JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1610–1613 Vol. 177, No. 6
0021-9193/95/$04.00+0

NOTES

Identification of the Domain Which Determines the g,m Serotype of the Flagellin of *Salmonella enteritidis*

A. J. A. M. VAN ASTEN,[1,2] K. A. ZWAAGSTRA,[1,2] M. F. BAAY,[2] J. G. KUSTERS,[1,2]
J. H. J. HUIS IN'T VELD,[2] AND B. A. M. VAN DER ZEIJST[1]*

Department of Bacteriology, Institute of Infectious Diseases and Immunology,[1] and Department of the Science of Food of Animal Origin,[2] Faculty of Veterinary Medicine, University of Utrecht, 3508 TD, Utrecht, The Netherlands

Received 5 August 1994/Accepted 29 December 1994

Clones expressing fragments of the flagellin protein of *Salmonella enteritidis* were constructed and screened with a g,m-specific monoclonal antibody. Results showed that the g,m epitope is localized between amino acids 258 and 348 of the flagellin. The *fliC* gene, encoding the flagellin of *S. enteritidis*, was proven to be the only flagellin gene present in *S. enteritidis*.

In recent years, the incidence of *Salmonella enteritidis* outbreaks, causing human gastroenteritis with fatal septicemia in the elderly and in young children, has risen sharply in the United States and in large parts of Europe (18, 21). Infection is often the result of consumption of poultry meat or food containing contaminated fresh eggs (21). Little is known about the factors of *S. enteritidis* that are involved in colonization of both humans and poultry. Among the candidate virulence factors are fimbrial structures (3, 23, 24), gene products involved in invasion of eukaryotic cells (5, 22), and lipopolysaccharides (16). An important feature of *Salmonella* and several other bacterial genera are the flagella, which confer motility to the bacterium and in this way contribute to colonization. The flagellar filament of members of the genus *Salmonella* is a multimer of a single protein, the flagellin. Comparison of the amino acid sequences of *Salmonella* flagellins has led to the definition of eight regions of different variability (27). The amino- and carboxy-terminal sequences (regions I and II and region VIII, respectively; see Fig. 1) are conserved. The middle part, however, especially regions IV, V, and VI, is (hyper)variable and determines the serotype-specific H antigen (15, 27). The properties of the regions become directly evident from a three-dimensional model for the *Salmonella typhimurium* flagellin (12). The essence of this model is that each of the several thousands of individual flagellins is folded in a horseshoe-like structure. The termini, the legs of the horseshoe, each consisting of the domains D1 and D2 as defined by Namba et al. (Fig. 1 and reference 12), are in the center of the flagellum near the central cavity. They are crucial to holding the flagellum together and, in contrast to the middle part of the flagellum, do not tolerate insertions and deletions (6, 8, 13–15). The middle part of the flagellin (D3) is at the surface of the flagellar filament. The H antigens, together with the O antigens, which are present in the lipopolysaccharides of the bacterium, are the basis of the Kaufmann-White typing scheme (9, 17). On the basis of this scheme, all salmonellae which have O antigens 1, 9, and 12 and H antigen g,m are *S. enteritidis*, although they may be evolutionarily distantly related (2). Since there is an early immune response to the antigenic determinants of the flagellum (1, 26), the serotype-specific determinants could be of great use for diagnosis. Therefore, we decided to characterize the flagellin of *S. enteritidis* and the region(s) of this flagellin responsible for the serotype.

With the primers H5 and H3, deduced from known flagellin sequences of other *Salmonella* strains (reference 4 and Table 1), and the DNA of boiled *S. enteritidis* 857, a randomly chosen Dutch field isolate, as template, a DNA fragment of about 1.5 kb was amplified. PCR conditions were 35 cycles of 1 min at 95°C, 2 min at 55°C, and 3 min at 72°C followed by a final step of 9.9 min of chain elongation at 72°C. *Taq* DNA polymerase (Promega, Madison, Wis.) was used as the enzyme. The fragment was cloned into pBluescript II KS (Stratagene, La Jolla, Calif.). The resulting clone, PE-6 (see Fig. 3) was sequenced either with the T7 sequencing kit (Pharmacia, Uppsala, Sweden) and [α-^{32}P]dATP or with the A.L.F. DNA sequencer (Pharmacia) together with the Auto-Read sequencing kit (Pharmacia). The sequence appeared to be identical to the flagellin sequences of *S. enteritidis* which became available during this study (11, 20). However, the PCR-amplified fragment lacked 34 nucleotides at the 5′ end of the flagellin gene, probably because of the technique used to make the fragment blunt ended (25). The flagellin gene was also, at the amino acid level, compared with a number of published *Salmonella* sequences (Fig. 1). Computer analyses were performed by the PC/Gene program (release 6.7; Genofit S.A., Geneva, Switzerland).

