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Production of Monoclonal Antibodies Specific for the *i* and *1,2* Flagellar Antigens of *Salmonella typhimurium* and Characterization of Their Respective Epitopes

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Salmonella typhimurium **expresses two antigenically distinct flagellins, each containing a different H antigen (*i* and *1,2*), the combination of which is highly specific for this serotype. In this study, overlapping recombinant flagellin fragments were constructed from the *fliC* (H:*i*) and *fljB* (H:*1,2*) flagellin genes, and the expression products were tested for binding to H antigen-specific monoclonal and polyclonal antibodies. A minimal area, 86 amino acids for H:*i* and 102 amino acids for H:*1,2*, located in the central variable domain of each flagellin was required for the binding of serotype-specific antibodies, providing further evidence for the presence of a discontinuous H epitope. Two peptides comprising these areas were shown to be highly suitable for application as antigens in an enzyme-linked immunosorbent assay detecting *S. typhimurium*-specific antibody.**

Worldwide, *Salmonella typhimurium* is a major cause of human food poisoning. The consumption of food products originating from infected animals is a primary source of human infection. To efficiently detect and ultimately eliminate the presence of *Salmonella* from animal reservoirs such as pigs and poultry, there is a need for a rapid and sensitive assay. Flagella are immunodominant antigenic surface structures of *Salmonella* that, upon infection, elicit an early short-lived humoral response in the host, including very young animals (19), making them ideal for the detection of infections in livestock of all ages. Flagellin is the major structural protein of flagella and carries the serotype-specific H-antigenic determinants (5). These H antigens are located in the central variable domain comprising flagellin regions IV, V, and VI (11, 20) and probably induce the production of serotype-specific antibodies in the infected host. On the other hand, antibodies against the conserved N- and C-terminal flagellin domains or regions I, II, and VIII give rise to cross-reactions between *Salmonella* serotypes and other *Enterobacteriaceae* in serological tests based on whole purified flagellum antigen (3). Furthermore, flagellar proteins are easily produced through heterologous expression systems and therefore can be obtained in large quantities that are relatively pure (7, 16). This is an advantage over the lipopolysaccharide (O antigens), the other commonly used antigen in *Salmonella*-specific serological assays. In a previous study we determined the location of the serotype-specific H:*gm* antigen of *Salmonella enteritidis* through the construction of overlapping recombinant peptides from its flagellin (16). Testing of these peptides with *gm*-specific antibodies resulted in the selection of a peptide that contained the H:*gm* epitope specific for *S. enteritidis* and allowed the development of an *S. enteritidis*-specific enzyme-linked immunosorbent assay (ELISA).

S. typhimurium (H:*i:1,2*) carries two distinct flagellin genes (*fliC* and *fljB*), and through phase variation it can express two antigenically different flagella on its surface (9). In the present study we have isolated both flagellin genes of *S. typhimurium* and cloned overlapping fragments of these genes in an expression vector. We produced monoclonal antibodies (MAbs) to both *S. typhimurium* flagellins and used these to select two recombinant flagellin fragments of 86 and 102 amino acids, specific to the H:*i* and H:*1,2* antigens, respectively. These peptides appeared to be highly suitable antigens in an ELISA detecting *S. typhimurium*-specific antibodies.

Production and validation of H:*i*- and H:*1,2*-specific MAbs. Purified *S. typhimurium* flagellins enriched in H:*i* or H:*1,2* were prepared as described by van Zijderveld et al. (19). MAb-producing hybridoma cell lines were obtained from mice immunized with the purified flagellin. Three H:*i*-specific MAbs (IH10, IC11, and VB5) and three H:*1,2*-specific MAb-producing cell lines (XA1, XA9, and VIID6) were selected (19) and used for further analyses. The H:*gm*-specific MAb (gm3) from our previous studies (16, 19) was used as a negative control.

