JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1993, p. 2263–2273
0095-1137/93/092263-11$02.00/0

Vol. 31, No. 9

DNA-Based Diagnostic Tests for *Salmonella* Species Targeting *agfA*, the Structural Gene for Thin, Aggregative Fimbriae

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Received 19 February 1993/Returned for modification 16 April 1993/Accepted 22 May 1993

***Salmonella enteritidis* 27655-3b and a few diarrheagenic *Escherichia coli* strains produce morphologically and antigenically related, thin, aggregative fimbriae, collectively named GVVPQ fimbriae (S. K. Collinson, L. Emödy, T. J. Trust, and W. W. Kay, J. Bacteriol. 174:4490–4495, 1992). To determine whether GVVPQ fimbriae are common to *Salmonella* spp. and other enteropathogenic members of the family *Enterobacteriaceae*, 113 isolates were phenotypically screened for Congo red binding and aggregative colony morphology. Presumptive positive and representative negative strains were examined by Western blotting (immunoblotting) by using antiserum to SEF 17, the native GVVPQ fimbria of *S. enteritidis*. Only four *S. enteritidis* strains and six *E. coli* isolates possessed substantial amounts of GVVPQ fimbriae after 24 h of incubation on T medium. Following 5 days of incubation, 56 of 93 *Salmonella* isolates (60%) and 1 of 7 additional *E. coli* clinical isolates possessed detectable levels of GVVPQ fimbriae. Since variable expression of GVVPQ fimbriae was observed among *Salmonella* isolates and some *E. coli* strains produced scant amounts, as revealed by immunoelectron microscopy, the ability to produce these fimbriae was evaluated by genotypic screening. The structural gene for the SEF 17 fimbrin, *agfA*, was amplified by the polymerase chain reaction, cloned, and sequenced to provide a characterized DNA probe. An *agfA* DNA fragment hybridized strongly to 603 of 604 (99.8%) *Salmonella* isolates but very weakly to 31 of 266 other members of the family *Enterobacteriaceae* including 26 of 137 *E. coli* strains, 3 of 14 *Citrobacter* spp., and single isolates of *Shigella sonnei* and *Enterobacter cloacae*. The *agfA* DNA probe proved to be a valuable diagnostic tool for *Salmonella* isolates arrayed on hydrophobic grid membrane filters. Unique *agfA* sequences were targeted in the development of a polymerase chain reaction assay specific for *Salmonella* spp.**

Salmonellae are invasive enteropathogens of humans and animals (13). Salmonellosis accounts for 2 million to 4 million cases of food-borne infection annually in the United States and contributes substantially to the estimated 1 billion annual cases of potentially fatal, food-borne diarrheal disease in children worldwide (7, 23). The increase in salmonellosis in industrialized countries over the past 35 years has accompanied the centralization of food production and processing, despite improvements in epidemiological and microbiological methods. During the past decade, a dramatic increase in the occurrence of *Salmonella enteritidis* infections was principally responsible for the rise of food-borne salmonellosis in the United States and Europe (35). Estimates of the economic costs of salmonellosis in the United States vary from $50 million to $23 billion annually, arising from lost productivity and medical treatment (7, 23, 46), with comparable per-capita costs reported for other industrialized countries (41, 45). The eradication of *Salmonella* isolates from the environment is practically impossible; therefore, the development of preventative and control measures are necessary, including improved diagnostics, vaccines, and hazard analysis (HACCP) education to limit the recurrence of disease.

Salmonella pathogenesis is poorly understood, but the molecular complexities are beginning to be appreciated (11, 12, 15, 19, 25, 26, 29). The role of bacterial cell surface structures remains unresolved. Similar to other pathogenic bacteria, salmonellae produce surface appendages called fimbriae (or pili) that are composed of a single structural protein species, fimbrins, arranged helically in fibrils that are 2 to 7 nm in width (31). Although certain types of *Escherichia coli* fimbriae are known to be important for virulence (21), only type 1 fimbriae have been implicated in *Salmonella* pathogenicity (18, 22).

S. enteritidis 27655-3b, a human isolate, produces three distinct fimbrial types, SEF 21 (type 1), SEF 17, and SEF 14 (9, 30). SEF 17 fimbriae mediate fibronectin binding and bacterial autoaggregation (8). SEF 17 are composed primarily of AgfA, a 17,000-molecular-weight (MW) fimbrin protein, and require depolymerization with 90% formic acid prior to analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (9). Certain clinical isolates of diarrheagenic *E. coli* produce thin, aggregative

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fimbriae composed of 18,000-MW fimbrins that are biochemically and serologically related to AgfA (10). The AgfA-related *E. coli* HB101 fimbrin was named "curlin," the gene encoding it was designated *csgA*, and the corresponding thin, aggregative fimbriae were named "curli" by Normark and coworkers (3). The class of fimbriae which includes *S. enteritidis* SEF 17 and *E. coli* curli has been termed GVVPQ fimbriae by Collinson et al. (10) in recognition of common fimbrin N-terminal sequences.

The present study was initiated to determine the extent of the distribution of GVVPQ fimbriae among *Salmonella* serovars and other enteropathogenic members of the family *Enterobacteriaceae*. Determination of the distributions of sequences homologous to the AgfA fimbrin gene, *agfA*, among isolates of *Salmonella* and other members of the family *Enterobacteriaceae* resulted in the development of novel DNA-based diagnostic tests for *Salmonella* spp.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *Salmonella*, other members of the family *Enterobacteriaceae*, and eubacteria used in the present study and their sources are provided in Table 1. Panels of *Enterobacteriaceae* arrayed on hydrophobic grid membrane filters (HGMFs; Gelman Sciences, Montreal, Quebec, Canada) were replicated (HGMF Replicator, Richard Brancker Research, Ottawa, Ontario, Canada) and were grown as described by Sharpe et al. (40). To promote the production of GVVPQ fimbriae and to assay Congo red binding ability (8, 10), the bacterial strains were grown on T medium. All bacteria except isolates of *Serratia marcescens*, *Erwinia caratovora*, *Aeromonas salmonicida*, and *Aeromonas hydrophila* were grown at 37°C; *S. marcescens* and *E. caratovora* were grown at 25°C, and *A. salmonicida* and *A. hydrophila* were grown at 20°C.

SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot (immunoblot) analyses of putative GVVPQ fimbrins were conducted as described by Collinson et al. (8, 10).

Electron microscopy. Bacteria were immunogold labeled with rabbit immune serum to SEF 17 and protein A-gold (Auroprobe; Pharmacia, Uppsala, Sweden), negatively stained, and observed with a Philips EM300 electron microscope operated at 60 kV (9).

