Novel Phase-Shift Marker in Cell Surface Proteins of Bordetella bronchiseptica

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Cell surface proteins of phase I cultures of Bordetella bronchiseptica strains from various species of animals were compared with those of isogenic phase III cultures by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein band with a molecular mass of 74 kilodaltons was found only in various phase I cultures. This protein disappeared in parallel with antigenic modulation and had strong antigenicity. We found it to be an appropriate phase-shift marker for two reasons: it was readily extracted and recognized, and it was specifically identified by its distinct antigenicity.

Bordetella bronchiseptica, a respiratory pathogen of animals, has been isolated from numerous species, including pigs, dogs, cats, monkeys, and laboratory animals, as well as from humans (5). The virulent phase I of Bordetella species degrades to avirulent phase III, with accompanying changes in colony morphology and antigenic properties (5, 19). As a result, it loses the various virulence factors such as diphtheroid toxin (6, 20), adenylate cyclase (2, 3), hemagglutinin (1), and surface components which facilitate its adherence to the respiratory epithelium (9, 22). Since most of these factors were found on the surface of phase I cells, changes in surface proteins are expected during phase degradation. However, little is known about this alteration of B. bronchiseptica (4, 18).

In this paper, we describe a novel surface protein with a molecular mass of 74 kilodaltons (kDa) that is applicable as a phase-shift marker of B. bronchiseptica.

The strains used in this study are listed in Table 1. The stable, nonreversible phase III variants of the strains were obtained after the strains had been cultured in the presence of crystal violet (8). The phase I-specific antiserum was obtained by immunization of rabbits. The rabbits were injected subcutaneously with the Formalin-inactivated phase I suspension of strain H-16 in Freund complete adjuvant (Nakarai Chemicals, Ltd., Kyoto, Japan). The resulting antiserum was absorbed by phase III cells of the same strain.

The strains and their variants were streaked onto Bordet-Gengou medium (Difco Laboratories, Detroit, Mich.) containing 15% defibrinated horse blood (BGA) and incubated at 37°C for 48 h. One loopful of cells from the BGA culture was inoculated into a 500-ml bottle containing 20 ml of modified Stainer-Scholte medium (MSS) (7). After incubation for 48 h at 37°C with shaking (200 rpm), 0.1 ml of the culture was added to 250 ml of MSS in a loosely capped 1-liter culture bottle, and the cells were cultured in the same manner. To induce antigenic modulation (i.e., reversible phenotypic or antigenic alteration), we grew the strains at 37°C for 48 h in MSS containing 20 mM MgSO4.

Cell surface extract (CSE) was prepared by the method which was generally used to extract the capsular or fimbrial antigens of Escherichia coli (10, 17). Briefly, cells were collected by centrifugation and then suspended in 0.1 M Tris hydrochloride buffer (pH 8.0). They were adjusted to 10^11 CFU/ml by measurement of the A_{620} with a spectrophotometer (model UV-240; Shimazu Corp., Kyoto, Japan) and heated at 60°C in a water bath for 30 min. The suspensions were cooled and centrifuged at 10,000 × g for 30 min at 4°C. The supernatants were saved as CSEs and used for the following analyses.

First, the CSEs from phase I and phase III cultures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% acrylamide gel (12). The gel was stained with Coomassie brilliant blue R-250 (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). Certain protein bands with a molecular mass range of 45 to 93 kDa were specific to phase I cultures when compared with isogenic phase III cultures (Fig. 1). One of them, with a molecular mass of 74 kDa, was the major band and was common to all phase I cultures. Although a weakly staining protein band in the region of the 74-kDa protein was observed in the phase III cultures of strains H-16, H-4, BC-10, and RT-1, its molecular mass was approximately 71 kDa. We studied the 74-kDa protein for two reasons: the extraction method for

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![FIG. 1. SDS-PAGE analysis of the CSEs from phase I and phase III cultures of various B. bronchiseptica strains. Molecular weight standards (MW) are indicated on the left in thousands (K). Lanes I show the CSEs from phase I cells of strains H-16, H-4, BC-10, GP-15, RB-18, and RT-1. Lanes III show the CSEs from phase III cells of the same strains as in lanes I.](http://jcm.asm.org/)

