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Antigenic Heterology Between Flagellin and Flagella of *Bacillus subtilis*[1]

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The antigenic properties of *Bacillus subtilis* flagella and periodate-treated flagellin were compared by using immobilization-inhibition and diethylaminoethyl cellulose binding assay. The results suggest that there are both unique and cross-reactive antibodies elicited by *B. subtilis* flagella and flagellin.

Several studies have demonstrated antigenic heterology between assembled proteins and their component subunit proteins. Schlesinger (16) demonstrated a difference in the serological specificity of alkaline phosphatase, which is enzymatically active only in a dimerized state, and the subunit protein of the enzyme. The antigenic determinants of tobacco mosaic virus (TMV) and its subunit protein (TMVP) have been extensively studied (reviewed by Rappaport, 15). Tobacco mosaic virus is composed of 2,180 protein subunits assembled around a ribonucleic acid core. Benjamini et al. (6) demonstrated that the tryptic peptide 93–112 possessed the antigenic determinant to TMVP. On the other hand, Anderer (4) showed four peptides 11–23, 62–68, 123–134 and 142–158 to play a role in the antigenicity of TMV. The difference in the results of the antigenicity of TMV and TMVP may be attributed to the exposure of a strong cryptotope in TMVP which resulted from the disaggregation of the TMV.

Ada et al. (2) demonstrated the serological homology among *Salmonella adelaide* flagella, their subunit protein flagellin, and polymerized flagellin as measured by immobilization-inhibition, the precipitin reaction, and immunodiffusion. Recently, Parish and Ada (*submitted for publication*) reported that *S. adelaide* flagellin cleaved with cyanogen bromide gave rise to four polypeptide fragments. The largest peptide, fragment "A" (molecular weight 18,000), possessed all of the antigenic determinants (Parish, Wistar, and Ada, *submitted for publication*) as determined by its inhibitory effect in three serological assay methods.

This report describes the serological specificity of *Bacillus subtilis* flagella and its subunit protein flagellin. The results demonstrate the existence of four types of antibodies elicited by flagella and flagellin: (i) antiflagella immobilizing antibodies which are neutralized only by flagella, (ii) antiflagellar antibodies which cross-react with flagellin, (iii) antiflagellin immobilizing antibodies which are neutralized by flagella and flagellin, and (iv) antiflagellin antibodies specific for flagellin.

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MATERIALS AND METHODS

Organisms and growth medium. The bacteria used were *B. subtilis* W23 (a prototrophic strain obtained from C. B. Thorne) and *B. subtilis* SB19 (a prototrophic strain obtained from W. R. Romig). The cells were grown on a rotary shaker at 37 C in a medium containing the following (in grams per liter): K_2HPO_4, 14; KH_2PO_4, 6; $(NH_4)_2SO_4$, 2; $MgSO_4$, 0.2; sodium citrate, 1; glucose, 5; N-Z case (Sheffield), 1. The cells were harvested in late exponential phase.

Purification of flagella. Flagella were isolated and purified by the diethylaminoethyl (DEAE) cellulose column procedure described by Martinez (13).

Preparation of flagellin. Purified flagella were dialyzed exhaustively against distilled water prior to dissociation. Preparations containing 0.5 to 10 mg of flagella per ml were dissociated to flagellin subunits by the careful addition of 3.5 N NaOH to give a final concentration of 0.1 N. After exactly 3 min of mixing, the samples were dialyzed against 4 liters of 0.002 M triethylammonium acetate—0.1 M NaCl (TEAS buffer), *p*H 7.0, for 2 hr at room temperature. The contents of the dialysis bag were centrifuged at 105,000 × *g* for 1 hr to remove any insoluble materials. Minute quantities of flocculent material were normally observed.

Preparation of periodate-treated flagellin. Flagellin was prepared by alkaline dissociation of flagella, dialyzed for 2 hr against distilled water which was adjusted to *p*H 8, and the centrifuged at 105,000 × *g* for 1 hr. Periodate treatment of the flagellin was by the method of Schlesinger (16); 5 ml of flagellin solution was adjusted to *p*H 2.0 with the addition of 0.5 ml

0.1 N HCl and placed in an ice bath; 0.5 ml of 0.1 M $NaIO_4$ was added. After 30 min, 1.25 ml of 0.1 M glycerol was added and the preparation was dialyzed against 2 liters of 0.01 M sodium phosphate—0.15 M NaCl (PBS buffer), pH 7.2, for 4 hr in the cold. The periodate-treated flagellin in PBS was found to be monodisperse in an analytical ultracentrifuge with an $S_{20,w}$ value of 1.9 even after storing for 2 days. Further, addition of ammonium sulfate to 40% saturation, a treatment known to induce assembly of flagellins to the ordered structure (2), did not cause the assembly of periodate-treated flagellin. Based on these observations, it was concluded that periodate-treated flagellin remains in its monomeric state in an environment where spontaneous reaggregation normally occurs (1).

