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Polysaccharides of the Genus *Bacillus* Cross-Reactive with the Capsular Polysaccharides of *Diplococcus pneumoniae* Type III, *Haemophilus influenzae* Type b, and *Neisseria meningitidis* Group A

RICHARD L. MYEROWITZ, RUTH E. GORDON, AND JOHN B. ROBBINS

Department of Pathology, Peter Bent Brigham Hospital, Harvard University Medical School, Boston, Massachusetts 02115; Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903; Developmental Immunology Branch, National Institute of Child Health and Human Development, Bethesda, Maryland 20014

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We studied 174 strains of the genus *Bacillus* for cross-reacting antigens to the capsular polysaccharides of groups A and C meningococcus, types I and III pneumococcus, and *Haemophilus influenzae* type b. Cross-reactions were detected by immunodiffusion in agarose gel by using type-specific antisera and confirmed by absorption and inhibition experiments. Of 20 *Bacillus pumilis* strains, six had an antigen cross-reacting with group A meningococcal polysaccharide. Other cross-reactions included one strain of *B. pumilis* with *H. influenzae* type b, one of *B. cereus* var. *mycoides* with pneumococcus type III, and one of *B. alvei* with both type b and SIII polysaccharides. These cross-reacting antigens are polysaccharides of vegetative cells and may be extracellular in location. Because these bacilli have antigens cross-reacting with the virulence factors of pyogenic bacteria, they may, as normal flora, be an antigenic stimulus for "natural" serum anti-capsular antibodies to the type b *Haemophilus* and group A meningococcus polysaccharides.

Bacteria of the genus *Bacillus* are gram-positive, gram-negative, or gram-variable, spore-forming rods found widely in soil and water (22). Because of the resistance of their spores to heat and other bactericidal agents, they are of economic importance in the processing of canned foods and the preparation of sterile products. Contrary to the general belief that only strains of *Bacillus anthracis* cause disease in humans and animals, evidence is accumulating that opportunistic human infections may occur due to strains of *Bacillus* species hitherto regarded as "nonpathogenic" (2, 4-6, 12, 16).

In the search to find enteric bacteria with cross-reacting antigens that may have induced the natural anti-capsular antibodies to meningococci, *Haemophilus influenzae* type b, and pneumococci, a cross-reacting antigen to the group A capsular polysaccharide of *Neisseria meningitidis* was found in a strain of *Bacillus pumilis*, designated as strain "Sh 17" (18). In addition, it was found that the ribitol phosphate polymer of the cell wall teichoic acids of gram-positive bacteria, including *Bacillus subtilis*, cross-reacted with the *H. influenzae* type b capsular polysaccharide (3). During the course of a study of the neonatal rabbit immune response to intestinal colonization with a cross-reacting *Escherichia coli*, a strain of *B. pumilis* (designated as strain Sh 18) was isolated from rabbit feces and was shown to contain an antigen that cross-reacts with the capsular polysaccharide of *H. influenzae* type b (14). These three findings prompted a systematic search among the described strains of the genus *Bacillus* for antigens that cross-react with the capsular polysaccharides of pyogenic bacteria.

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MATERIALS AND METHODS

Bacterial strains. We studied 174 strains of the genus *Bacillus* from a previously described reference collection (22). They are listed in Table 1. The

TABLE 1. *Strains of the genus bacillus surveyed for cross-reacting antigens*

Bacillus species	No. of strains examined
pumilis	20
megaterium	10
subtilis	10
licheniformis	10
firmus	10
cereus var. *mycoides*	5
cereus var. *thuringiensis*	5
cereus var. *anthracis*	5
cereus	10
circulans	15
macerans	9
polymyxa	10
laterosporus	10
alvei	9
sphaericus	8
brevis	8
miscellaneous sp.	20

bacteria were cultivated in tryptic soy broth (Difco Laboratories) and stored on heart infusion agar slants (Difco Laboratories) in partly stoppered vials at 4 C.

Serological reagents. Hyperimmune animal sera were prepared in burros and rabbits by the intravenous injection of formaldehyde (pneumococci and *H. influenzae* type b) or glutaraldehyde (meningococci)-fixed bacteria from a 6-h broth culture according to a reported schedule (1). The blood was collected under sterile conditions from the external jugular vein in the burro and from the central ear artery in the rabbit. The serum was decanted from blood, centrifuged at 10,000 × *g* at 4 C for 30 min, and stored in sterile vials without preservative at −20 C until used. The anti-capsular antibody content of the serum used to detect the cross-reactions was approximately 2 to 3 mg of antibody per ml, as determined by the quantitative precipitin reaction (10) for the capsular polysaccharides of meningococcus groups A and C and pneumococcus types I and III, and by radioimmunoassay for *H. influenzae* type b (19).