Amplification, cloning, and sequencing of *agfA*. A 394-bp *agfA* DNA fragment was amplified from *S. enteritidis* 27655-3b Tn*phoA* mutant 2-7f by the polymerase chain reaction (PCR) as described previously (8). To facilitate subcloning of the *agfA* PCR fragment, primers were constructed to introduce terminal restriction endonuclease cleavage sites. A 34-bp biased, oligonucleotide mixture, TAF1 (dGGCGGAAGCTTGAATTCGT[A/C/T]GT[A/C/T]CC[A/G/T]CA[A/G]TGGGG), was designed from DNA sequences corresponding to amino acid residues 2 to 7 of the AgfA N terminus (9) and incorporated a hexanucleotide sequence (underlined) which is required to create an *Eco*RI cleavage site in the PCR product. The opposite-strand primer, TAF2 (dGGGAAAGGTTGAATTCAGGACGCTACTTGTG), into which three nucleotide changes (underlined) were introduced to create an *Eco*RI site in the PCR product, was complementary to the IS*50*$_L$ sequence residing at the junction of Tn*phoA*-generated alkaline phosphatase gene fusions (27).

The amplified 394-bp *agfA* fragment was isolated by agarose (1.5%) gel electrophoresis in Tris-acetate-EDTA

TABLE 1. Screening of *Salmonella* isolates and other members of the family *Enterobacteriaceae* for ability to produce GVVPQ fimbriae

Bacterial species	Strain	Source[a]	Serogroup	AgfA cross-reactive protein[b]	Hybridization to *agfA* probe[c]
Salmonella spp.					
S. agona	BTR II-1	TJT	B	–	nd
	Rosy-1	TJT	B	–	nd
		HPB	B		13/13
S. alachua		HPB	O		1/1
S. albany		TJT	C3		+
		HPB	C3		6/6
S. anatum		UVIC	E1	+	+
		HPB	E1		14/14
S. arizonae		TJT		–	+
		HPB			4/4
S. arkansas		HPB	E3		4/4
S. bardo		HPB	C3		1/1
S. barielly		HPB	C1		12/12
S. berta	8392	ATCC	D1	–	+
	89-4065	PVL	D1	++	+
	90-1271	PVL	D1	++	+
		HPB	D1		4/4
S. binza		HPB	E2		6/6
S. blockley		HPB	C2		5/5
S. bovismorbificans		TJT	C2	+++	nd
S. braenderup		HPB	C1		5/5
S. brandenburg		HPB	B		3/3
S. bredeney		HPB	B		8/8
S. brunei		HPB	C3		1/1
S. burnepta		UVIC		++	nd
S. california		HPB	B		2/2
S. cerro		TJT	K	++	+
		HPB	K		7/7
S. chester		HPB	B		1/1
S. choleraesuis		BBF	C1	–	+
		HPB	C1		1/1
S. colindale		HPB	C1		1/1
S. cubana		TJT	G2	+[d]	nd
		HPB	G2		2/2
S. dahomey		TJT	X	–	+
S. derby		HPB	B		3/3
S. drypool		UVIC	E2	++	+
		HPB	E2		1/1
S. dublin	15480	ATCC	D1	–	+
	89-3349	PVL	D1	–	+
	89-4189	PVL	D1	–	+
	90-243	PVL	D1	–	+
	90-1176	PVL	D1	–	+
	89-3320	PVL	D1		+
		HPB	D1		1/1
S. ealing		HPB	O		1/1
S. eastbourne		UVIC	D1	–	+
		HPB	D1		2/2
S. eimsbuettel		HPB	C4		2/2
S. elisabethville		HPB	E1		1/1
S. enteritidis	27655-3b	TW	D1	+++	+
	27036 2I	TW	D1	++	+
	27036 2II	TW	D1	+++	+
	13076	ATCC	D1	+++	+
	4931	ATCC	D1	–	+
	31194	ATCC	D1	+	+
	801	LCDC	D1	+++	nd
	809	LCDC	D1	–	+
	813	LCDC	D1	++	+
	907	LCDC	D1	++	+
	913	LCDC	D1	++	+
	914	LCDC	D1	++	+

