Diversity of Vi-Related Antigens in the Microcapsule of *Salmonella typhi*

BOGUSŁAW SZEWCZYK AND ALINA TAYLOR*

Department of Biochemistry, University of Gdańsk, PL-80-822 Gdańsk, Poland

Two new antigenic acidic polysaccharides, Vi-P and Vi-C, were isolated from the microcapsule of *Salmonella typhi* by a very mild procedure. The antigens were purified to serological homogeneity, and it was found that each of them shares with Vi antigen one of its two serological determinants (a different one in each case). One of the antigens, Vi-C, was also isolated from *Escherichia coli* B, which is not a Vi-producing strain.

Studies on *Salmonella typhi* microcapsular polysaccharides have been almost completely confined to Vi antigen, due to its manifold biological roles. Vi antigen has been purified (1, 8, 16, 18), and its structure has been elucidated (5). It is a highly acidic polysaccharide composed of *O*- and *N*-acetylated galactosaminouronic acid units connected through $\alpha(1-4)$ - linkages. Its structure appears to be relatively simple; however, Martin et al. (10) pointed out that polydispersion of Vi antigen is observed after ultrasonic treatment, which probably destroys postulated interchain ester bonds. It is possible that the same bonds are responsible for the presence of two fractions during immunoelectrophoresis of chemically pure Vi antigen (17). These fractions appear to be immunochemically identical, though they differ in Vi phage receptor properties (17).

We here describe the purification and properties of two antigens from *S. typhi* which are immunochemically related to, but not identical with, Vi antigen. Their cross-reactivity with Vi antigen is explained on the basis of the immunochemistry of this antigen, which has two antigenic determinants (15). One of the Vi determinants has an *O*-acetyl moiety as the immunodominant group; in the second, both *N*-acetyl and carboxyl groups play a dominant role.

MATERIALS AND METHODS

Bacteria. *S. typhi* Ty2, *S. typhi* 21802, and *S. typhi* 88 (Vi⁻ strain) were obtained from the National Reference Laboratory for Enteric Phage Typing, Gdańsk, Poland. *S. typhi* strains and *Escherichia coli* B were cultivated on a standard agar medium supplemented with 1% peptone. *S. typhi* Ty2 was also grown in a liquid medium (aerated casein hydrolysate broth containing 0.1% glucose). Harvested cells were dried with acetone.

Isolation of the crude antigens. Acetone-dried cells were shaken with 0.9% NaCl solution for 1 h (1 g

of dried cells in 50 ml of saline), and the bacteria were centrifuged off. Three volumes of acetone were added to the supernatant, and the precipitate was collected by centrifugation and dried in acetone. It was digested first with ribonuclease and deoxyribonuclease (7) and then with pancreatin (16). After extensive dialysis, the non-dialyzable material was subjected to affinity chromatography on erythrocyte stroma in order to remove the Vi antigen (16). The material not absorbed on the column was fractionated with Cetavlon according to Hungerer et al. (6), yielding a crude mixture of polysaccharides. In the case of *E. coli* B extracts, the affinity chromatography on erythrocyte stroma was omitted, and polysaccharides were directly fractionated with Cetavlon.

Analytical methods. Total carbohydrates were determined with phenol-sulfuric acid (4). Hydrolysis of polysaccharides was done with either 6 N HCl at 100°C for 12 h (for detection of aminogalacturonic acid) or 0.25 N H₂SO₄ at 100°C for 12 h (for detection of neutral monosaccharides). Thin-layer chromatography of the former hydrolysate was performed on silica gel plates (Merck, catalog no. 5567) in the *n*-butanol-acetic acid-water (4:1:5 by volume) solvent system. Thin-layer chromatography plates were developed twice and sprayed with 0.2% ninhydrin in acetone. The latter hydrolysate was chromatographed on cellulose plastic plates (Merck, catalog no. 5578) in an ethyl acetate-pyridine-water (2:1:2, upper phase) solvent system. Sugars were detected with 0.1 M anisidine-phthalate reagent in ethanol. Agarose gel electrophoresis for testing the purity of polysaccharides was performed in 50 mM barbital buffer, pH 9.6 and 8.6, and in 50 mM sodium acetate, pH 6.0, at 10 to 15 V/cm, using cresol red as the indicator. After precipitation of the polysaccharides with Cetavlon (3), the agarose plates were dried and stained with amido black (2).