Most *Salmonella* species are capable of flagellin phase variation. They contain two flagellar genes at different locations on the chromosome. There is a regulatory mechanism that ensures that only one gene is expressed at a given time (28). Only one serotype of *S. enteritidis* flagellin is known, but the possibility that we had amplified and sequenced a silent flagellin gene could not be excluded. To ensure that a functional *fliC* gene rather than such a (silent) additional flagellin gene was amplified, a Southern blot with DNA of *S. enteritidis* 857 and *S. typhimurium* C52 (obtained from L. Norel, Institute Pasteur, Paris, France), digested with several restriction enzymes, was

* Corresponding author. Mailing address: Institute of Infectious Diseases and Immunology, Department of Bacteriology, Faculty of Veterinary Medicine, University of Utrecht, P.O. Box 80.165, 3508 TD, Utrecht, The Netherlands. Phone: (31)-(0)30-534888. Fax: (31)-(0)30-540784. Electronic mail address: Zeijst@vetmic.dgk.ruu.nl.

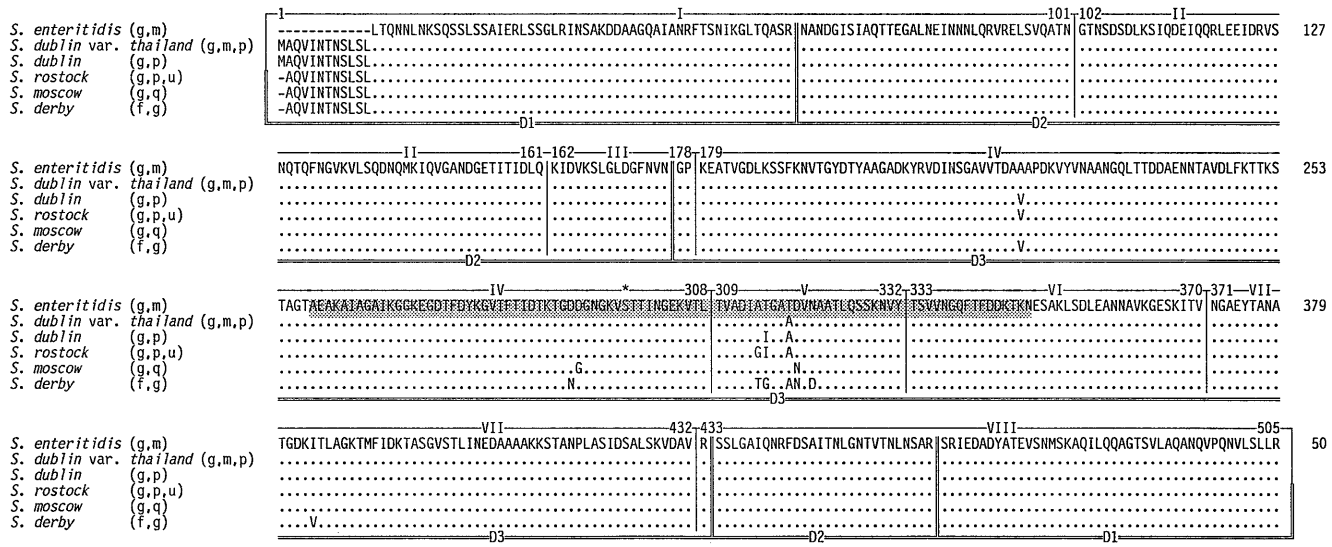

FIG. 1. Alignment of the amino acid sequences of flagellins. The *S. enteritidis* line shows the translation of the newly determined *fliC* sequence. The other sequences were translated from DNA sequences with accession numbers as follows: *S. dublin* var. *thailand*, M84973 (20); *S. dublin*, 84972 (20); *S. rostock*, Z15071 (11); *S. moscow*, Z15086 (11); *S. derby*, Z15066 (11). The H type of each strain is indicated. Identical amino acids are indicated by a dot. The structural domains recognized by Namba et al. (12) in the electron density map of the flagellum (12) are demarcated by double lines. The eight regions described by Wei and Joys on the basis of amino acid homology (27) are demarcated by single lines. The first and last amino acid of each region are given. The shaded part of *S. enteritidis* represents the smallest part tested which still reacts with the g,m-specific MAb. The serine at position 298 which is replaced by proline in clone C1-3* is indicated by an asterisk.