Continued on following page

TABLE 1—*Continued*

Bacterial species	Strain	Source[a]	Sero-group	AgfA cross-reactive protein[b]	Hybrid-ization to *agfA* probe[c]
	930	LCDC	D1	−	+
	939	LCDC	D1	++	+
	955	LCDC	D1	−	+
	972	LCDC	D1	++	+
	989	LCDC	D1	++	nd
	89-2749	PVL	D1	−	nd
	JTSe1	JT	D1	++	+
	JTSe2	JT	D1	++	nd
	JTSe3	JT	D1	++	nd
	JTSe4	JT	D1	++	nd
	27655-3a	TW	D1		+
		HPB	D1		7/7
S. flint		HPB	Z		1/1
S. florida		TJT	H	++	+
S. gallinarum	9184	ATCC	D1	−	+
		HPB	D1		1/1
S. gaminara		TJT	I		+
S. give		HPB	E1		1/1
S. godesberg		HPB	N		1/1
S. haardt		HPB	C3		9/9
S. hadar	F9-1	TJT	C2	+++	nd
		HPB	C2		56/56
S. halmstad		HPB	E2		1/1
S. hamburg		TJT	B	++	+
S. havana		TJT	G2	+++	+
		HPB	G2		5/5
S. heidelberg	8326	ATCC	B	+	nd
		HPB	B		26/26
S. indiana		HPB	B		10/10
S. infantis	S41-16	TJT	C1	+++	nd
	JTSi1	JT	C1	++	+
	JTSi2	JT	C1	++	nd
	JTSi3	JT	C1	+	nd
	JTSi4	JT	C1	−	nd
		HPB	C1		25/25
S. isangi		HPB	C1		1/1
S. javiana	10721	ATCC	D1	−	nd
		HPB	D1		1/1
S. johannesburg		HPB	R		22/22
S. kentucky		HPB	C3		11/11
S. landau		HPB	N		1/1
S. lexington		HPB	E1		1/1
S. lille		HPB	C1		2/2
S. litchfield		HPB	C2		1/1
S. livingstone		HPB	C1		7/7
S. london		HPB	E1		4/4
S. manhattan		TJT	C2	+++	nd
		HPB	C2		1/1
S. mbandaka		TJT	C1	+++	nd
		HPB	C1		27/27
S. meleagridis		HPB	E1		2/2
S. mikawasima		UVIC	C1	++	nd
S. minnesota		TJT	L	++	+
		HPB	L		2/2
S. montevideo		HPB	C1		14/14
S. muenchen	Rosy-3	TJT	C2	++	nd
	Bowmer-9	TJT	C2	−	nd
		HPB	C2		1/1
S. muenster		HPB	E1		6/6
S. negev		TJT	S	−	nd
S. newbrunswick		HPB	E2		2/2
S. newington		HPB	E2		13/13
S. newport		TJT	C2	+[d]	+
		HPB	C2		5/5
S. nienstedten		TJT	C4	++	+
		HPB	C4		1/1
S. ohio		HPB	C1		11/11
S. oranienburg	9239	ATCC	C1	−	nd
		HPB	C1		6/6
S. orion		HPB	E1		2/2
S. panama		HPB	D1		2/2
S. paratyphi A subsp. *durazzo*	11511	ATCC	A	++	+
S. paratyphi B		TJT	B	++	nd
		HPB	B		3/3
S. poona		HPB	G1		2/2
S. pullorum	9120	ATCC	D1	++	+
	10398	ATCC	D1	−	+
	19945	ATCC	D1	−	+
	89-2331	PVL	D1	−	+
	90-1175	PVL	D1	−	+
		HPB	D1		2/2
S. reading		HPB	B		2/2
S. saint-paul		TJT	B	++	nd
		HPB	B		6/7
S. sandiego		TJT	B	++	nd
		HPB	B		1/1
S. schwarzengrund		TJT	B	++	nd
		HPB	B		18/18
S. senftenberg		HPB	E4		28/28
S. stanley		TJT	B	−	+
S. taksony		HPB	E4		1/1
S. tennessee		TJT	C1	++	+
		HPB	C1		14/14
S. thomasville		HPB	E3		3/3
S. thompson		HPB	C1		18/18
S. typhi		UVIC	D1	−	nd
		HPB	D1		2/2
S. typhimurium	F18-1	TJT	B	++	+
	F103-29	TJT	B	++	nd
	F112-2	TJT	B	++	+
	S736	TJT	B	++	+
	Bowmer-10	TJT	B	−	nd
	Bowmer-12	TJT	B	−	nd
	3Pork 1	TJT	B	−	nd
	962	TJT	B	−	+
	10GC1	TJT	B	−	nd
	JTst2	JT	B	++	+
	JTst3	JT	B	−	+
	JTst4	JT	B	++	nd
		HPB	B		34/34
S. urbana		HPB	N		1/1
S. wassenaar		HPB	Z		1/1
S. weltevreden		HPB	E1		1/1
S. westhampton		HPB	E1		1/1
S. widemarsh		TJT	O	++	nd
S. worthington		TJT	G2	++	+
		HPB	G2		6/6
Other *Enterobacteriaceae*					
Citrobacter freundii	8090	UVIC		−	−
		HPB			2[e]/11
Citrobacter sp.		GS			+[e]
		HPB			0/1
Enterobacter aerogenes		UVIC		+[d]	−
		HPB			0/2
Enterobacter agglomerans		HPB			0/6
Enterobacter cloacae		HPB			1[e]/8

Continued

TABLE 1—*Continued*

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TABLE 1—*Continued*

Bacterial species	Strain	Source[a]	Sero-group	AgfA cross-reactive protein[b]	Hybrid-ization to *agfA* probe[c]
Erwinia caratovora		UVIC		–	–
		HPB			0/2
Escherichia coli	11775	ATCC		–	–
	C600	ATCC		–	+[e]
	HB101	ATCC		–	+[e]
	E1049a-13	TJT		–	+[e]
	B41M	LE		–	–
	HM1475	LE		–	+[e]
	438 Hf	TJT		++[d]	+[e]
	B4	GS		++[d]	+[e]
	Vietnam I/1	LE		+++[d]	+[e]
	Viet G	LE		PF[f]	+[e]
	Gambia G3	LE		+++[d]	+[e]
	NG7c	LE		PF[f]	+[e]
	NG7c1	LE		PF[f]	+[e]
	135+	GS		++[d]	+[e]
	314-H	GS		++[d]	+[e]
	654-H	GS		++[d]	+[e]
	H2	TJT			+[e]
		HPB			11[e]/120
Hafnia alvei		UVIC		–	–
		HPB			0/5
Klebsiella oxytoca		HPB			0/1
Klebsiella pneumoniae	13883	ATCC		–	–
		HPB			0/6
Proteus mirabilis		HPB			0/4
Proteus morganii		HPB			0/1
Proteus rettgeri		HPB			0/4
Proteus vulgaris		UVIC		–	–
		HPB			0/5
Proteus sp.		HPB			0/1
Providencia rettgeri		UVIC		–	–
Providencia alcalifaciens		HPB			0/1
Providencia sp.		HPB			0/2
Serratia fonticola		HPB			0/1
Serratia marcescens		UVIC		–	–
		HPB			0/4
Shigella boydii		HPB			0/2
Shigella dysenteriae		HPB			0/3
Shigella flexneri		HPB			0/14
Shigella sonnei		UVIC		–	+[e]
		HPB			0/4
Yersinia enterocolitica		HPB			0/27
Yersinia frederiksenii		HPB			0/1
Yersinia intermedia		HPB			0/3
Yersinia pseudotuberculosis		HPB			0/1
Other eubacteria					
Achromobacter spp.		HPB			0/2
Acinetobacter calcoaceticus		HPB			0/2
Aeromonas hydrophila		TJT			0/1
		HPB			0/4
Aeromonas salmonicida		WWK			0/1
Alcaligenes faecalis		HPB			0/2
Bacillus subtilis		UVIC			0/1
Bordetella bronchiseptica		HPB			0/1
Pseudomonas aeruginosa		UVIC			0/1
		HPB			0/5
Pseudomonas fluorescens		HPB			0/2
Pseudomonas putida		HPB			0/2
Pseudomonas stutzeri		HPB			0/1
Pseudomonas sp.		HPB			0/1

Continued

TABLE 1—*Continued*

[a] ATCC, American Type Culture Collection; BBF, B. B. Finlay, Biotechnology Laboratory and Departments of Biochemistry and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada; GS, G. Sarlós, University Medical School, Institute of Microbiology, Pécs, Hungary; HPB, Health Protection Branch of Health and Welfare Canada, Ottawa, Ontario, Canada; JT, J. Tomas, Departement Microbiologie, Universitat de Barcelona, Barcelona, Spain; LCDC, H. Lior, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada; LE, L. Emödy, University Medical School, Institute of Microbiology, Pécs, Hungary; PVL, G. Thiele, Provincial Veterinary Laboratory of British Columbia, Abbotsford, British Columbia, Canada; TJT, T. J. Trust, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada; TW, T. Wadström, University of Lund, Lund, Sweden; UVIC, Culture Collection of the Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada; WWK, W. W. Kay, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada. The strains designated as originating from source HPB (Health Protection Branch of Health and Welfare Canada, Ottawa, Ontario, Canada) were screened as panels of colony blots on HGMF membranes (32, 40) for hybridization to the *agfA* DNA probe only.