Antigens. Native Vi antigen, *O*-deacetylated Vi antigen, and 2,3-di-*O*-acetylpolysaccharide were obtained by methods described previously (15).

Antisera. Rabbit antisera against *S. typhi* 21802 and *Citrobacter ballerup* 107 (Vi antiserum) were obtained from the National Reference Laboratory for Enteric Phage Typing.

Serological techniques. Precipitin reactions in

agarose gels were performed by the Ouchterlony technique (12). One-dimensional immunoelectrophoresis was done by the Scheidegger method (13), and crossed immunoelectrophoresis was performed according to Laurell (9). The dried gels were stained with 0.5% solution of Coomassie blue in acetic acid-methanol-water (1:5:5 by volume) for 20 min at room temperature. The excess stain was removed by washing the gels with the same solution without Coomassie blue.

Indirect hemagglutination was performed with formalized sheep erythrocytes (SRBC). For sensitizing SRBC with antigens, 0.1 mg of antigenic material was added to 10 ml of 1% washed-SRBC suspension in saline. After incubation at 37°C for 30 min, the excess antigen was washed off and the sensitized SRBC were resuspended in saline to give a 1% suspension. The SRBC suspension was added to series of antiserum dilutions in microtiter plates as described previously (11).

To measure the inhibiting capacity of a substance, the hemagglutination inhibition test was used. Serial dilutions (0.2 ml) of the inhibitor were incubated at 37°C for 1 h with 0.2 ml of a serum dilution containing 2 hemagglutination units. Then 0.2 ml of the sensitized SRBC was added, and the plates were again incubated for the same time. The lowest inhibitor concentration giving a total inhibition of hemagglutination was recorded after 2 h at room temperature.

Quantitative precipitin assays were performed according to Hungerer et al. (6).

RESULTS

Choice of antisera. The results of previous quantitative precipitation tests for the native and *O*-deacetylated Vi antigens in various Vi antisera showed that the ratios of antibodies precipitated by these compounds are not constant (15). The amounts of antibodies in the tested antisera precipitated by the *O*-deacetylated antigen in relation to those precipitated by the native Vi antigen ranged from 0 to 65%. Three antisera were chosen for studies of the immunochemical properties of Vi-related antigens: two unabsorbed *S. typhi* 21802 antisera and one *C. ballerup* 107 antiserum absorbed with a Vi⁻ strain of *C. ballerup*. The antisera contained 0% (antiserum I), less than 20% (antiserum II), and about 65% (antiserum III), respectively, of the antibodies against *O*-deacetylated Vi antigen as compared with the native Vi antigen.

Purification of Vi-related antigens from *S. typhi*. The crude mixture of acidic polysaccharides obtained from *S. typhi* cells (see Materials and Methods) was fractionated on a diethylaminoethyl-Sephadex A-25 column. The pattern of separation for the polysaccharides of *S. typhi* 21802 grown on agar is shown in Fig. 1. A similar pattern was obtained for the polysaccharides of *S. typhi* Ty2 grown in the same medium. Fractions eluted from the column (frac-

tions 1 through 9) were analyzed by immunoelectrophoresis with all three antisera. Fractions 1 through 4 and 7 through 9 did not react with any antiserum. Fraction 5 contained two antigens, denoted Vi-P (higher electrophoretic mobility) and Vi-C (lower electrophoretic mobility), whereas fraction 6 contained immunochemically pure Vi-C. Figure 2 represents the results of crossed immunoelectrophoresis for fraction 5. Antigen Vi-P was precipitated in antisera II and III, whereas antigen Vi-C was precipitated in antisera I and II. The electrophoretic mobility of Vi-P was equal to that of the faster fraction of the native Vi antigen. The electrophoretic mobility of Vi-C was less than that of the slower fraction of the Vi antigen.