Polysaccharides. The bacterial polysaccharides were isolated from 6-h broth cultures of the bacilli as previously described (7, 17). The capsular polysaccharides of *H. influenzae* type b and *N. meningitidis* groups A and C were prepared as described (7, 20). We are grateful, to B. Prescott of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., for his generous gifts of pneumococcus types I and III capsular polysaccharides, to K. Amiraian, New York State Public Health Laboratories, Albany, N.Y., and to E. C. Gotschlich, Rockefeller University, N.Y., N.Y., for the pneumococcal and meningococcal antisera, respectively.

Serological methods. The cross-reactions of the 174 strains of the genus *Bacillus* were studied by immunodiffusion by use of overnight broth cultures of the bacilli and type-specific antiserum (18). All strains were tested with each of the antisera. The immunodiffusion gels were observed for precipitin bands after incubation at 4 C for 48 h and again after staining with amido Schwartz (18). Absorbed serum was prepared by incubation with the purified polysaccharides at 37 C for 1 h and for 48 h at 4 C. The absorbed serum was centrifuged for 1 h at 20,000 × *g* at 4 C and tested for its reactivity by immunodiffusion gels (16) with 0.15 M D-glucuronic acid and 0.15 M *N*-acetyl-D-mannosamine (Sigma Chemical Co., St. Louis, Mo.). Controls included D-glucose and ribose-5-phosphate (Sigma Chemical Co., St. Louis, Mo.).

Quantitative measurement of serum *H. influenzae* type b (19) and pneumococcal type III (G. Schiffman, M. Robin, and R. Austrian, in press) anti-capsular antibodies in immunized animals was made by radioimmunoassay. Complement-dependent bactericidal assay was carried out by using *H. influenzae* type b strain "rab" (8) according to a previously described method (11).

RESULTS

Table 2 summarizes the results of this survey. Of the 174 strains tested, nine bacilli (5.2%) had cross-reacting antigens, including one strain with type III pneumococcus, six strains with group A meningococcus, one strain with *H. influenzae* type b, and one strain with both type III pneumococcus and *H. influenzae* type b. No cross-reactions to the capsular polysaccharides of meningococcus group C or pneumococcus type I were observed in this collection.

H. influenzae type b: One *B. pumilus* strain [American Type Culture Collection (ATCC) no. 72], precipitated with *H. influenzae* type b antiserum, yielding a reaction of complete identity with the previously described cross-reacting purified polysaccharide of *B. pumilis* strain Sh 18 (14) and a reaction of partial identity with the type b capsular polysaccharide (Fig. 1). Absorption of the *H. influenzae* type b antiserum with either of the two *B. pumilis* antigens completely removed the precipitating activity toward both of these cross-reacting antigens and reduced its reaction with the *H. influenzae* type b polysaccharide. Absorption

TABLE 2. *Strains of the genus bacillus with cross-reactive antigens*

Species	ATCC no.	Cross-reaction
B. cereus var. *mycoides*	10206	*D. pneumoniae* type III
B. pumilis	7065	*N. meningitidis* Group A
B. pumilis	7061r	*N. meningitidis* Group A
B. pumilis	7061s	*N. meningitidis* Group A
B. pumilis	6632	*N. meningitidis* Group A
B. pumilis	14884	*N. meningitidis* Group A
B. pumilis	12140	*N. meningitidis* Group A
B. pumilis	72	*H. influenzae* type b
B. alvei	6348	*D. pneumoniae* type III and *H. influenzae* type b

