rather than other types of protein stains.

Note: The optimal level of purified protein for loading is ~ 1 pmol for Western analysis and ~25 pmol for gel staining

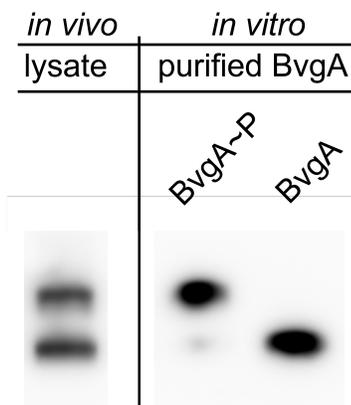


Figure 1. Separation of phosphorylated BvgA from unphosphorylated BvgA by Phos-tag™ SDS-PAGE, analyzed by Western blot. First lane shows the *in vivo* lysate of *B. pertussis*, which contains both phosphorylated BvgA (BvgA~P) and nonphosphorylated BvgA (BvgA). Lanes 2 and 3 show purified BvgA, which has been phosphorylated *in vitro* by the addition of acetyl phosphate or incubated in the absence of acetyl phosphate, respectively.

Recipes

- 1 M formic acid
5 ml of formic acid (> 95%) in 95 ml of H₂O
Stored at 4 °C
- 5 N NaOH
5 ml 10 N NaOH
5 ml H₂O
Stored at 4 °C
- 1 M Tris-Cl solutions (for 1 L)
121.14 g Tris base adjusted to pH 6.8 or to pH 8.0 with HCl at 4 °C
Filter sterilized
- 1% Bromophenol blue
1 g bromophenol blue in 100 ml H₂O
- 5x Loading Solution
1% SDS
65 mM Tris-Cl (pH 6.8)
25% glycerol
0.02% bromophenol blue
Stored at 4 °C

6. Water-saturated butanol
 - 4 ml water added to 40 ml butanol
 - Mix by shaking
 - Butanol will be the top layer
7. 5 mM Phos-tagTM acrylamide
 - 10 mg Phos-tagTM acrylamide dissolved in 100 μ l methanol
 - Solution is pipetted repeatedly to dissolve the acrylamide
 - Add 3.2 ml H₂O
 - Solution is centrifuged at 2,000 x g for 10 min to remove white precipitate
 - Stored at 4 °C in dark
8. 10 mM Zn(NO₃)₂
 - 2.97 g Zn(NO₃)₂ dissolved in 1 L H₂O
 - Filter sterilized
9. 10% APS
 - 1 g ammonium persulfate dissolved in 10 ml H₂O
 - Solution is freshly prepared
10. 1x MOPS Running Buffer, pH 7.8
 - 100 mM Tris-CL, pH 7.8
 - 100 mM MOPS, pH 7.8
 - 0.1% SDS
 - 5 mM sodium metabisulfite
 - For 1 L
 - 12.1 g Tris base
 - 20.9 g MOPS
 - 10 ml 10% SDS
 - 0.95 g sodium metabisulfite (Na₂S₂O₅)
 - Solution is adjusted to pH 7.8 with 10 N HCl at 4 °C
 - Stored at 4 °C
11. Transfer Buffer
 - 3 g Tris base
 - 14.4 g glycine
 - 200 ml of methanol
 - 800 ml of H₂O
12. BG agar plates
 - 10 g DifcoTM Bordet Gengou agar base
 - 3.3 g BactoTM proteose peptone
 - 3.3 ml glycerol added to 350 ml H₂O

- The mixture is autoclaved
Cooled to 46 °C before adding 50 ml defibrinated sheep blood
Pour warm solution into petri dishes and allow to cool to room temperature.
Stored at 4 °C
13. 200 mM acetyl phosphate
0.0039 g lithium potassium acetyl phosphate dissolved in 105.7 µl 20 mM Tris-Cl, pH 8
Solution is freshly made
Stored on ice
14. 1% or 5% BSA in PBS
1 g or 5 g BSA
100 ml PBS
15. 0.05% Tween-20 in PBS
0.05 ml of Tween-20
100 ml of PBS
16. 1% non-fat milk in PBS
1 g non-fat dry milk
100 ml of PBS

Acknowledgments

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