In order to confirm the specificities of the anti-*i* and anti-*1,2* MAbs for their respective H antigens, serotyping of isolates of various biphasic *Salmonella* serotypes (Table 1) was performed on Western blots, both with MAbs and with commercially obtained H:*i*- H:*1,2*-, or H:*gm*-specific serotyping sera (absorbed agglutinating rabbit sera; Murex Diagnostics, Dartford, United Kingdom). *Salmonella* strains were cultured overnight at 37°C in Luria-Bertani broth, and whole-protein cell extracts were separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and then electrophoretically transferred (1) onto a Hybond C nitrocellulose filter (Amersham, Buckinghamshire, United Kingdom). Binding of commercial typing antiserum (1:400) or MAbs (IH10, 1:4,000; IC11 and VB5, 1:1,000; XA1, XA9, and VIID6, 1:4) to different *Salmonella* serotypes was analyzed by a Western blot assay performed essentially as described by Ausubel et al. (1).

As expected, the commercial serotyping antisera showed only weak nonspecific binding to heterologous flagellins or other unrelated protein bands (Fig. 1C and Table 1). When we

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TABLE 1. Validation of H antigen-specific MAbs compared to commercial serotyping antisera (Murex) with whole-protein cell extracts of various *Salmonella* serotypes in Western blots

Common H antigen	*Salmonella* strain (serotype)	H:*i*-specific antibody binding[d]			H:*1,2*-specific antibody binding[d]		
		Serotyping antisera		MAbs	Serotyping antisera		MAbs
		1[b]	2[b]		1[c]	2[c]	
H:*i:1,2*	*S. typhimurium* (*i:1,2*) human isolates[a]	+	+	+	+	+	+
	S. typhimurium (*i*) human isolates[a]	+	+	+	+	+	+
	S. typhimurium (*1,2*) human isolates[a]	±	+	±	+	+	+
	S. typhimurium (*1,2*) human isolates[a]	−	−	−	+	+	+
	S. typhimurium (*i*) ATCC 14028[a]	+	+	+	±	−	−
	S. aberdeen[a] (*i*)	+	+	+	±	±	−
H:*i*	*S. bonariensis* (*i:enx*)	+	+	+	−	−	−
	S. kentucky (*i:z*$_6$)	+	+	+	±	±	−
	S. bergen, *S. jukestown* (*i:enz*$_{15}$)	+	+	+	−	±	−
	S. takoradi (*i:1,5*)	+	+	+	±	−	−
	S. bandia, *S. kedougou* (*i:lw*)	+	+	+	±	−	−
	S. schalkwijk (*i:en*)	+	+	+	±	±	−
H:*1,2*	*S. heidelberg*, *S. virchow* (*r:1,2*)	±	±	−	+	+	+
	S. muenchen (*d:1,2*)	±	±	−	+	±	±
	S. newport (*eh:1,2*)	±	±	−	+	+	+
H:*gm*	*S. enteritidis* (*gm:*−)	−	−	−	−	−	−

[a] The dominant H antigen, determined by agglutination, is shown in parentheses.
[b] Serum batch 1, lot no. K002510; serum batch 2, lot no. K973010.
[c] Serum batch 1, lot no. 846810; serum batch 2, lot no. K506610.
[d] Antibody binding by Western blotting: +, strong; ±, weak; −, negative.

tested different batches of commercial typing H antigen-specific serum, the H:*1,2* antigen-specific sera in particular showed some differences in their binding patterns to the *Salmonella* cell extracts (Table 1). All selected MAbs showed clear positive and negative recognition of their corresponding H antigens in whole-cell extracts of diverse *Salmonella* serotypes, with no detectable nonspecific binding (Table 1 and Fig. 1A and B). No H:*1,2* flagellin was recognized in *S. typhimurium* ATCC 14028 or *Salmonella aberdeen* preparations (both serotype H:*i:1,2*) by H:*1,2*-specific MAbs, while H:*i*-specific MAbs strongly bound to both serotypes (Table 1). Subsequent agglutination of both strains with commercial typing sera confirmed the absence of H:*1,2* flagellin. MAbs were highly suitable for the detection of H:*i* or H:*1,2 Salmonella* serotypes and, compared to serotyping sera MAbs, more specific in recognizing their H antigens, since they did not bind at all to heterologous flagellins or other proteins (Table 1 and Fig. 1).