Preparation of antisera. Antisera to flagella and periodate-treated flagellin were prepared by the methods of Ada et al. (2) in which 500 μg of antigen is injected subcutaneously with Freund's complete adjuvant into rabbits. This was followed by four or five intravenous injections of 500 μg at 3-week intervals. The rabbits were bled 1 week after the last injection.

Immobilization assay. The immobilization assay of the antiflagellar or antiflagellin serum was performed by the method of Nossal (14) and Ada et al. (2). The method consists of diluting the serum twofold serially; a suspension of 10^6 actively motile bacteria in 0.1 ml is added to the serum dilutions. The mixture is allowed to incubate at 37 C for 15 min. The reciprocal of the dilution at which 90% of the bacteria are immobilized, as observed by phase-contrast microscopy at 100×, is taken as the end point of the titration.

The ability of antigens to neutralize the immobilizing activity of the sera was determined by first absorbing the titered antisera with various amounts of antigen. The antigen-antibody reaction mixture was serially diluted and the excess antiserum was estimated by the reduction in immobilization titer.

DEAE cellulose binding assay. The DEAE cellulose binding method was essentially that of Grant and Simon (8) in which ^{125}I-labeled antibody is complexed with antigen. The amount of labeled antibody bound to increasing amounts of antigen is determined by filtering the mixture through DEAE cellulose filter pads and counting the ^{125}I.

Three-component-modified assay. The method of Askonas and Rhodes (5), with ^{125}I-labeled flagellin as the antigen, was used. The labeled antigen which binds to antibody is precipitated with anti-rabbit γ-globulin serum. This assay was the only one used for the detection of flagellin, since antiflagellin was found to be a poor precipitating antibody.

Antiflagellin was adsorbed with various amounts of flagella, and the residual flagellin-binding activity was determined after centrifuging the flagella-antiflagellin complex at 105,000 × g for 1 hr.

^{125}I-labeling of antigen and antibody. Antiflagella antibody and periodate-treated flagellin were labeled with ^{125}I by the direct oxidation method of Ada, Nossal, and Pye (3). Between 0.5 and 2.0 mg of protein per ml in PBS was mixed with 2 mc of carrier-free ^{125}I. Chloramine-T (150 μg in 0.15 ml of PBS) was added quickly in a fine stream to the mixing reaction mixture by means of a tuberculin syringe with a no. 26 needle. After 5 min of mixing at room temperature, sodium metabisulfite (200 μg in 0.2 ml of PBS) was added to terminate the iodination. The entire reaction mixture was applied to a column (1 by 30 cm) of Sephadex G-25 and eluted with PBS to remove unbound iodine from the protein mixture.

RESULTS

Properties of antiflagellar antibodies. The immobilizing and binding properties of antiflagellar antibodies were first determined. Various quantities of flagella or flagellin were complexed with antiflagellar sera and the residual immobilizing titer was determined (Fig. 1). The antiserum used had an immobilizing titer of 10,240. About 20 μg of flagella was needed to fully neutralize the immobilizing activity. Flagellin, on the other hand, even at 128 μg of protein, did not reduce the immobilizing antibody titer of the antiflagellar sera. This initial observation led us to question the antigenic homology between *B. subtilis* flagella and flagellin.