[b] Strains grown for 5 days on T medium were screened for immunoreactive AgfA proteins by Western blot analysis as described in the text. nd, not determined. Production of AgfA cross-reactive proteins was qualitatively compared with the levels of production by *S. enteritidis* 27655-3b, which was scored as +++. A moderately strong band was scored as ++, and a weak band was scored as +. No production of a cross-reactive protein was scored as –.

[c] Hybridization of samples of membrane-bound bacterial DNA to the 394-bp *agfA* gene probe. The results of hybridization to dot blots of purified DNA are reported as positive (+), negative (–), or not determined (nd). Hybridization of the *agfA* probe to DNA samples represented as colony blots on HGMF (Health Protection Branch of Health and Welfare Canada) panels are reported as the number of positively hybridizing strains per total number of strains tested.

[d] AgfA immunologically cross-reactive band migrated with an apparent MW of 18,000 to 19,000 rather than with an apparent MW of 17,000, as observed with *S. enteritidis* 27655-3b AgfA fimbrin.

[e] DNAs from these strains hybridized very weakly to the 394-bp *agfA* gene probe.

[f] AgfA cross-reactive protein previously found (10).

buffer (37) and was purified by using Gene Clean II glassmilk following the double Gene Clean protocol recommended by the manufacturer (Bio 101, Inc., La Jolla, Calif.). The amplified fragment was cleaved with *Eco*RI (Bethesda Research Laboratories, Bethesda, Md.) and was cloned into pUC19 (49). The sequences of both strands were determined by using the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio) for enzymatic, dideoxy termination sequencing (38). The results were confirmed by analysis of two independent clones by using an Applied Biosystems model 373A automated DNA sequencer and the associated reagents, protocols, and software (version 1.10) for cycle sequencing (Applied Biosystems Canada Inc., Mississauga, Ontario, Canada). Computer analysis of the DNA sequence for open reading frames and gene translation was performed by using DNA Strider version 1.1 (28).

***agfA* DNA probe hybridization.** DNA dot blot hybridiza-

tion was used to screen 58 *Salmonella* isolates, 28 other members of the family *Enterobacteriaceae*, and 4 other eubacteria. Chromosomal DNA was purified from proteinase K-treated cell lysates by repeated phenol-chloroform extraction and ethanol precipitation (37) or by the method of Alm et al. (1). The DNA was quantified by spectroscopy, and 0.5-μg samples were applied to HyBond-N^+ nylon membranes (Amersham Canada Inc., Oakville, Ontario, Canada) by using a dot blot manifold. A total of 546 *Salmonella* serovars, 239 other *Enterobacteriaceae* strains, and 22 isolates of other eubacteria were screened for *agfA*-related genes as colony blots prepared on HGMF panels as described previously (32). The presence of *Salmonella* DNA on the HGMF panels was verified by hybridization with a mixture of total DNA prepared from *S. enteritidis*, *S. typhimurium*, and *S. berta* which was labeled with [α-^{32}P] dATP by random primer-directed DNA synthesis (37). An *agfA* gene probe was similarly constructed from the 394-bp *agfA* PCR fragment described above. DNA blots were prehybridized at 65°C in prehybridization buffer (37) containing 200 μg of herring sperm DNA per ml. Following hybridization at 65°C, the membranes were washed at a high stringency (0.2× SSPE buffer [1× SSPE is 0.18 M NaCl, 10 mM Na_3PO_4, and 1 mM EDTA; pH 7.7] [37], 0.1% SDS, 63°C) to maximize the signal difference between strongly and weakly hybridizing samples. The results were recorded by autoradiography on X-Omat AR5 film (Kodak, Rochester, N.Y.).

N-terminal protein sequencing. The 17,000-MW AgfA band of *S. enteritidis* 27655-3b and the 18,000-MW curlin bands of three clinical *E. coli* strains were isolated by SDS-PAGE, electrophoretically transferred to Immobilon membranes (Millipore Corp., Bedford, Mass.), and excised for direct determination of N-terminal amino acid sequences as described previously (9, 10).

***agfA*-based PCR assays.** Two sets of oligonucleotide primers were designed, taking advantage of the potential similarities or differences in the sequences of *agfA* and *csgA* predicted by N-terminal protein sequencing and the fact that fimbrins with similar N-terminal sequences often have dissimilar C-terminal sequences (31). Accordingly, primers TAF3 and TAF4 were selected to provide *Salmonella*-specific PCR amplification of a 261-bp *agfA* DNA fragment, whereas TAF5 and TAF6 were designed to amplify 92-bp DNA fragments from both *agfA* and *csgA*. Specifically, PCR primers TAF3 (dTCCGGCCCGGACTCAACG) and TAF4 (dCAGCGCGGCGTTATTACCG) targeted complementary strands of *agfA* in regions corresponding to AgfA amino acid residues 19 to 24 and 100 to 105, respectively (see Fig. 3). A second set consisted of TAF5 (dGGCGGCGGCAATA[G/A] TTCCGGCCCG), which targeted the *agfA* sequence corresponding to amino acid residues 14 to 21 of AgfA, and TAF6 (dCGGGCATCG[C/G]TTTGCAGAGCAAGCGC), which was derived from the opposite-strand sequence corresponding to amino acid residues 36 to 44 (see Fig. 3). To favor amplification of a 92-bp fragment from both *Salmonella* and *E. coli* isolates, single-amino-acid differences in the corresponding regions of the AgfA and curlin fimbrins (see Fig. 4) were taken into consideration in the synthesis of TAF5 and TAF6 as mixtures of two oligonucleotides.

Bacterial DNA samples for PCR analyses were prepared from cells (20 mg [wet weight]) that were resuspended in 1 ml of distilled, deionized H_2O and lysed by boiling for 5 min. Cell lysates containing DNA were clarified by centrifugation (16,000 × *g*, 5 min, 25°C) and stored at −20°C. To conduct the PCR, a 10-μl reaction volume contained 1 μl of heat-denatured bacterial DNA solution, 5 pmol of each primer, the four deoxynucleotide triphosphates at final concentrations of 0.5 mM, and 0.4 U of *Taq* DNA polymerase (Stratagene, La Jolla, Calif.) in a reaction buffer consisting of 50 mM Tris-HCl [pH 8.5], 20 mM KCl, 15 mM $MgCl_2$, and 0.5 mg of bovine serum albumin per ml. Thermocycling was performed on samples contained in sealed glass capillary tubes inserted in an Idaho Technology air-driven thermocycler for 30 cycles of denaturation (95°C, 5 s), annealing (55, 62, or 68°C, 1 s), and elongation (74°C, 30 s). Annealing temperatures of 68 to 70°C were used to provide *Salmonella*-specific PCR amplification of an internal *agfA* fragment. The products of DNA amplification were separated by electrophoresis in 15% polyacrylamide gels and were visualized by UV illumination following ethidium bromide staining (37).