Construction of flagellin gene clones. The *fliC* gene (Fig. 2) was amplified from chromosomal DNA of *S. typhimurium* SL3261 (*i,1,2* [14]) with the conserved flagellin domain primers E1 and E2 (Table 2 and Fig. 2). PCR was performed in a 50-μl reaction volume with 1 U of DNA polymerase (PrimeZyme kit; Biometra, Göttingen, Germany) and 25 pmol of each primer. For each reaction, 35 cycles, each consisting of three steps (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min), were carried out, followed by a single step of 10 min at 72°C to complete elongation of the products. The amplified *fliC* gene was cloned into pEX11 (6) and electroporated into *Escherichia coli* POP2136 (8).

The *Hin*dIII site typically present in the *fliB* gene (13) could not be detected in the PCR product obtained with primers E1 and E2. Therefore, a central part of both the *fliC* and *fliB* flagellin genes was amplified from *S. typhimurium* SL3261 chromosomal DNA with primers P1 and P2 (13) (Table 2 and Fig. 2). *Hin*dIII digestion produced a 750-bp fragment containing the variable domain and part of the downstream conserved domain of the *fljB* gene. This probe was ^{32}P labeled (1) and used in Southern blotting, where it identified two larger *fljB* fragments; a 980-bp *Hin*dIII-*Eco*RI fragment and a 999-bp *Pst*I-*Pst*I fragment (PP-1,2 [Fig. 2B]). These fragments were cloned into pUC18 and used for further subcloning of *fljB* flagellar fragments. The amino acid sequences deduced from the *fliC* and *fljB* genes had an overall identity of 75% (data not

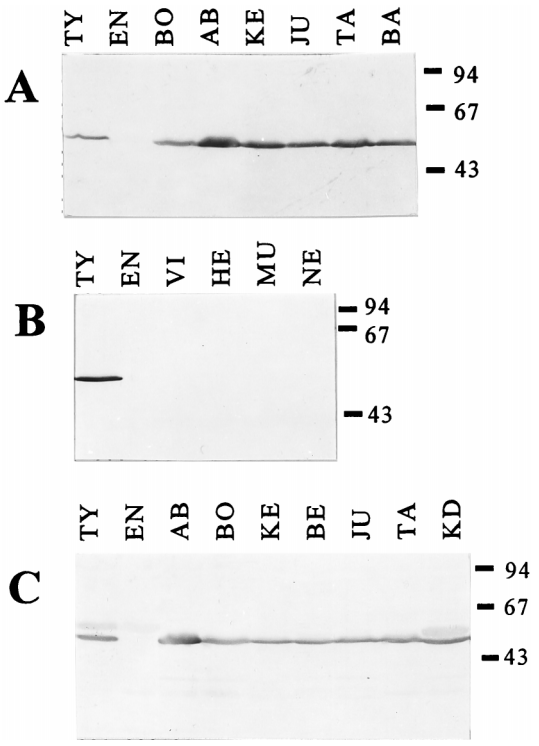

FIG. 1. Serotyping of *Salmonella* strains with MAb VB5 and commercial serotyping serum (batch 2) on Western blots. (A) H:*i* flagellin-containing *Salmonella* stained with VB5. (B) H:*1,2* flagellin-containing *Salmonella* stained with VB5. (C) H:*i* flagellin-containing *Salmonella* stained with commercial H:*i*-specific serotyping serum. The *Salmonella* strains used (with the H serotype in parentheses) are as follows: AB, *S. aberdeen* (*i:1,2*); BA, *S. bandia* (*i:lw*); BE, *S. bergen* (*i:enz15*); BO, *S. bonariensis* (*i:enx*); EN, *S. enteritidis* (*gm*:-); HE, *S. heidelberg* (*r:1,2*); JU, *S. jukestown* (*i:enz15*); KE, *S. kentucky* (*i:z6*); KD, *S. kedougou* (*i:lw*); NE, *S. newport* (*eh:1,2*); MU, *S. muenchen* (*d:1,2*); TA, *S. takoradi* (*i:1,5*); TY, *S. typhimurium* (*i:1,2*); VI, *S. virchow* (*r:1,2*). Molecular size markers in kilodaltons are shown at the right.