Binding experiments were performed in which the association between flagella or periodate-treated flagellin and ^{125}I-antiflagella was determined (Fig. 2). The two antigens gave rise to distinctively different curves. The flagellin-binding curve was essentially identical to that seen with periodate-treated flagellin. These experiments were carried out in TEAS buffer with conditions under which no aggregagation of flagellin occurred. On the other hand, the binding curve of

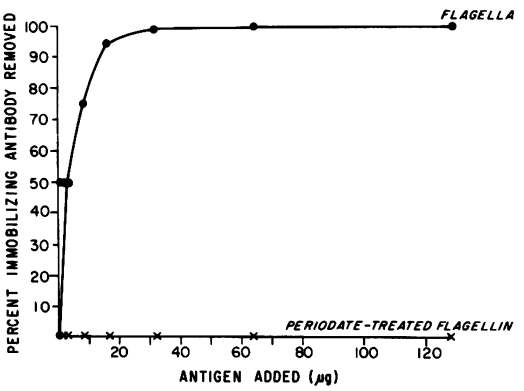

FIG. 1. *Adsorption of immobilizing antiflagellar antibody by purified flagella (●) and periodate-treated flagellin (×). Anti-W23 flagellar antibody (0.1 ml) was treated with various concentrations of homologous flagella and periodate-treated flagellin for 15 min at 37 C. Motile W23 cells (10^6) in 0.1 ml were added and the percentage of residual motile cells was determined by phase-contrast microscopy.*

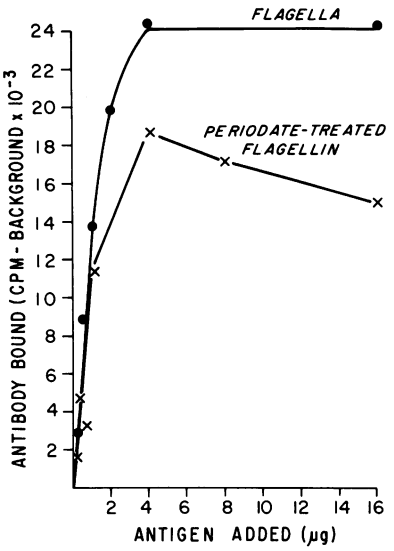

FIG. 2. *Binding of ^{125}I-labeled purified anti-W23 flagellar antibody by purified flagella (●) and periodate-treated flagellin (×). An 0.1-ml amount of purified anti-W23 flagellar antibody (2 μg of labeled protein) was allowed to react with increasing concentrations of purified flagella and periodate-treated flagellin for 30 min at room temperature; the mixture was filtered through DEAE paper, washed, and counted.*

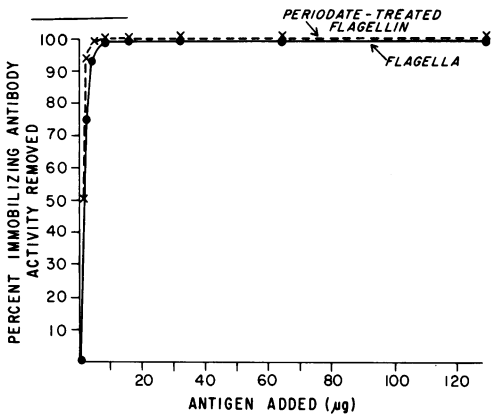

FIG. 3. *Adsorption of immobilizing antiflagellin antibody by purified flagella (●) and periodate-treated flagellin (×). Anti-W23 periodate-treated flagellin antibody (0.1 ml) was treated with various concentrations of homologous flagella and periodate-treated flagellin for 15 min at 37 C. Motile W23 cells (10^6) in 0.1 ml were added, and the percentage of residual motile cells was determined by phase-contrast microscopy.*

aggregated flagellin resembles that observed with flagella.

These results show that the cross-reacting antibodies observable in this binding assay cannot be detected by the immobilization reaction.

Properties of antiperiodate-treated flagellin antibodies. Experiments similar to those described above were performed by using antiflagellin antisera. Figure 3 represents a curve obtained by the immobilizing method when antiperiodate-treated flagellin antisera were adsorbed with flagella or periodate-treated flagellin. Both antigens neutralized the immobilizing antibody equally well. The antiserum used had a titer of 20,000, and about 5 μg of flagella or periodate-treated flagellin was needed to fully neutralize the immobilizing activity.