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TABLE 1. B. bronchiseptica strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>H-16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Pig (nasal)</td>
</tr>
<tr>
<td>H-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Pig (nasal)</td>
</tr>
<tr>
<td>BC-10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Cat (pneumonic)</td>
</tr>
<tr>
<td>GP-15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Guinea pig (pneumonic)</td>
</tr>
<tr>
<td>Rb-18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rabbit (nasal)</td>
</tr>
<tr>
<td>RT-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Rat (pneumonic)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All strains are phase I.
<sup>b</sup> Cultures obtained from K. Koshimizu, University of Tokyo, Tokyo, Japan.
<sup>c</sup> Cultures obtained from M. Nakagawa, National Institute of Health, Tokyo, Japan.

this protein was very simple, and it was the major band in phase I CSEs irrespective of the difference in the bacterial strains and the animal species from which these strains were isolated.

Lacey (11) reported that reversible phenotypic or antigenic alteration of Bordetella species was induced by culturing at 25°C or in the presence of high concentrations of magnesium ions. This shift is termed antigenic modulation. Ezzell et al. (4) demonstrated that the modulated phase I (C-mode) cultures of B. bronchiseptica lost two cell envelope proteins (molecular masses, 30 and 28 kDa) of normal phase I (X-mode) cultures during the antigenic modulation. In light of this observation, the CSEs of X-mode and C-mode cultures were prepared and analyzed by SDS-PAGE. The staining pattern is shown in Fig. 2. A limited change was located in the molecular mass range of 28 to 30 kDa. On the other hand, the 74-kDa protein was detected only in X-mode cultures. The discrepancy between their observation (4) and ours may be attributed to the difference in the methods used for isolation of cell surface proteins.

The antigenicity of the 74-kDa protein was subsequently analyzed. The CSEs were subjected to SDS-PAGE and then electrophoretically transferred to a Durapore membrane sheet (Nihon Millipore Kogyo K. K., Yonezawa, Japan) by the method of Towbin et al. (21). The sheet was blocked and exposed to phase I-specific antiserum. After reacting with peroxidase-conjugated anti-rabbit immunoglobulin G antibody bodies (Organon Teknika, Malvern, Pa.), the sheet was washed and treated with the substrate solution (4-chloro-1-naphthol; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The 74-kDa protein strongly reacted with the antiserum (Fig. 3). This characteristic is convenient for a phase-shift marker. For example, when the 74-kDa protein presents as a weakly staining band or when many bands are present near this protein, it can be identified by using the phase I-specific antiserum.

Our results suggest that the 74-kDa protein may be useful as a phase-shift marker of B. bronchiseptica.

Ezzell et al. (4) reported the presence of a phase-shift marker in cell envelope proteins of B. bronchiseptica. However, their method for isolation of cell envelope proteins requires several steps and is time-consuming. Our 74-kDa protein is simply and quickly extracted. When the cell envelope proteins of strains used in this study were prepared by the method of Ezzell et al. (4) and analyzed, the 74-kDa protein was a minor band and was confused with other bands (data not shown). Therefore, our method is both suitable and necessary for the visualization of the 74-kDa protein.

Lugtenberg et al. (13) reported that a nonpathogenic swine isolate of B. bronchiseptica lacked the doublet proteins with molecular masses of 70 and 67 kDa in its cell envelope. The 74-kDa protein may be similar to these proteins in molecular mass. However, Lugtenberg et al. did not describe these as phase-shift markers, nor did they describe their antigenicity. On the other hand, Montaraz et al. (14) recently noted that the 68-kDa outer membrane protein of virulent B. bronchiseptica was the protective antigen against intraperitoneal and aerosol challenge with the virulent strain in mice. Novotny et al. (15, 16) also reported that it was absent in atrophic rhinitis nonpathogenic strains. This protein seems to be similar to the 74-kDa protein, but Novotny et al. did not describe it as a phase-shift marker. Although they demonstrated that the 68-kDa protein disappeared upon modulation of the culture conditions, the nature of the modulation was not fully defined.

In this paper, we present the 74-kDa protein as a novel and useful phase-shift marker of B. bronchiseptica. Since the protein is specific to phase I cultures, it will be necessary to clarify its relationship to the surface components, including the virulence factor.
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LITERATURE CITED