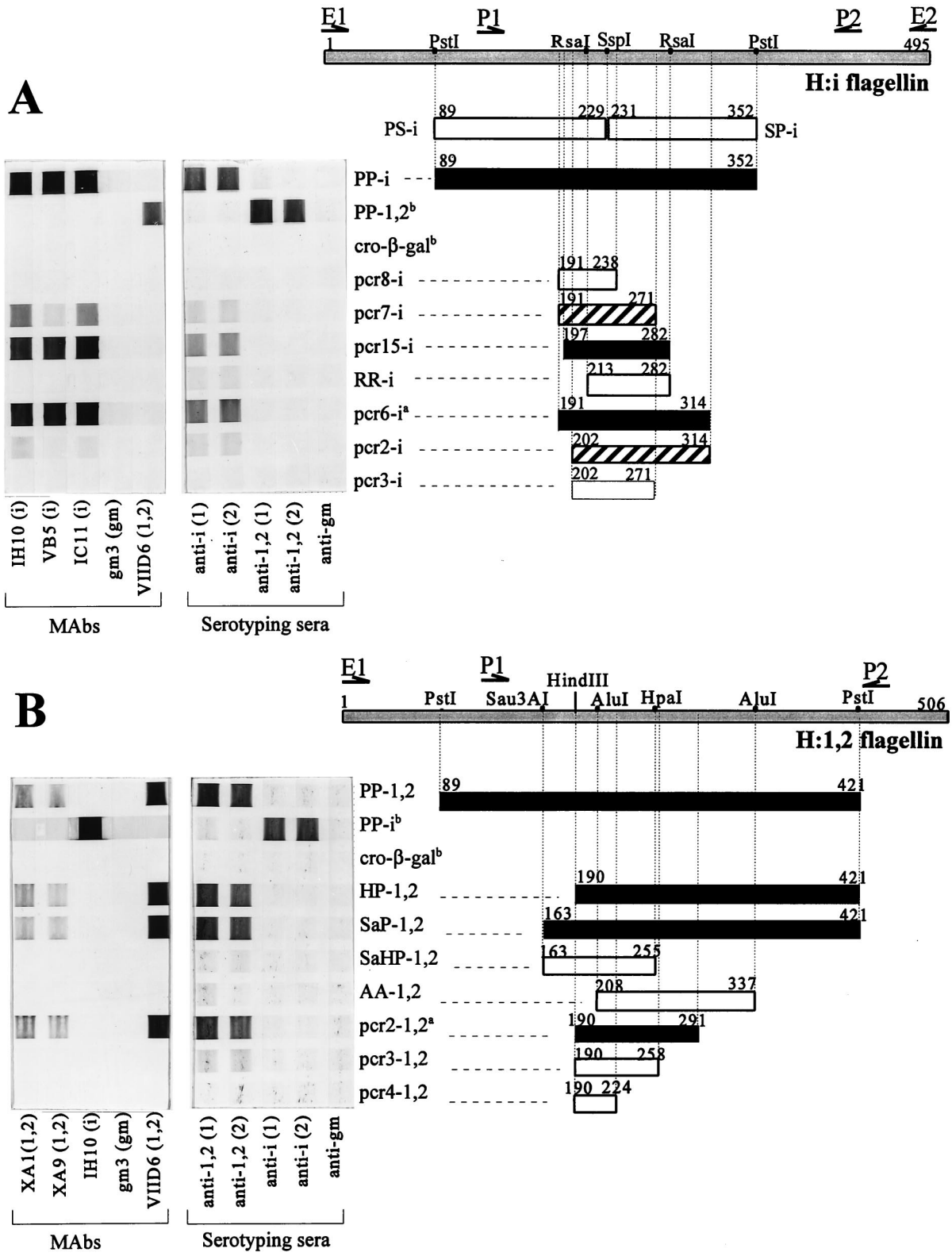

FIG. 2. Immunostained cross blots showing the binding of H-specific antibodies to recombinant flagellin expression products. Binding of the H:*i* (A) and H:*1,2* (B) flagellins with H-specific MAbs and commercial serotyping sera. Binding of the MAbs is indicated by the bar (filled, strong; hatched, weak; open, negative). Restriction sites used for the creation of fragments are indicated on the top bar of each panel, which represents the complete flagellin protein. The positions of the primers used for the cloning of the flagellin genes (E1, E2, P1, and P2) are depicted by arrows. Fragments indicated by superscript a were also cloned in pGEX and applied in an ELISA. Both a large fragment of the heterologous flagellin and cro-β-galactosidase (cro-β-gal) expressed from the empty vector pEX13 (indicated by superscript b) were always included as negative control fragments.