carried out. As a probe, a 239-bp *Hin*dIII-*Pst*I fragment of PE-6 containing bp 35 to 269 of the conserved 5′ region of the flagellin gene, which was labelled by random primer extension (19), was used. Hybridization was performed overnight at 60°C in 5× Denhardt's solution (19), 0.5% sodium dodecyl sulfate (SDS), and 5× SSPE containing 100 μg of denatured herring sperm DNA per ml (1× SSPE is 0.01 M NaH_2PO_4 [pH 7.0], 0.18 M NaCl, and 0.001 M EDTA). Two 15-min posthybridization washes were performed at 60°C in 0.2% SDS–2× SSPE. Bands were visualized by autoradiography. Chromosomal DNA of *S. enteritidis* always contained only one fragment reacting with the probe, whereas digested chromosomal DNA of *S. typhimurium* showed two bands (Fig. 2). The single band of *S. enteritidis* was as intense as either band of *S. typhimurium*. The result of this experiment, which was confirmed by an experiment with four other *S. enteritidis* strains (data not shown), showed that *S. enteritidis* has only one flagellin gene.

Previous attempts to map the serospecific epitopes of *Salmonella* flagellins have been based on sequence comparison, Pepscan, and the generation of deletion mutants. This approach has been successful for the d serotype. This antigen appears to be determined mainly by region IV (4, 15), although contributions from regions V and VI were also found (7). Attempts to identify the epitopes responsible for the g,m antigen have been unsuccessful so far (11). We decided to screen expression products representing parts of the flagellin of *S. enteritidis* for their reactivity with poly- and monoclonal anti-

TABLE 1. Nucleotide sequences of primers used

Primer	Sequence[a]	Position[b]
H5	AAGGAAAAGATCATGGCA	−12 to 6
H3	TTAACGCAGTAAAGAGAG[c]	1515 to 1532
1L	tgcgaattCCGCTGAAGCCAAAGCG	771 to 786
2L	taagaaTTCTACTACCATCAAATGGT	891 to 909
2R	TATTTTGGTTTTTGCcggccgc	1031 to 1045
3R	TGCGGTGCCCACTAgcggccgc	1133 to 1146
839	cgtgaattcTACTATTGATAC	849 to 860

[a] Uppercase characters represent the *S. enteritidis fliC* derived sequence; lowercase characters stand for nucleotides added in order to facilitate cloning.
[b] A in the first ATG of the entire *S. enteritidis* flagellin gene is nucleotide 1.
[c] The stop codon of the flagellin gene is underlined.

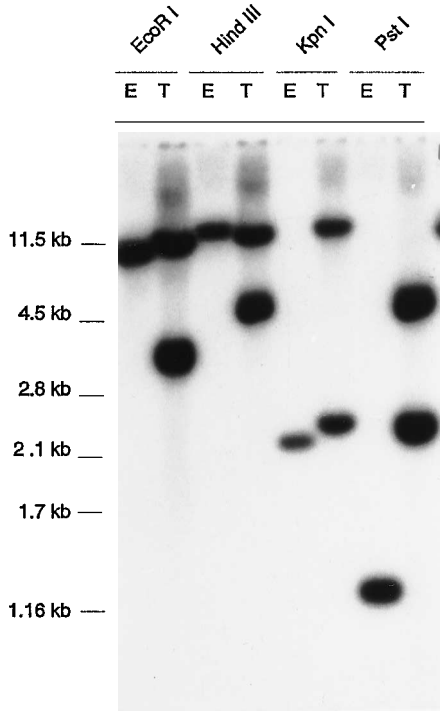

FIG. 2. Southern blot analysis of DNA of *S. enteritidis* (E) and *S. typhimurium* (T). DNA was digested with the restriction enzymes indicated and probed with a 235-bp fragment of the conserved 5′ end of the *S. enteritidis* flagellin gene.