Antigen Vi-P was further purified by preparative gel electrophoresis on glass plates (20 by 20 cm) with a 0.4-cm-thick agarose (Fluka) layer in 50 mM barbital buffer, pH 8.6, at 10 V/cm. The position of the antigen was spotted by immunoelectrophoresis in the second dimension of the thin agarose strip cut longitudinally. Polysaccharide Vi-P was washed out of the agarose with water and then precipitated with Cetavlon and ethanol (6). Soluble polysaccharide components of the agarose were eliminated during the precipitation. Antigen Vi-P purified by this method gave a single, well-defined spot on electrophoresis in three buffer systems (pH 6.0, 8.6, and 9.6). When *S. typhi* Ty2 was grown in liquid medium, the step of electrophoretic purification could be omitted, as antigen Vi-C is not pro-

FIG. 1. Diethylaminoethyl-Sephadex separation of polysaccharides from *S. typhi* 21802 precipitated with Cetavlon. The mixture of polysaccharides (100 mg) was loaded on a diethylaminoethyl-Sephadex A-25 column (1.6 by 100 cm), and fractions (10 ml) (solid line) were eluted with a gradient of KCl (0 to 1.0 M) (broken line) in 0.1 M phosphate buffer, pH 6.0. Total carbohydrates were estimated by the phenol-sulfuric acid assay (4). Combined fractions 1 through 9 were tested for antigenic activity with Vi antisera.

FIG. 2. Antigen-antibody crossed immunoelectrophoresis of 30 μ l (150 μ g) of fraction 5 from diethylaminoethyl-Sephadex separation. Different Vi antisera (25 μ l/cm²) were used in the second-dimension gels; antiserum I (A), antiserum II (B), and antiserum III (C). (D) Crossed immunoelectrophoresis of 50 μ g of Vi antigen (included for comparison) with antiserum III (25 μ l/cm²) used in the second dimension. Technical details: dimensions of the plates, 10 by 10 cm; 1% (wt/vol) agarose gel in barbital buffer (pH 8.6, ionic strength, 0.05); thickness of gel, 1 mm; first-dimension electrophoresis, 10 V/cm for 1 h at 5°C, anode to the right; second-dimension electrophoresis, 2 V/cm for 24 h at 5°C, anode at the top, staining; Coomassie brilliant blue.

duced under these conditions.

The Vi⁻ strain of *S. typhi* (*S. typhi* 88) produced exocellular polysaccharides which were eluted from the diethylaminoethyl-Sephadex column in the NaCl concentration range of 0.8 to 1.0 M. On electrophoresis they gave a badly defined spot roughly corresponding to the position of antigen Vi-C. These polysaccharides did not react in any Vi antiserum; nevertheless, they may contaminate the serologically pure antigen Vi-C if they are produced by the Vi⁺ strains of *S. typhi*.

Isolation of antigen Vi-C from *E. coli* B cells. Polysaccharides Vi-P and Vi-C inhibit the activity of lysozymes of Vi and T4 phages (un-

published data). We therefore searched for these polysaccharides in *E. coli* B, which is a natural host of T4 phages. Indeed, it was observed that the fraction of acidic polysaccharides from *E. coli* B precipitated by Cetavlon contained an antigen reacting in antisera I and II. Crossed immunoelectrophoresis showed that this antigen had the same electrophoretic mobility as antigen Vi-C of *S. typhi* (Fig. 3). When studied by the agar immunodiffusion method, these two antigens were found to be identical (see Fig. 5 and 6).