RESULTS

Expression of GVVPQ fimbriae. Bacteria were grown on solid T medium to rapidly screen for Congo red binding and aggregative colony morphology indicative of GVVPQ fimbria production. After 24 h, only 4 isolates of *S. enteritidis*, from among 93 *Salmonella* isolates representing 43 serovars, bound significant amounts of the hydrophobic dye and demonstrated aggregative colonial morphology. Western blot analysis of 38 representative *Salmonella* strains confirmed that only autoaggregative, dye-binding *S. enteritidis* 27655-3b, 27036 2II, ATCC 13076, and LCDC 801 produced substantial amounts of the 17,000-MW GVVPQ fimbrin protein AgfA during 24 h of incubation. Six *E. coli* clinical isolates, B4, Vietnam I/1, Gambia G3, 135+, 314-H, and 654-H, selected for Congo red binding produced detectable levels of an 18,000-MW protein that was cross-reactive with AgfA. Seven *E. coli* strains and strains of *Citrobacter freundii*, *Enterobacter aerogenes*, *Erwinia caratovora*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Providencia rettgeri*, *Serratia marcescens*, and *Shigella sonnei* did not bind Congo red or produce a cross-reactive GVVPQ fimbrin following 24 h of incubation. Incubation for 2 to 5 days resulted in an increase in Congo red binding by all *Enterobacteriaceae* family strains such that, after 48 h, dye binding by GVVPQ fimbria-producing strains was not readily distinguished from the amount of dye otherwise absorbed by aged colonies. Following 5 days of incubation, 56 of 93 (60%) *Salmonella* isolates produced GVVPQ fimbriae detectable by Western blotting (Table 1; Fig. 1). Prolonged incubation also resulted in the expression by an additional *E. coli* clinical isolate of an 18,000-MW protein that was cross-reactive with AgfA and the production of a cross-reactive, 19,000-MW protein by a strain of *E. aerogenes* (Table 1; Fig. 1).

Variation in GVVPQ fimbrin production was evident within serovars of *Salmonella* as exemplified by 5 of 22 *S. enteritidis* isolates, 1 of 5 *S. infantis* isolates, 1 of 2 *S. muenchen* isolates, 4 of 5 *S. pullorum* isolates, and 6 of 12 *S. typhimurium* isolates which did not produce detectable levels of AgfA (Table 1). Similarly, there was evidence of variable expression of GVVPQ fimbriae among *E. coli* isolates, because 6 of 13 strains did not possess quantities of GVVPQ fimbrin detectable by Western blot analysis (Table 1). Several *E. coli* strains considered to be nonproducers of GVVPQ fimbriae were examined by immunoelectron microscopy by using rabbit antiserum generated to the SEF 17 fimbriae of *S. enteritidis*. *E. coli* ATCC 11775, B41M, E1049a-13, and C600, which were grown at 37°C, infrequently possessed thin fimbriae decorated with anti-SEF 17

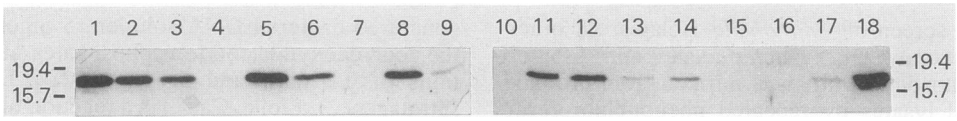

FIG. 1. Western blot identification of proteins from *Salmonella* strains and other members of the family *Enterobacteriaceae* that are immunologically cross-reactive with AgfA of *S. enteritidis* 27655-3b. Lanes: 1, *S. enteritidis* 27655-3b; 2, *S. hadar* F9-1; 3, *S. hamburg*; 4, *S. heidelberg*; 5, *S. infantis* S41-16; 6, *S. paratyphi* B; 7, *S. javiana*; 8, *S. mbandaka*; 9, *S. mikawasima*; 10, *S. typhi*; 11, *S. typhimurium* JTst4; 12, *S. typhimurium* JTst2; 13, *S. infantis* JTsi2; 14, *S. infantis* JTsi1; 15, *S. sonnei*; 16, *E. aerogenes*; 17, *S. worthington*; 18, *S. enteritidis* 27655-3b. Aggregative fimbriae from colonies grown on T medium for 5 days were solubilized and subjected to Western blot analysis as described by Collinson et al. (8). Anti-AgfA immune serum served as the primary antibody. Visualization of proteins that were immunologically cross-reactive with AgfA was accomplished by using goat, anti-rabbit, immunoglobulin G-alkaline phosphatase conjugates (Caltag Laboratories, San Francisco, Calif.), the substrate 5-bromo-4-chloro-3-indolyl phosphate, and the enhancer Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, Mo.). The molecular weights (in thousands) of comigrating, prestained protein standards (Bethesda Research Laboratories are noted.

antibody–protein A–gold conjugates as well as much more numerous unlabeled fimbriae with different morphologies (Fig. 2).

Amplification and sequencing of an *agfA* DNA probe. Since GVVPQ fimbriae were widely distributed among *Salmonella* serovars and were common to several *E. coli* strains yet were produced in variable amounts, a GVVPQ fimbrin gene probe was considered to be a useful tool for determining the distribution of GVVPQ fimbriae. The *S. enteritidis* 27655-3b Tn*phoA* insertion mutant, strain 2-7f, which produced an AgfA-PhoA fusion protein of approximately 70,000 MW (8), was selected for use in the isolation of an *agfA* DNA probe. A DNA fragment spanning the *agfA* gene from the sequence corresponding to the mRNA triplet encoding the second amino acid residue of the mature AgfA fimbrin to the downstream $IS50_L$ linker region flanking the *agfA*-Tn*phoA* junction was amplified by PCR by using primers TAF1 and TAF2. The amplified 394-bp DNA fragment was repeatedly subcloned into pUC19 to generate the plasmids pAGF1, pAGF3, and pAGF4. DNA sequencing confirmed that the PCR fragment contained a 333-bp region originating from the *S. enteritidis agfA* gene (Fig. 3). The N-terminal region of the translated DNA sequence of the single open reading frame corresponded precisely to amino acid residues 2 to 31, as determined by N-terminal sequencing of AgfA (Fig. 4). The amino acid composition of the translated sequence had a similar high glycine content (16%), high combined alanine, serine, plus glycine content (37%), low basic amino acid content (4.5%), and nearly 30% asparagine plus aspartic acid content, consistent with the total amino acid analysis of native AgfA fimbrin (9). Minimally, three-quarters of the SEF 17 fimbrin was represented in the 333-bp region of *agfA*, considering that AgfA has an apparent MW of 17,000, as estimated by SDS-PAGE.