TABLE 2. Reactivities of MAbs with *Salmonella* strains of various flagellar serotypes

MAb	Reactivity with indicated strain[a]							
	S. agona (f,g,s:−)	*S. derby* (f,g:[1,2])	*S. dublin* (g,p:−)	*S. enteritidis* (g,m:−)	*S. gallinarum* (−:−)	*S. godesberg* (g,m:−)	*S. monschaui* (m,t:−)	*S. typhimurium* (i:1,2)
2	−	−	−	+	−	+	−	−
3	−	−	−	+	−	+	−	−
5	−	−	+	+	−	+	−	−
8	−	+	+	+	−	+	−	−
10	−	+	+	+	−	+	−	−
16	+	+	+	+	−	+	+	−
18	+	+	+	+	−	+	+	+

[a] The H serotype of each strain is shown in parentheses.

sera and in this way locate the part of the flagellin which contains the g,m-specific determinant(s). We first characterized monoclonal antibodies (MAbs; kindly donated by F. G. van Zijderveld, ID-DLO, Lelystad, The Netherlands) against the flagellin protein of *S. enteritidis* for their reactivity in Western blots (immunoblots) with flagellin proteins of the following *Salmonella* strains: *S. agona* (f,g,s:−), *S. derby* (f,g:[1,2]), *S. dublin* (g,p:−), *S. enteritidis* (g,m:−), *S. gallinarum* (−:−), *S. godesberg* (g,m:), *S. monschaui* (m,t:−), and *S. typhimurium* C52 (i:1,2). With the exception of *S. enteritidis* and *S. typhimurium* C52, all strains were of our own collection. The results of these Western blots with the MAbs are given in Table 2. From these results, it could be concluded that MAb 2 and MAb 3 reacted identically and that both were specific for the serotype g,m. MAb 8 and MAb 10 also reacted identically and recognize a g epitope, whereas MAb 5 recognizes another g epitope. MAb 16 and MAb 18 seemed to be directed against parts of the flagellin which are conserved among serotypes. Because of the type of experimental procedure, these results differ from the results previously obtained with agglutination tests (26).

A construct was made in the expression vector pEX12 (10), containing the insert of clone PE-6, and this construct was introduced into *Escherichia coli* POP2136 by electroporation with the Gene Pulser system (Bio-Rad Laboratories, Hercules, Calif.). The resulting clone was designated 12E3 and expressed the almost complete *fliC* gene as a fusion protein. This protein reacted with MAb 2 in a Western blot. Then, five other pEX clones were made, expressing fragments of the flagellin protein, particularly from those regions in which the flagellins differed most (Fig. 1) and flanking regions. To be sure that the constructs were in the right reading frame, the pEX clones were sequenced, after which the expression products of the clones were screened in Western blots for reactivity with MAb 2. An overview of the exact locations of the fragments as well as their reactivities with MAb 2 is given in Fig. 3. All of the expression products which reacted positively with MAb 2 also reacted with two polyclonal serum samples specific for H type g,m, one of which was obtained from Wellcome Diagnostics (Dartford, England) and the other of which was a kind gift of W. H. Jansen (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). From these experiments, it could be concluded that the epitope which is recognized by MAb 2 is located between amino acids 256 and 382 of the flagellin. Two of the differences in region IV and all of the differences in region V were situated in this stretch (Fig. 1). In order to find out which of the differences are responsible for the variation in the flagellar serotype between different *Salmonella* strains, we amplified subregions of the part of the flagellin gene which encoded amino acids 258 through 382. Primers used for the amplification are given in Table 1; PCR conditions were as given above. PCR products were cloned into expression vector pGEX-4T-3 (Pharmacia) with *E. coli* PC2495 as the host strain, and expression products were tested for reactivity with MAb 2 (Fig. 3). The part of the flagellin gene which encoded amino acids 258 through 382 was amplified and cloned into pGEX-4T-3 as a positive control (clone C1-3). The inserts of all pGEX clones were sequenced to ascertain that they were in the proper reading frame and that no PCR artifacts were present. With the exception of C1-3, only the expression product of clone C1-2 (amino acids 258 through 348) reacted with MAb 2. The expression product of clone C2 (amino acids 298 to 348) did not react with MAb 2, which shows that region V (amino acids 308 to 332) alone does not contain the epitope. The product of clone C2P (amino acids 284 to 348), which was 26 amino acids shorter at the amino-terminal side than clone C1-2 but still contained the part of region IV in which *Salmonella moscow* and *S. derby* differ from the other four *Salmonella* strains, was also negative. Thus, the region between amino acids 258 and 284 appears to contribute to the epitope, although it is not responsible for the difference in serotype between *S. enteritidis*, *S. dublin* var. *thailand*, *S. dublin*, *Salmonella rostock*, *S. moscow*, and *S. derby*. For the first four strains, the difference in serotype is wholly determined by the sequence of region V, whereas the differences in region IV may play a role in the case of the last two strains. Another indication of a contribution of the carboxy-terminal