Immunochemical characterization of antigens. (i) Immunodiffusion tests. Precipitin reaction in agarose gel was used to confirm the

FIG. 3. Antigen-antibody crossed immunoelectrophoresis of 30 μ l (150 μ g) of *E. coli* B extract fractionated with Cetavlon. Antiserum II (25 μ l/cm²) was used in the second-dimension gel. Technical details were the same as in the legend of Fig. 2.

serological identity of purified Vi-P preparations from different sources. The reaction between antiserum III and Vi-P antigens isolated from *S. typhi* strains Ty2 and 21802 is demonstrated in Fig. 4. The precipitin lines of these antigens and the precipitin line of O-deacetylated Vi antigen fused; thus, their immunochemical identity was established.

The immunodiffusion pattern for antigen Vi-C and native and O-deacetylated Vi antigen is shown in Fig. 5. The mutual arrangement of precipitin lines indicated that antigen Vi-C had at least one antigenic determinant less than native Vi antigen. However, the immunodiffusion pattern for Vi-C and 2,3-di-O-acetylpolysaccharuronic acid (Fig. 6) showed that they were serologically identical. As shown previously (15), 2,3-di-O-acetylpolysaccharuronic acid shares one antigenic determinant with Vi antigen.

The absorption of any Vi antiserum used in our studies with native Vi antigen rendered it inactive with both antigens, Vi-P and Vi-C.

(ii) **Immunoelectrophoresis.** Purified Vi antigen consisted of two fractions which could be separated by electrophoresis. The fraction with greater electrophoretic mobility comigrated with antigen Vi-P. When a mixture of Vi antigen and antigen Vi-P was subjected to immunoelectrophoresis using antiserum II or III, the antigens gave precipitin lines which formed a spur when they converged (the results for antiserum III are shown in Fig. 7). Antigen Vi-C formed a spur with the slower fraction of Vi antigen when antiserum II was used (data not shown), though in this case the mobility of Vi-C

was lower than that of the slower fraction of Vi antigen. These facts by no means manifested the identity of Vi-P and Vi-C with two fractions of Vi antigen, because it was only in antiserum II that both Vi-P and Vi-C antigens were precipitated. As mentioned earlier, antigen Vi-P did not react in antiserum I, whereas antigen Vi-C did not form a precipitate when antiserum III was used. Nevertheless, the native Vi antigen had the same profile of precipitin lines irrespective of the antiserum used.

(iii) Passive hemagglutination and inhi-

FIG. 4. Double immunodiffusion pattern, showing the serological identity of O-deacetylated Vi antigen and antigen Vi-P isolated from different sources. (1) Vi-P (0.5 mg/ml) isolated from *S. typhi* 21802 grown on agar; (2) Vi-P (0.5 mg/ml) isolated from *S. typhi* Ty2 grown in agar; (3) O-deacetylated Vi antigen (0.1 mg/ml); (4) Vi-P (0.5 mg/ml) isolated from *S. typhi* Ty2 grown in liquid medium; (5) Vi-C (0.5 mg/ml) isolated from *S. typhi* 21802 grown on agar. The center well (A) contains antiserum III.

FIG. 5. Double immunodiffusion pattern, showing the relationship between antigen Vi-C, native Vi antigen, and O-deacetylated Vi antigen. (1) O-Deacetylated Vi antigen (0.1 mg/ml); (2) antigen Vi-C (0.5 mg/ml) from *S. typhi* 21802; (3) antigen Vi-C from *E. coli* B (1 mg/ml), (4) native Vi antigen (0.05 mg/ml). The center well (A) contains antiserum II.

bition of hemagglutination of Vi-coated SRBC. Antigen Vi-P, like *O*-deacetylated Vi antigen (15), did not coat formalized SRBC nor did it inhibit the hemagglutination of Vi-coated SRBC in any of the antisera studied. Antigen Vi-C sensitized SRBC for hemagglutination with each of the antisera studied (e.g., antiserum III showed a titer of 320 against SRBC coated with Vi-C, in comparison with a titer of 2,560 for the native Vi antigen). This indicates that antigen Vi-C may contain *O*-acetyl groups, because, as shown previously (15), the presence of *O*-acetyl groups is an indispensable condition for hemagglutination with Vi antiserum. The amounts of