In a typical binding experiment (Fig. 4) in which ^{125}I-labeled antiflagellin γ-globulin was used with flagella and periodate-treated flagellin, the two antigens gave rise to curves similar to those obtained in the binding experiments using ^{125}I-labeled antiflagellar γ-globulin (Fig. 2). When flagellin in TEAS buffer was used as antigen, its binding to antibody was similar to that obtained for the periodate-treated flagellin. These data suggested that a minimum of two types of antibodies were elicited by periodate-

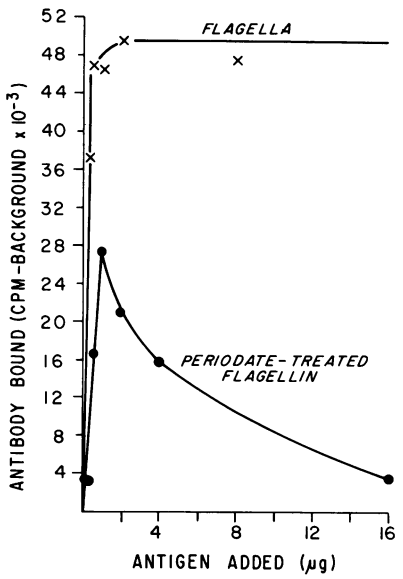

FIG. 4. *Binding of ^{125}I-labeled immunoglobulin G fraction of anti-W23 flagellin antiserum by purified flagella (×) and periodate-treated flagellin (●). An 0.1-ml amount of immunoglobulin G fraction of anti-W23 flagellin antiserum (5 μg of labeled protein) was allowed to react with increasing concentrations of purified flagella and periodate-treated flagellin for 30 min at room temperature; the mixture was filtered through DEAE paper, washed, and counted.*

treated flagellin. This possibility was tested by the following adsorption experiment. A preparation of antiperiodate-treated flagellin γ-globulin (100 μg/ml) was adsorbed with flagella at two concentrations, one approaching equivalence (160 μg/ml), and the other with slight antigen excess (640 μg/ml). The antigen-antibody complex formed upon incubation at 37 C for 2 hr and refrigeration overnight was centrifuged at 105,000 $\times$ g for 1 hr; the residual γ-globulin was reacted with ^{125}I-labeled periodate-treated flagellin. The resulting complex was precipitated with anti-rabbit γ-globulin serum (Fig. 5). The data indicate that, after adsorbing the antiflagellin γ-globulin with flagella, a population of antibody remains which is specific for periodate-treated flagellin.

To determine whether the residual flagellin-specific antibody level is a significant one, an antiflagellin immunoglobulin G preparation (DEAE-cellulose chromatography) was adsorbed with flagella in antigen excess (640 μg of flagella),

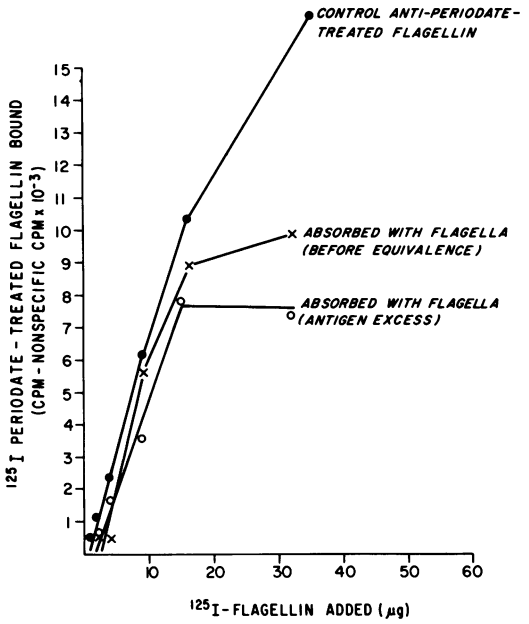

FIG. 5. *Binding of ^{125}I-labeled flagellin to anti-periodate-treated flagellin which was adsorbed with flagella. Immunoglobulin G fraction of anti-W23 flagellin antiserum (100 μg of protein/ml) was adsorbed with 160 μg of flagella/ml, which was before equivalence (×), and 640 μg of flagella/ml, which was in antigen excess (○). The adsorbed antibody was centrifuged and then allowed to react with increasing concentrations of ^{125}I-labeled periodate-treated flagellin at 37 C for 1 hr and in the cold overnight; it was then allowed to react with goat anti-rabbit γ-globulin at 37 C for 1 hr, then overnight in the cold. The precipitate was centrifuged, washed, and counted.*

and the complex was centrifuged and readsorbed with the same amount of flagella. Readsorption of the antiflagellin γ-globulin with an excess of flagella did not alter the level of flagellin-specific antibody. It appears, then, that a portion of the antiflagellin antibodies cross-reacts with flagella, whereas some antiflagellin binds specifically to flagellin.