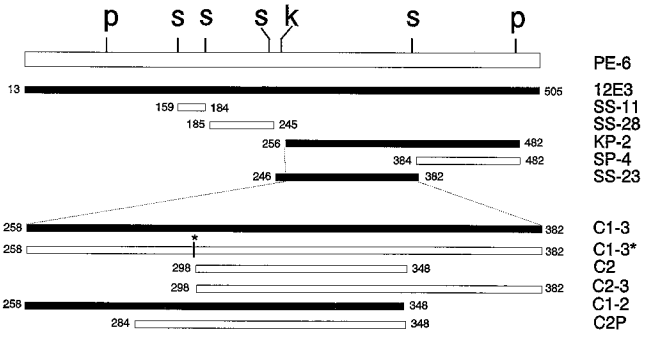

FIG. 3. Relative locations of the flagellin fragments expressed by the *fliC*-derived inserts cloned into either the expression vector pEX (upper panel) or pGEX (lower panel) and their reactivities with MAb 2. The upper open bar represents the originating clone. The positions of the restriction sites used for cloning into pEX are indicated as follows: P, *Pst*I; S, *Sau*3AI; K, *Kpn*I. Expression products encoded by pEX and pGEX clones are shown as thinner bars, together with the name of the clone and the position numbers of the first and the last amino acid. Expression products represented by a black bar react with g,m-specific MAb 2. Position 298, in which C1-3* is mutated, is indicated by an asterisk.

part of region IV to the g,m epitope comes from clone C1-3*. This clone, the result of a PCR artifact, differs from clone C1-3 by just 1 nucleotide, at position 893 (T→C). The resulting change from Ser to Pro (amino acid 298) destroys the reactivity of the protein with MAb 2.

Our results show that fusion proteins 12E3, KP-2, SS-23, C1-3, and C1-2 contain the g,m epitope, which makes them functionally equivalent to denatured protein with regard to reactivity to MAb 2. This indicates that the g,m epitope recognized by MAb 2 is conformation independent. On the other hand, the need for the presence of the region containing amino acids 258 through 283 suggests that some conformation may play a role in determining the epitope.

The localization of the g,m-specific epitope of *S. enteritidis* flagella may be used to develop a specific serological test for *S. enteritidis*. Our approach to the expression of parts of the flagellin gene can also be used to map the determinants of other H antigens.

Nucleotide sequence accession number. The nucleotide sequence of the *fliC* gene of *S. enteritidis* 857 has been assigned GenBank number U12963.

The authors thank Jana Kerver for technical assistance and Arnoud H. M. van Vliet for his interest and advice.