Distribution of *agfA* and related fimbrin genes. The PCR-amplified *agfA* gene fragment was hybridized to DNA from a total of 896 bacterial strains represented as colony blots assembled on HGMF panels or as DNA dot blots on nylon membranes (Table 1; Fig. 5). The *agfA* gene probe detected 603 of 604 (99.8%) *Salmonella* isolates representing 95 serovars. A single isolate of *S. saint-paul* did not hybridize to the *agfA* probe, although six other *S. saint-paul* isolates hybridized strongly. DNA preparations from 235 of 266 strains of other genera of the family *Enterobacteriaceae* and DNA samples from 26 unrelated eubacteria did not hybridize to the *agfA* probe. The *agfA* gene probe hybridized extremely weakly to heterologous DNA on HGMF membranes derived from 11 of 120 *E. coli* isolates, 2 of 12 *Citrobacter*

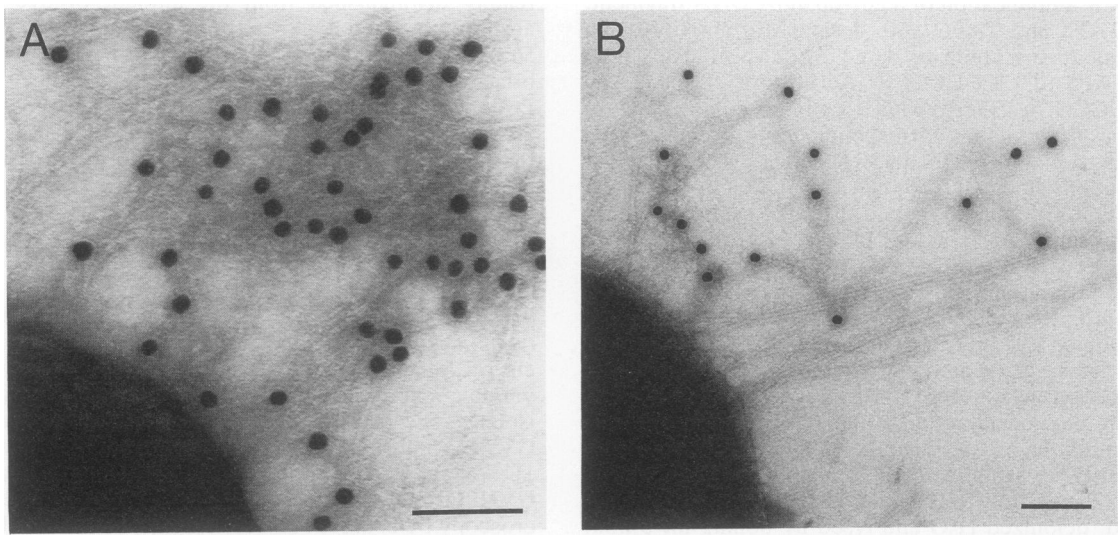

FIG. 2. Electron micrographs of immunogold-labeled GVVPQ fimbriae from *S. enteritidis* 27655-3b and *E. coli* C600. The fimbriae on the cells were labeled with protein A-gold after incubation with rabbit immune serum generated to *S. enteritidis* 27655-3b SEF 17 fimbriae (9). (A) SEF 17 fimbriae of *S. enteritidis* 27655-3b. (B) *E. coli* C600. Bars, 100 nm.