TABLE 2. Nucleotide sequences of primers used for amplification of flagellin genes, sequencing analysis of gene *fljB*, and construction of overlapping fragments

Primer function[c] and name	Sequence[a]	Primer position[b]	Gene
FS E1	5′AAG**GAATTC**ATCATGGCACAAG-3′	>−12	*fliC* or *fljB*
F E2	5′-GAA**GAATTC**AACGCAGTAAAGAGAG-3′	<1471	*fliC*
F P1	5′-AAAGTCCTGGCGCAGGACAACA-3′	>406	*fliC* or *fljB*
F P2	5′-ATAGCGGAGTTGAAACGGTTCT-3′	<1289	*fliC*
		<1322	*fliB*
S S1,2	5′-TGGATGGAGTCGAGGTCAGACT-3′	<317	*fljB*
O ST i-191D2	5′-TGT**GAATTC**ATATGCCGATACTACGATTG-3′	>561	*fliC*
O ST i-197D	5′-TAC**GAATTC**TGCTTTAGACAATAGTACTT-3′	>582	*fliC*
O ST i-202D	5′-GAC**GAATTC**ACTTTTAAAGCCTCGGCT-3′	>595	*fliC*
O ST i-238R	5′-CCA**GTCGAC**CCCGTAACGGTAACTTT-3′	<697	*fliC*
O ST i-274R	5′-TTT**GTCGAC**TCCTCAGTTGCTGTCGCAGGTAGT-3′	<810	*fliC*
O ST i-282R	5′-AAC**GTCGAC**ATTTTTCACATCCTCAGT-3′	<826	*fliC*
O ST i-314R	5′-ACC**GTCGAC**ATCAGTATAAGACATCTTAAC-3′	<922	*fliC*
O ST1,2-190D	5′-ACA**GAATTC**GCTTATGCCAATAATGGT-3′	>559	*fljB*
O ST1,2-224R	5′-TTT**GTCGAC**ACCACCGGTTACAGAAGC-3′	<655	*fljB*
O ST1,2-258R	5′-ACC**GTCGAC**AGCAACGTTAACTTCATA-3′	<757	*fljB*
O ST1,2-291R	5′-ACA**GTCGAC**GGTGTATCTTTTAACTC-3′	<856	*fljB*

[a] The *Eco*RI (GAATTC) and *Sal*I (GTCGAC) sites are indicated in bold. Start or stop codons are underlined.

[b] Numbering refers to position of the 5′ end of the last perfect matching nucleotide in forward primers (indicated by >) or the last matching nucleotide in the 3′ end in reverse primers (indicated by <) and is according to an alignment performed with Clustal-W (15) with sequence D13689 from the NCBI database.

[c] Primers were used for amplification of flagellin genes (F), sequencing analysis of gene *fljB* (S), and construction of overlapping fragments (O). All primers were ordered from Pharmacia.

shown). As expected, the highest variability in amino acids was found in the central domain of the flagellin protein.

Subcloning and expression of flagellin fragments. The *fliC* and *fljB* gene fragments were digested in several single and double digestions as outlined in Fig. 2, separated on an agarose gel, and isolated from the gel (QIAEX kit; Qiagen, London, United Kingdom). Smaller fragments were created by PCR amplification of either a *Pst*I-*Pst*I *fliC* fragment (PP-i [Fig. 2A]) or the 980-bp *Hin*dIII-*Eco*RI *fljB* fragment. All fragments were cloned into expression vector pEX11, pEX12, or pEX13 (6), and clones in *E. coli* POP2136 were selected by culturing at 30°C on agar supplemented with 100 μg of ampicillin per ml. Subclones containing the correct inserts were identified by restriction enzyme analysis of the plasmids. The expression and purification of fusion proteins were carried out as described previously (8). The expression products were separated by SDS-polyacrylamide gel electrophoresis and tested for recognition by specific MAbs and typing sera in Western blots. For each fragment, one representative subclone was selected for further analysis.