REFERENCES

1. **Baay, M. F. D., and J. H. J. Huis in 't Veld.** 1993. Alternative antigens reduce cross-reactions in an ELISA for the detection of *Salmonella enteritidis* in poultry. J. Appl. Bacteriol. **774:**243–247.
2. **Beltran, P., J. M. Musser, R. Helmuth, J. J. Farmer III, W. M. Frerichs, I. K. Wachsmuth, K. Ferris, A. C. McWhorter, J. G. Wells, A. Cravioto, and R. K. Selander.** 1988. Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes *S. cholerasuis*, *S. derby*, *S. dublin*, *S. enteritidis*, *S. heidelberg*, *S. infantis*, *S. newport*, and *S. typhimurium*. Proc. Natl. Acad. Sci. USA **85:**7753–7757.
3. **Clouthier, S. C., K.-H. Müller, J. L. Doran, S. K. Collinson, and W. W. Kay.** 1993. Characterization of three fimbrial genes, *sefABC*, of *Salmonella enteritidis*. J. Bacteriol. **175:**2523–2533.
4. **Frankel, G., S. M. C. Newton, G. K. Schoolnik, and B. A. D. Stocker.** 1989. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. EMBO J. **8:**3249–3252.
5. **Galan, E., and R. Curtiss.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
6. **Hyman, H. C., and S. Trachtenberg.** 1991. Point mutations that lock *Salmonella typhimurium* flagellar filaments in the straight right-handed and left-handed forms and their relation to filament superhelicity. J. Mol. Biol. **220:**79–88.
7. **Joys, T. M., and F. Schodel.** 1991. Epitope mapping of the *d* flagellar antigen of *Salmonella muenchen*. Infect. Immun. **59:**3330–3332.
8. **Kanto, S., H. Okino, S. I. Aizawa, and S. Yamaguchi.** 1991. Amino acids responsible for flagellar shape are distributed in terminal regions of flagellin. J. Mol. Biol. **219:**471–480.
9. **Kauffmann, F.** 1964. Das Kauffmann-White-Scheme, p. 21–66. *In* E. van Oye (ed.), The world problem of salmonellosis. W. Junk Publishers, The Hague, The Netherlands.
10. **Kusters, J. G., E. J. Jager, and B. A. M. van der Zeijst.** 1989. Improvement of the cloning linker of the bacterial expression vector pEX. Nucleic Acids Res. **17:**8007.
11. **Masten, B. J., and T. M. Joys.** 1993. Molecular analysis of the *Salmonella g . . .* flagellar antigen complex. J. Bacteriol. **175:**5359–5365.
12. **Namba, K., I. Yamashita, and F. Vonderviszt.** 1989. Structure of the core and central channel of bacterial flagella. Nature (London) **342:**648–654.
13. **Newton, S. M. C., C. O. Jacob, and B. A. D. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70–72.
14. **Newton, S. M. C., M. Kotb, T. P. Poirier, B. A. D. Stocker, and E. H. Beachey.** 1991. Expression and immunogenicity of a streptococcal M protein epitope inserted in *Salmonella* flagellin. Infect. Immun. **59:**2158–2165.
15. **Newton, S. M. C., R. D. Wasley, A. Wilson, L. T. Rosenberg, J. F. Miller, and B. A. D. Stocker.** 1991. Segment-IV of a *Salmonella*-flagellin gene specifies flagellar antigen epitopes. Mol. Microbiol. **5:**419–425.
16. **Petter, J. G.** 1993. Detection of two smooth colony phenotypes in a *Salmonella enteritidis* isolate which vary in their ability to contaminate eggs. Appl. Environ. Microbiol. **59:**2884–2890.
17. **Popoff, M. Y., J. Bockemuhl, and A. McWhorter-Murlin.** 1993. Supplement 1990 (no. 34) to the Kaufmann-White scheme. Res. Microbiol. **142:**1029–1033.
18. **Rodrigue, D. C., R. V. Tauxe, and B. Rowe.** 1990. International increase in *Salmonella enteritidis*: a new pandemic? Epidemiol. Infect. **105:**21–27.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Selander, R. K., N. H. Smith, J. Li, P. Beltran, K. E. Ferris, D. J. Kopecko, and F. A. Rubin.** 1992. Molecular evolutionary genetics of the cattle-adapted serovar *Salmonella dublin*. J. Bacteriol. **174:**3587–3592.
21. **St. Louis, M. E., D. L. Morse, M. E. Potter, T. M. DeMelfi, J. J. Guzewich, R. V. Tauxe, and P. A. Blake.** 1988. The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections. JAMA **259:**2103–2107.
22. **Stone, B. J., C. M. Garcia, J. L. Badger, T. Hassett, R. I. F. Smith, and V. L. Miller.** 1992. Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. J. Bacteriol. **174:**3945–3952.
23. **Thorns, C. J., M. G. Sojka, and D. Chasey.** 1990. Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* by using a monoclonal antibody. J. Clin. Microbiol. **28:**2409–2414.
24. **Turcotte, C., and M. J. Woodward.** 1993. Cloning, DNA nucleotide sequence and distribution of the gene encoding the SEF14 fimbrial antigen of *Salmonella enteritidis*. J. Gen. Microbiol. **139:**1477–1485.
25. **van Vliet, A. H. M., F. Jongejan, and B. A. M. van der Zeijst.** 1992. Phylogenetic position of *Cowdria ruminantium* (*Rickettsiales*) determined by analysis of amplified 16S ribosomal DNA sequences. Int. J. Syst. Bacteriol. **42:**494–498.
26. **van Zijderveld, F. G., A. M. van Zijderveld-van Bemmel, and J. Anakotta.** 1992. Comparison of four different enzyme-linked immunosorbent assays for serological diagnosis of *Salmonella enteritidis* infections in experimentally infected chickens. J. Clin. Microbiol. **30:**2560–2566.
27. **Wei, L. N., and T. M. Joys.** 1985. Covalent structure of three phase-1 flagellar filament proteins of *Salmonella*. J. Mol. Biol. **186:**791–803.
28. **Wilcox, G., J. Abelson, and C. F. Fox.** 1977. Molecular approaches to eukaryotic genetic systems. Academic Press, New York.