Vi-C necessary to inhibit the hemagglutination of Vi-coated SRBC with antisera I and III observed the relation described previously for the inhibition of hemagglutination of Vi-coated erythrocytes with 2,3-di-*O*-acetylpolysaccharuronic acid (15); that is, 16 times more Vi-C was necessary to produce the inhibition in antiserum III than in antiserum I (Table 1). The absolute inhibitory amounts of Vi-C were relatively high (25.6 μ g of Vi-C per ml in antiserum III in comparison with 3.2 μ g/ml for 2,3-di-*O*-acetyl polysaccharuronic acid and 0.1 μ g/ml for the native Vi antigen), which may indicate that antigen Vi-C was not chemically pure or that a monosaccharide bearing an antigenic determinant was separated by a unit of other monosaccharides.

Chemical characterization of the antigens. Detailed chemical analyses of the electrophoretically pure Vi-P will be presented in a separate paper (B. Lindberg, P. E. Jansson, B. Szewczyk, and A. Taylor, manuscript in preparation). Antigen Vi-P was found to be composed mainly (about 70% by weight) of *N*-acetylaminogalacturonic acid linked by α (1-4)-glycosidic bonds. Galactose made up the remaining 30%. Hydrolysis of Vi-P with a diluted acid (0.1 M trifluoroacetic acid, 30 min, 100°C) removed the galactose residues, leaving the intact *N*-acetylaminogalacturonic acid chains. When antigen Vi-P was passed over a Dowex 50Wx8 (H^+) column, two fractions were obtained, the insoluble one consisting of the pure *N*-acetylaminogalacturonic acid chains and the soluble one containing more than 80% of the non-dialyzable galactan. The soluble fraction presumably also contained some *N*-acetylaminogalacturonic

FIG. 6. Double immunodiffusion pattern, showing the identity of antigen Vi-C and 2,3-di-*O*-acetylpolysaccharuronic acid. (1, 3, 7) 2,3-Di-*O*-acetylpolysaccharuronic acid (0.1 mg/ml); (2, 5, 6) antigen Vi-C (0.5 mg/ml) from *S. typhi* 21802; (4) antigen Vi-C (1 mg/ml) from *E. coli* B. The center well (A) contains antiserum II.

FIG. 7. One-dimensional immunoelectrophoresis (A, C) and electrophoresis (B) under the same conditions (50 mM barbital buffer, pH 8.6, 10 V/cm), comparing the mobilities of native Vi antigen and pure antigen Vi-P. (A) 1, Vi antigen (10 μ g); 2, Vi-P (10 μ g); center trough, antiserum III (300 μ l). (B) 1, Vi-P (50 μ g); 2, Vi antigen (50 μ g). (C) 1, Mixture of Vi antigen (10 μ g) and Vi-P (10 μ g); 2, Vi-P (10 μ g); center trough, antiserum III (300 μ l).

TABLE 1. Serological activities of the native Vi antigen and Vi-related antigens with different Vi antisera^a

Antigen	Precipitation in agar gel with antiserum:			Passive hemagglutination with antiserum:			Inhibition of hemagglutination of Vi-coated SRBC (minimal inhibiting dose, µg/ml) with antiserum:	
	I	II	III	I	II	III	I	III
Native Vi	+	+	+	+	+	+	0.2	0.1
Vi-P	+	+	+	+	+	+	None	None
Vi-C	+	+	+	+	+	+	1.6	25.6
O-Deacetylated Vi	+	+	+	+	+	+	None	None
2,3-Di-O-acetyl polygalacturonic acid	+	+	+	+	+	+	0.2	3.2

^a Ratios of antibodies precipitated by O-deacetylated Vi antigen to antibodies precipitated by the native Vi antigen were as follows: antiserum I, 0.0; antiserum II, 0.2, and antiserum III, 0.65.