Epitope mapping of the H:*i* and H:*1,2* flagellins. The purified recombinant flagellin fragments were screened for binding to H:*i*- and H:*1,2*-specific typing sera and MAbs in cross blots. For this procedure, a Hybond C nitrocellulose filter (Amersham) was soaked in 0.5 M Tris-HCl (pH 6.8) and then placed in a Miniblotter 16 (Immunetics, Cambridge, Mass.) according to the manufacturer's instructions. Purified *fliC* and *fliB* flagellin fragments (Fig. 2) were resuspended in 1 M Tris-HCl (pH 6.8)–6% SDS–30% glycerol–0.75% dithiothreitol and boiled for 5 min. Each of a maximum 10 channels of the Miniblotter was filled with 110 μl of a suspension containing 15 μg of protein and incubated for 1 h at room temperature. SDS was removed by electroelution for 10 min at 50 V in 25 mM Tris-HCl–192 mM glycine–20% methanol. Immunostaining was performed essentially as with Western blotting, except that incubation with typing antisera and MAbs was carried out with the filter placed crosswise in the immunoblotter.

The smallest H:*i* and H:*1,2* flagellin fragments that were still strongly recognized by H antigen-specific MAbs were 86 (pcr15-i) and 102 (pcr2-1,2) amino acids, respectively (Fig. 2), and contained 67 to 70% of the variable residues identified by the above-mentioned sequence comparison of the H:*i* and H:*1,2* flagellins. When 5 N-terminal (pcr2-i) or 11 C-terminal (pcr7-i) amino acids were deleted from pcr15-i, a significant decrease in antibody binding was observed (Fig. 2A). The simultaneous removal of both these ends (pcr3-i) or the deletion of 17 amino acids from the N terminus (RR-i) resulted in a complete loss of all detectable binding. When a larger fragment, PP-i (89-352), was cut into two parts at amino acid position 230, no binding by specific MAbs to fragment PS-i or SP-i could be detected (Fig. 2A). However, SP-i (data not shown) and RR-i (Fig. 2) were still weakly recognized by specific typing sera. Removing 18 N-terminal (AA-1,2) or 33 C-terminal (pcr3-1,2) amino acids from the minimal fragment pcr2-1,2 eliminated binding of all specific MAbs (Fig. 2B). However, pcr3-1,2 was still recognized weakly by commercial typing sera, but this binding disappeared after the removal of 34 additional C-terminal amino acids (pcr4-1,2).

Obviously, the most critical parts of the *i* and *1,2* antigenic determinants of *S. typhimurium* are contained within the pcr15-i and pcr2-1,2 fragments and are located between positions 190 and 291 on the H:*i* and H:*1,2* flagellins (Fig. 2), a site which is in the hypervariable flagellin region IV (20). The localization of the H:*i*- and H:*1,2*-specific antigenic determinants in this part of the flagellin is in accordance with the results of a study of Yoshioka et al. (22). These authors showed that a spontaneous deletion mutant, lacking a part of the flagellin that corresponded to our fragment pcr15-i, was motile in the presence of H:*i*-specific antisera, in contrast to wild-type *S. typhimurium*. A similar location for the H:*d* antigen was inferred for *Salmonella muenchen* by Newton et al. (11), who presented evidence that region IV codes for the major determinants.

Flagellin fragments were recognized exclusively by homologous MAbs and not by heterologous MAbs (Fig. 2). The binding by H-specific typing antisera showed similar results. Apart from the overall pattern of strongly positive and clearly negative antibody binding to flagellin fragments, two H:*i* fragments (pcr2-i and pcr7-i) bound only weakly to both MAbs and serotyping sera. Since these two fragments were slightly smaller or shifted in position with respect to the smallest strongly