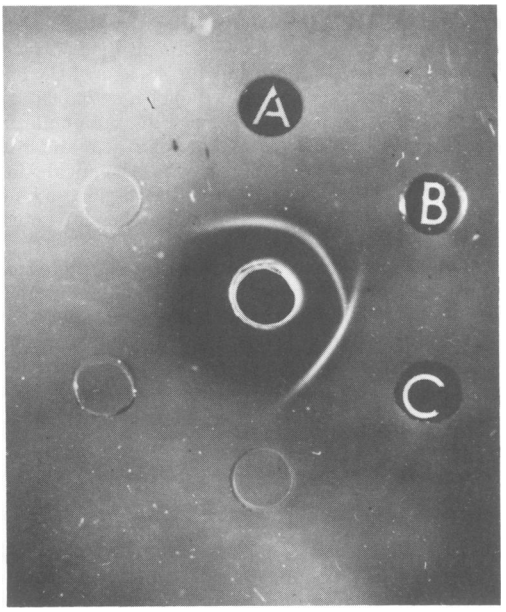

FIG. 1. *Precipitin reactions of partially purified antigen of B. pumilis (ATCC no. 72) in the well labeled B (1 mg/ml) with H. influenzae type b antiserum (central well). A reaction of complete identity is seen with the antigen of B. pumilis strain Sh 18 in the well labeled A (1 mg/ml) and a reaction of partial identity with the type b capsular polysaccharide is seen in the well labeled C (0.1 mg/ml).*

with the type b polysaccharide abolished reactivity with all three antigens. Immunization of rabbits with either *B. pumilis* strain resulted in the formation of precipitating antibodies to the *H. influenzae* type b capsular polysaccharide and complement-dependent bactericidal activity toward *H. influenzae* type b strain "rab" (Table 3).

D. pneumoniae type III. One strain, *B. cereus* var. *mycoides* (ATCC no. 10201), precipitated with pneumococcus type III antiserum and yielded a reaction of partial identity with the pneumococcus type III capsular polysaccharide (SIII) when either the whole broth culture or the partially purified *B. cereus* var. *mycoides* polysaccharide was used. Incubation of the gel in 0.15 M D-glucuronic acid for 15 min at room temperature completely dissolved the precipitin line between *B. cereus* var. *mycoides* and the pneumococcal type III antiserum. Neither the overnight broth culture nor the *B. cereus* var. *mycoides* antigen reacted with the SIII-absorbed pneumococcal type III antiserum. The type III pneumococcus capsular polysaccharide is reported to consist of a polymer of the disaccharide cellobiuronic acid, which is D-glucose and D-glucuronic acid linked in an $\alpha 1,4$ glycosidic bond (13). The inhibition experiments suggest that the cross-reactivity of *B. cereus* var. *mycoides* polysaccharide is due to its content of glucuronic acid.

Immunization of rabbits with *B. cereus* var. *mycoides* produced a serum that contained low levels of anti-SIII antibodies as measured by radioimmunoassay and mouse protective activity (Table 3). Note that, although the *B. cereus* var. *mycoides* antigen did not precipitate with the *H. influenzae* type to antiserum, a small but detectable immune response to *H. influenzae* type b capsule was induced. The possibility that this response is due to the presence of an ubiquitous ribitol phosphate teichoic acid present in the cell walls of most bacilli is presently under investigation.

B. alvei (ATCC no. 6348). One strain of B. alvei (ATCC no. 6348) contained an antigen that precipitated weakly with both *H. influenzae* type b and pneumococcus type III antisera. After absorption of both antisera with the appropriate homologous capsular polysaccharide, the precipitin reactivity of these antisera toward *B. alvei* (ATCC no. 6348) was removed. Immunization of rabbits with *B. alvei* strain resulted in the production of small but detectable amounts of anti-capsular and complement-dependent bactericidal antibody to *H. influenzae* type b as well as anti-SIII and mouse protective antibodies toward pneumococcus type III.

N. meningitidis group A. A cross-reaction was observed with meningococcus group A polysaccharide in 6 of 20 strains of *B. pumilus* when either rabbit or burro meningococcus group A antiserum was used. The cross-reacting antigens obtained from these 6 strains of *B. pumilis* showed an identity reaction by immunodiffusion analysis with each other as well as with the purified polysaccharide of another previously described strain of *B. pumilis* (Sh 17) (17) (Fig. 2). Antisera raised with Sh 17 including sera from one burro and three rabbits, previously shown to precipitate with meningococcus group A capsular polysaccharide, precipitated with all 6 *B. pumilis* strains with a reaction of complete identity. *N*-acetyl-D-mannosamine completely dissolved the precipitin reaction of the purified Sh 17 polysaccharide and the 6 other *B. pumilis* strains with the meningococcus group A antiserum.