^b At a high concentration of the antigen.

acid, as it precipitated antibodies from antisera II and III. Therefore, we observed the production of acetylated (Vi) and O-deacetylated (Vi-P) polysaccharide polymers by the same bacterial strain. This is not an exceptional situation among bacteria, as other microbial polysaccharides, such as colanic acid, alginates, or *Xanthomonas* polysaccharides are also produced in acetylated and deacetylated forms (14).

Aminogalacturonic acid was released from native Vi and from Vi-P during a very drastic hydrolysis with concentrated HCl. However, it was not detected in the hydrolysis products of antigen Vi-C hydrolyzed in the same conditions. The hydrolysis of Vi-C with sulfuric acid (0.25 N, 12 h, 100°C) yielded galactose as the only neutral sugar component. More detailed chemical analyses of Vi-C will be undertaken after its chemical purity is ascertained.

DISCUSSION

Two new Vi-related antigens were isolated from an *S. typhi* microcapsule in serologically pure forms. The content of the first, Vi-P, in dry bacteria amounted to about 0.1 to 0.2% by weight. This polysaccharide is produced on agar, as well as in a liquid medium. Bacteria grown on agar produce one more Vi-related antigen, Vi-C, though in much lower amounts (0.01 to 0.03% of dry bacterial weight).

Studies on the immunochemistry of Vi antigen reported previously (15) throw some light on the behavior of these antigens in different rabbit Vi antisera. Vi antigen possesses two antigenic determinants; one has O-acetyl as the immunodeterminant group, and the other, larger one con-

tains N-acetyl and carboxyl groups in the immunodominant region. The first of the antisera used in our studies (antiserum I) possessed only the antibodies against the O-acetyl groups of Vi antigen. The lack of reaction of Vi-P in antiserum I therefore may be easily explained by the fact that Vi-P is immunochemically identical with O-deacetylated Vi antigen. More puzzling is the behavior of antigen Vi-C in Vi antisera. The immunodiffusion and immunoelectrophoresis of this antigen reveal that, unlike Vi-P, it precipitates Vi antibodies from antiserum I, but not from antiserum III, where the ratio of the antibodies precipitated by O-deacetylated Vi antigen to the antibodies precipitated by native Vi antigen is equal to 0.65. On the other hand, antigen Vi-C is active in the passive hemagglutination test, and it also inhibits the hemagglutination of Vi-coated SRBC in each of the antisera studied (Table 1). The immunodiffusion tests show that Vi-C is serologically identical with 2,3-di-O-acetylpolysaccharide. On the basis of these data, we assume that antigen Vi-C, like 2,3-di-O-acetylpolysaccharide, precipitates only those Vi antibodies which react with that part of the Vi antigen which has O-acetyl as the immunodeterminant group. This assumption helps us to explain the reactions of Vi-C in antiserum III. The results of the quantitative precipitin tests for 2,3-di-O-acetylpolysaccharide acid reported previously (15) indicated that over 15 times more of this polysaccharide is needed to precipitate the same amount of antibodies from antiserum III than from antiserum I. This may be explained by the inhibitory action of 2,3-di-O-acetylpolysaccharide acid on the antibodies directed against O-deacetylated Vi antigen. The same experiments could not be performed for antigen Vi-C because of its low solubility (less than 1 mg/ml), but on the basis of the comparative hemagglutination inhibition studies we presume that antigen Vi-C may also inhibit these antibodies. Hence, the precipitate is visible only when the ratio of the antibodies precipitated by O-deacetylated Vi antigen to the antibodies precipitated by native Vi antigen is sufficiently low (e.g., as in antiserum II).

The relatively low specificity of the antibodies against the O-acetyl determinant of Vi antigen, demonstrated by their reaction with 2,3-di-O-acetylpolysaccharide acid, with the carboxyl reduced acetylated Vi antigen, and with the Vi antigen with only one O-acetyl residue per five monosaccharide units, described in detail previously (15), may be responsible for the reactions of *E. coli* B extracts in antisera I and II. It appears to be much easier to produce and purify

antigen Vi-C from this microorganism, so we hope to be able to elucidate its structure and to establish its relation to the other known, common polysaccharide antigens.

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