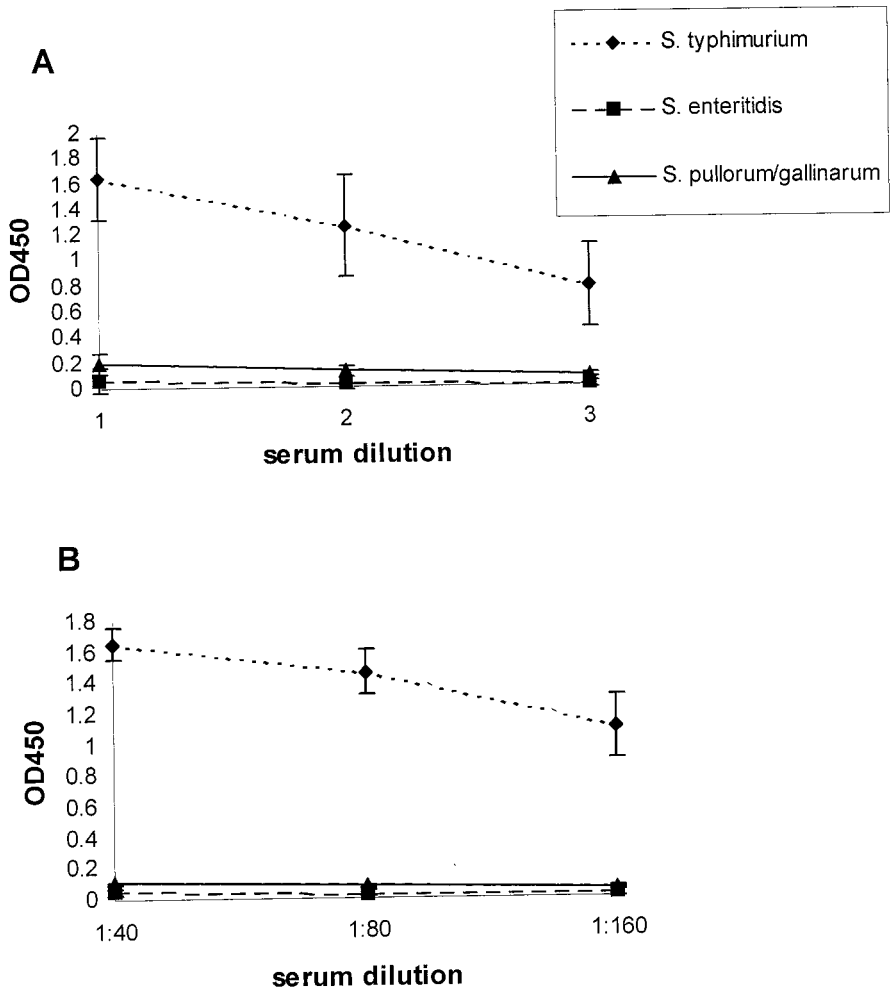

FIG. 3. Results of an indirect ELISA with experimental sera from chickens orally infected with *S. typhimurium* ($n = 8$), *S. enteritidis* ($n = 5$), or *S. enterica* Pullorum and Gallinarum ($n = 7$) on H:*i*-specific fragment pcr6-i (A) or H:*1,2*-specific fragment pcr2-1,2 (B). OD, optical density.

positive fragment pcr15-i, we assume that they contain only part of the antigenic determinants. Furthermore, H:*i* and H:*1,2* typing sera bound to some flagellin fragments (SP-i, RR-i, and pcr3-1,2), none of which was recognized by the corresponding MAbs (Fig. 2). Although we do not have conclusive evidence, we believe that due to incomplete absorption of the commercial typing sera, residual antibodies that recognize flagellin epitopes unrelated to the H antigen are still present and are probably the cause of this weak binding. All other flagellin fragments, however, showed similar binding of both MAbs and serotyping sera. It is evident that both MAbs and serotyping sera have one antibody binding site on the *S. typhimurium* flagellin which probably consists of a single epitope.