DISCUSSION

Serological differences between flagella and their homologous flagellins have been observed by several investigators. Gard et al. (7) demonstrated, by agar-diffusion studies with *Proteus* antiflagellar sera, a precipitin line specific for flagella. A second line of precipitation was observed with acid- or heat-dissociated flagella. They further found that the flagellin-specific line disappeared when the flagellin was stored in the frozen state. Based on our present knowledge of flagellar proteins, these results may be interpreted by assuming that the flagellin preparation spontaneously reaggregated, forming polymerized flagellin, which reacted antigenically like flagella, forming only the flagella-specific line (2).

Read (Ph.D.Thesis, Purdue Univ., 1957) and Koffler (10) showed that heat-dissociated *P. vulgaris* flagella were able to precipitate only 20% of the homologous antiflagellar antibodies. However, flagella reacted quantitatively almost the same as flagellin with antisera prepared against flagellin. Their interpretation of these results was that there are some antigenic determinants on the flagella not found or accessible on flagellin, whereas flagella have all the flagellin antigenic determinants.

Kerridge et al. (9) compared the serological properties of *S. typhimurium* flagella and ultrasonically disrupted flagella. They prepared antisera to flagella as well as the ultrasonically disrupted flagella (2.2*S*) and found fewer ultrasonically disrupted flagella precipitated with both antisera than flagella.

Ada et al. (2) investigated the antigenic properties of *S. adelaide* flagellar proteins, comparing flagella, acid-dissociated flagella, and ammonium sulfate-polymerized flagellin. Their immobilization-inhibition results showed that two to four times more flagellin was required to neutralize antiflagellar and antipolymerized flagellin antisera than flagella and polymerized flagellin. On the other hand, they found more flagellin than flagella or polymerized flagellin precipitated by antiserum in the antigen-excess region with all their antisera. They concluded that flagella, flagellin, and polymerized flagellin contained the same protein subunit, but the differences were due to the structural organization of the various antigens.

The data we present demonstrate that *B. subtilis* flagellin does not neutralize antisera from its homologous flagella. Our data differ quantitatively from those of Ada et al. (2) using *S. adelaide* in which flagellin is capable of adsorbing homologous antisera against flagella. A possible explanation is the gross differences in the fine structure of *B. subtilis* and *S. adelaide* flagella. Lowy and Hanson (11) demonstrated two distinctive types of subunit arrangements in flagella: a "beaded" surface consisting of helically connected globular subunits in a hexagonal pattern, as in *S. typhimurium* flagella, and a "lined" surface arrangement showing longitudinal lines running parallel to the flagellar axis, as in the *B. subtilis* flagella. From X-ray diffraction studies, Lowy and Spencer (12) proposed differences in the structure of subunits in flagella of *S. typhimurium* (beaded type) and *B. pumilus* (line type). The expression of antigenic determinants specific for flagella and flagellin in different species may be due to the differences in the arrangement of the subunits in the flagella. The difference in the structure of the subunits or the differences in the surface structure of the flagella of *B. subtilis* ("line" form) and *S. adelaide* ("beaded" form), or both, may correlate with their different serological behavior.

The immobilizing activity of antisera from rabbits immunized with flagella was shown to be neutralized by flagella but not flagellin (Fig. 1). However, labeled flagellar antibody did bind flagellin, as was shown by the ^{125}I-labeled antibody-binding assay (Fig. 2). This binding, however, did not saturate the antibody sites for flagella. This suggests that antiflagella contains a species of immobilizing antibodies which is not neutralized by flagellin and a species which cross-reacts with flagellin. Flagellin, then, appears unable to neutralize the antiflagellar immobilizing activity, presumably due to an altered conformation of the subunit. However, it is capable of eliciting immobilizing antibodies which can be neutralized by both flagella and flagellin, as well as flagellin-specific antibodies.

Figure 6 represents our view of the antigenic structure of flagella and flagellin. The immobilizing antibody is anti-A; only flagella are able to neutralize anti-A. Anti-F will bind flagellin, which contains F antigen. On the other hand, A/2 can engender effective immobilizing antibody and anti-A/2 can be neutralized by the homologous flagellin and also cross-reacts with A. However, anti-A does not have sufficient affinity for A/2 to be inhibited by it because of the conformational change resulting from the dissociation of the structure. The *N* determinant is a cryptotope specific for flagellin.

FIG. 6. *Schematic representation of the antigenic structure of flagella and flagellin. F = Cross-reacting antigenic determinant; A = antigenic determinant specific for flagellar immobilizing activity; N = unique antigenic determinant for flagellin.*

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