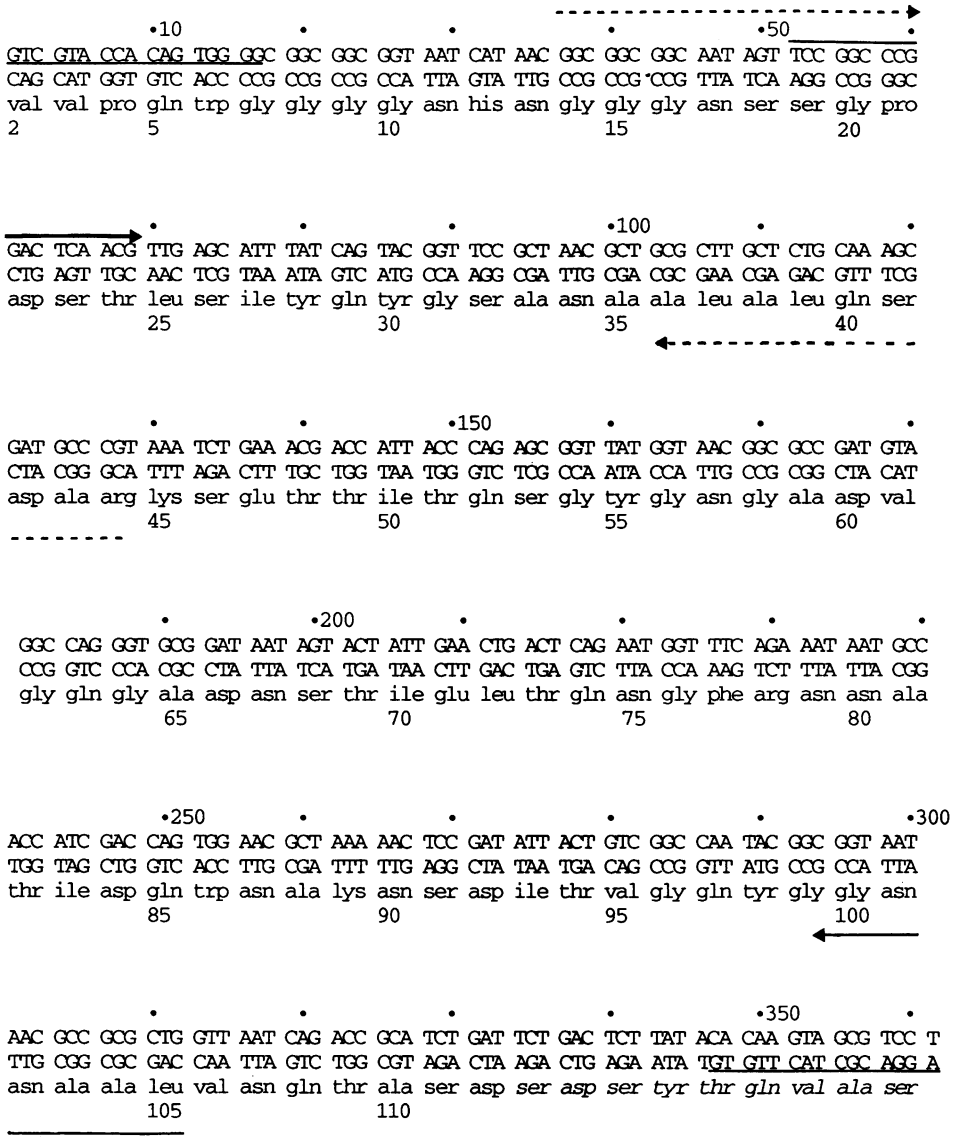

FIG. 3. DNA sequence of the *agfA* fimbrin gene fragment from the *S. enteritidis* 27655-3b Tn*phoA* mutant strain 2-7f amplified by PCR and cloned into pUC19. The bases underlined in the *agfA* sequence are common to portions of the PCR primers TAF1 and TAF2, which were used in the amplification of this fragment. Below the *agfA* sequence is the translated amino acid sequence from residues 2 to 112. The amino acid residues following the junction between AgfA and the truncated PhoA peptide (27) are italicized. The sequences targeted by the diagnostic PCR primer pairs TAF3 and TAF4 (solid arrows) and TAF5 and TAF6 (dashed arrows) are indicated.

spp., and 1 of 16 *Enterobacter* spp. No hybridization to members of other genera of the family *Enterobacteriaceae*, *Erwinia*, *Hafnia*, *Klebsiella*, *Proteus*, *Providencia*, *Serratia*, *Shigella*, or *Yersinia* or to several other eubacteria was detected. DNA dot blots prepared from 0.5-μg amounts of purified genomic DNA provided a more sensitive format. Accordingly, we detected weak hybridization to DNA from 15 of 17 *E. coli* isolates, 10 of which produced GVVPQ fimbrin detectable by Western blotting, and to a single strain of *S. sonnei*, but not to other DNA samples including that

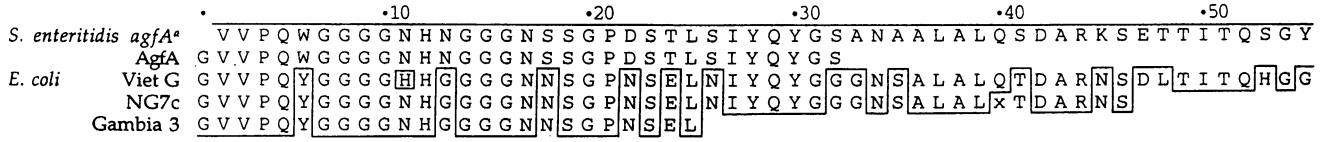

FIG. 4. Comparison of N-terminal amino acid sequences of AgfA from *S. enteritidis* 27655-3b and GVVPQ fimbrins from *E. coli* Viet G, NG7c, and Gambia 3 with the sequence of AgfA predicted by the *agfA* sequence (Fig. 3). Amino acid residues shared by AgfA and *E. coli* GVVPQ fimbrins are boxed. [a] indicates translation of the *agfA* sequence.