Evidently, H:*i* (pcr15-i) and H:*1,2* (pcr2-1,2) flagellin fragments, with minimum lengths of 86 and 102 amino acids, respectively, are required for the strong binding of H-specific MAbs. The lengths of these fragments correspond well to the length of our previously found flagellin fragment, 91 amino acids, the minimal fragment for the H:*gm* flagellar determinant of *S. enteritidis* (16). Since the binding site of an antibody cannot contain more than 15 amino acid residues (18), it is obvious from our data that the residues involved in the formation of the H epitope are dispersed over a flagellin area of approximately 100 amino acids and therefore must represent a discontinuous epitope. Support for this position comes from two other studies in which sequence comparisons of *Salmonella* flagellins could not identify a single stretch of amino acid residues as a possible epitope site for the H antigen (10, 17). Furthermore, Joys and Schödel (4) showed that synthetic octameric peptides recognized by specific antibodies originated from sites widely dispersed on the flagellin of *S. muenchen*. The presence of such a discontinuous epitope suggests that some conformation of the recombinant flagellin fragments is required for antibody binding. Apparently, this conformation is maintained during or recovered after exposure to the denaturing conditions of our assays. Indeed, in our cross blots, a significant reduction of antibody binding to the recombinant flagellin fragments was observed when the electroelution of the denaturing agent SDS was omitted (data not shown), indicating that regaining the conformation of the epitope is necessary for optimal binding. An important consequence of the discontinuity of the H-specific epitope is that we could determine only the flagellin region in which a significant portion of the H-antigenic determinants was dispersed. No conclusion could be drawn about the individual amino acid residues that interact with the antibody. Also, the outer termini of the smallest binding fragments do not necessarily represent amino acid residues that are part of the antibody binding site, since their sole function could well be to maintain the flagellin fragment in the conformation required for the formation of the three-dimen-

sional antibody binding site. In an attempt to identify the amino acids that are involved in the binding of specific antibodies, alignments between flagellar amino acid sequences of various *Salmonella* serotypes present in the National Center for Biotechnology Information (NCBI) database were determined (data not shown). This analysis resulted in the identification of a considerable number of hypervariable residues, which may be important for the differences in antigenic properties of flagellins of different serotypes. Aside from the fact that the majority of these residues were located on the H:*i*- and H:*1,2*-containing flagellin fragments, no solid conclusions could be drawn from this alignment, and further studies are needed to establish which amino acid residues contribute to the formation of the H antigen-determining epitope.

Use of H-specific flagellin fragments in ELISA for the detection of antibodies in chickens. To obtain a soluble protein antigen suitable for use in ELISA, two small flagellin fragments of *fliC* (pcr6-i) and *fljB* (pcr2-1,2) (Fig. 2) were cloned into pGEX4T-1 (12) (Pharmacia, Uppsala, Sweden). Expression in *E. coli* PC2495 (21) and purification of the fusion proteins were carried out as described before (12) (Pharmacia). The wells of a microtiter plate (Greiner, Alphen a/d Rijn, The Netherlands) were each coated with 100 μl of a solution containing 5 μg of either the pcr6-i or the pcr2-1,2 flagellin peptide per ml. ELISAs were performed as described previously (2). Sixteen-week-old SPF White Leghorn chickens were infected orally with *S. typhimurium*, *S. enteritidis*, or *Salmonella enterica* Pullorum and Gallinarum, and serum samples were obtained on day 14, 21, or 28 after infection. Only sera from *S. typhimurium*-infected chickens bound to both recombinant flagellin fragments by ELISA (Fig. 3).

In conclusion, this study provides evidence that all H-specific MAbs and commercial typing sera recognize the same epitope on their corresponding flagellin. This antibody binding site most probably consists of a single epitope, since both MAbs and serotyping sera showed the same binding to the various flagellin fragments. The finding that relatively large flagellin peptides are minimally required for binding of H:*i* and H:*1,2* serotype-specific antibodies provides further evidence for the existence of a discontinuous H-specific epitope. Furthermore, ELISA results indicate that H-specific flagellin fragments bind specifically to antibodies from *S. typhimurium*-infected chickens. This indicates that the use of these fragments in an ELISA is a convenient method for the detection of *S. typhimurium* antibodies in animal reservoirs. A clear advantage is that the use of separate H:*i* and H:*1,2* antigens in a test allows discrimination between infections with *S. typhimurium* and other *Salmonella* serotypes that have only one of these two H antigens in common with *S. typhimurium*. The application of these flagellar antigens as a diagnostic tool for the detection of *S. typhimurium* antibodies in commercial flocks of chicken and swine is currently being evaluated.

Nucleotide sequence accession number. The amino acid sequences deduced from the *fliC* gene and the *fliB* gene were deposited in GenBank under accession no. AF045151.

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