These data indicate the antigenic identity of the cross-reacting polysaccharide of the 6 *B. pumilis* strains and the Sh 17 strain. The chemical analysis of the Sh 17 cross-reacting antigen as well as quantitative immunochemical data indicate that the cross-reacting moiety in these *Bacillus* strains contains *N*-acetyl-D-mannosamine phosphate (T. Y. Liu, E. C.

TABLE 3. *Results of rabbit immunization with cross-reacting bacilli*

Immunizing strain	Average of serum antibodies[a]							
	Microgram antibody per ml of serum				Mouse protective activity		Bactericidal activity	
	H. influenzae type b		SSS III		*D. pneumoniae* III		*H. influenzae* type b	
	Pre-immune	Immune	Pre-immune	Immune	Pre-immune	Immune	Pre-immune	Immune
B. pumilis (ATCC no. 72)	0.2	84.8	<0.1	<0.1	<1/1	<1/1	1/2	1/800
B. cereus var. mycoides (ATCC no. 10206)	0.1	3.2	<0.1	8.4	<1/1	1/5	1/4	1/16
B. alvei (ATCC no. 6348)	0.2	7.2	<0.1	6.8	<1/1	1/2	1/2	1/64

[a] Each value represents the average of three rabbits.

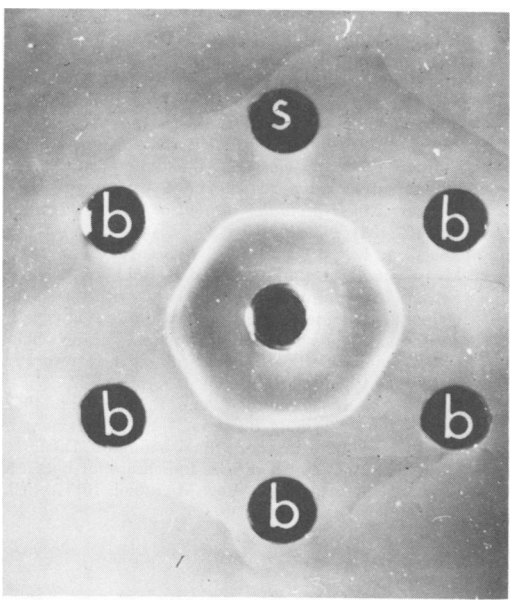

FIG. 2. *Precipitin reaction of partially purified antigens of five B. pumilis strains in wells labeled b (1 mg/ml) with meningococcus group A antiserum (central well). A reaction of complete identity is observed between all five antigens as well as the antigen of B. pumilis strain Sh 17 in the well labeled S (1 mg/ml).*

Gotschlich, R. L. Myerowitz, and J. B. Robbins, manuscript in preparation).

DISCUSSION

Cross-reactions among the polysaccharides of nonpathogenic enteric bacteria with the capsular polysaccharides of pyogenic bacteria have been previously reported (8, 9, 18, 19, 21). This report cites yet another genus of bacteria yielding frequent cross-reactions with the capsular polysaccharides of pyogenic bacteria. These capsular polysaccharides are believed to be the "virulence factors" responsible for the pathogenicity of pneumococci, meningococci, and *H. influenzae* type b. The possibility that cross-reacting antigens may play a role in virulence of otherwise noninvasive bacteria such as bacilli is the subject of ongoing investigation (15). These findings suggest that a study of the immunogenicity of these bacilli as residents of the gastrointestinal tract or other "normal flora" be instituted as a possible antigenic source for "natural" serum antibodies observed in most adults to encapsulated pyogenic organisms such as meningococci, pneumococci, and *H. influenzae* type b.

The structural localization of these cross-reacting antigens of the genus *Bacillus* has not been identified. The production of the antigen in fresh broth culture identifies the antigen as a constituent of the vegetative cell. The isolation of the cross-reactive antigen from cell-free supernatants is consistent with the notion that these polysaccharides may be extracellular. Further work, however, is needed to confirm the designation of the cross-reacting antigens as an extracellular polysaccharide and to investigate the possibility that these antigens are also present on the spores of these bacilli.

It is of interest that, among seven strains of *B. pumilis* from the collection under study, cross-reactions were observed with the capsular polysaccharides of two pyogenic bacteria, meningococcus group A and *H. influenzae* type b. The antigenic variability within any single species of *Bacillus* has been previously stressed (23) and is again emphasized by these data.

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