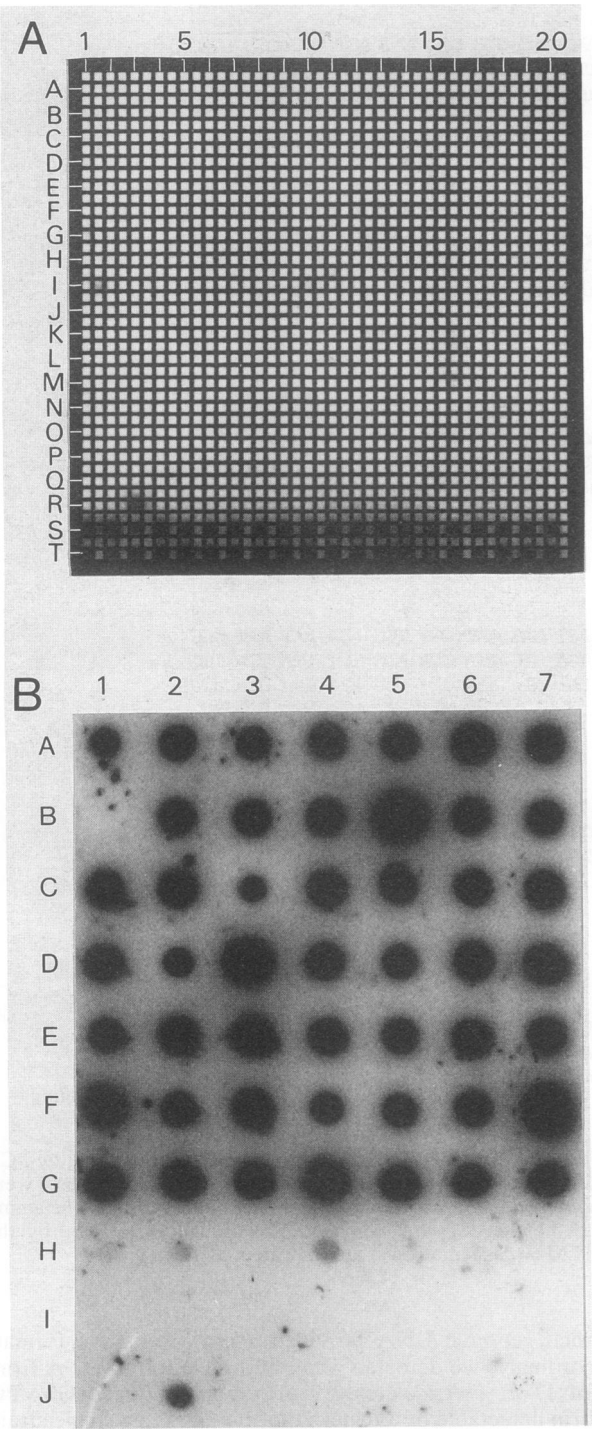

FIG. 5. Autoradiograph of hybridization of ^{32}P-labelled *agfA* fimbrin gene probe derived by PCR from *S. enteritidis* 27655-3b Tn*phoA* mutant 2-7f to representative panels of HGMF colony blots (A) and DNA dot blots (B). The bacterial strains represented are as follows: for panel A, positions A1 to F20, *E. coli* strains; G1 to G12, *Citrobacter* spp.; I1 to I16, *Enterobacter* spp.; J1 to J5, *Hafnia* spp.; K1 to K15, *Proteus* spp.; L1 to L7, *Klebsiella* spp.; M1 to N3, *Shigella* spp.; N9 to P20, *Yersinia* spp.; Q1 to Q4, *Aeromonas* spp.; Q5, *Borellia* sp.; Q6 and Q7, *Erwinia* spp.; Q8 and Q9, *Providencia* spp.; Q10 to Q13, *Serratia* spp.; Q14 and Q15, *Acinetobacter* spp.; Q16 and Q17, *Achromobacter* spp.; Q18 and Q19, *Alcaligenes* spp.; Q20, *S. marcescens*; R1 and R2 and R4 to R12, *Pseudomonas* spp.; R3 and S1 to T20, *Salmonella* spp.; G13 to H20, I17 to I20, J6 to J20, K16 to K20, L8 to L20, N4 to N8, and R13 to R20, no bacteria applied; for panel B, positions A1 to A7 and B2 to G7, *Salmonella* spp.; A1 to A7 and B2 to C3, *S. enteritidis* isolates; C4 to C6, *S. berta* isolates; C7 to D4, *S. pullorum* isolates; D5, *S. gallinarum*; D6 to E4, *S. dublin* isolates; E5, *S. drypool*; E6, *S. eastbourne*; E7, *S. albany*; F1, *S. anatum*; F2, *S. arizonae*; F3, *S. cerro*; F4, *S. choleraesuis*; F5, *S. dahomey*; F6, *S. florida*; F7, *S. gaminara*; G1, *S. havana*; G2, *S. minnesota*; G3, *S. newport*; G4, *S. neinstedten*; G5, *S. tennessee*; G6, *S. typhimurium*; G7, *S. worthington*; H1, *C. freundii* 8090; H2, *E. coli*; H3, *S. marcescens*; H4, *S. sonnei*; H5, *K. pneumoniae* 13883; H6, *E. aerogenes*; H7, *H. alvei*; I1, *E. caratovora*; I2, *P. vulgaris*; I3, *Providencia* sp.; I4, *P. aeruginosa*; I5, *A. hydrophila*; I6, *A. salmonicida*; I7, *Bacillus subtilis*; J1, herring sperm DNA; J2, *S. paratyphi* A; B1 and J3 to J7, blank.

prepared from the *E. aerogenes* strain which produced a 19,000-MW protein that was weakly, immunologically cross-reactive with AgfA.

PCR detection of GVVPQ fimbriae-producing *Enterobacteriaceae*. Extended N-terminal amino acid sequencing of the AgfA fimbrin of *S. enteritidis* and GVVPQ fimbrins of *E. coli* Viet G, NG7c, and Gambia 3 revealed significant sequence similarity (Fig. 4). However, comparative *agfA* hybridization indicated a lesser degree of similarity in the *agfA* and *csgA* gene sequences (Table 1; Fig. 5). In a survey of *Salmonella* spp., including strains of *S. agona*, *S. choleraesuis*, *S. enteritidis* 27655-3b, *S. hadar* F9-1, *S. heidelberg* ATCC 8326, *S. infantis* S41-16, *S. newport*, *S. paratyphi* A ATCC 11511, *S. typhi*, and *S. typhimurium* SU453, the PCR primers TAF3 and TAF4 directed *Salmonella*-specific amplification of an *agfA* DNA fragment of approximately 260 bp at an annealing temperature of 62°C (Fig. 6A). The fragment size was consistent with the predicted size of 261 bp on the basis of the size of the *agfA* sequence. The *agfA* PCR fragment was consistently generated in lesser amounts from *S. typhi* than from any of the other *Salmonella* serovars examined (Fig. 6A). No PCR-amplified DNA fragments were generated from strains of *E. coli*, *S. sonnei*, *C. freundii*, *E. aerogenes*, *E. caratovora*, *H. alvei*, *K. pneumoniae*, *P. vulgaris*, *S. marcescens*, or a *Providencia* sp. at annealing temperatures above 55°C. At an annealing temperature of 68°C, the PCR primers TAF5 and TAF6 directed amplification of an *agfA* fragment of approximately 92 bp from all *Salmonella* serovars listed above which comigrated with the 92-bp fragment amplified from *S. enteritidis* (Fig. 6B). An approximately 92-bp DNA fragment was amplified efficiently from *E. coli* NG7c but was inefficiently amplified from *E. coli* HB101, C600, Gambia 3, and Viet G as well as from a strain of *S. sonnei* (Fig. 6B). Under the same conditions, no PCR products were generated from *E. coli* clinical isolates 438Hf, B41M, or Vietnam I-1 or from *C. freundii* 8090, *E. aerogenes*, *E. caratovora*, *H. alvei*, *K. pneumoniae* 13883, *P. vulgaris*, *S. marcescens*, or a *Providencia* sp. At an annealing temperature of 55°C, this set of primers promoted amplification of a 92-bp fragment from *Salmonella* spp., *E. coli* HB101, C600, 438Hf, B41M, NG7c, Gambia 3, Vietnam I-1, and Viet G, and *S. sonnei* (Fig. 6C). No PCR fragments were generated from *C. freundii*, *E. aerogenes*, *E. caratovora*, *H. alvei*, *K. pneumoniae* 13883, *P. vulgaris*, a *Providencia* sp., or *S. marcescens* at the lower annealing temperature.

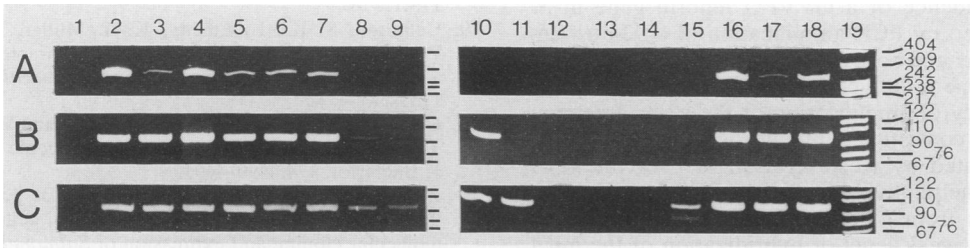

FIG. 6. PCR amplification of DNA fragments from various members of the family *Enterobacteriaceae* by using oligonucleotide primers designed from the *agfA* gene of *S. enteritidis* 27655-3b. (A) DNA products of PCR assays directed by the primers TAF3 (dTCCGGCCCG-GACTCAACG) and TAF4 (dCAGCGCGGCGTTATTACCG) at an annealing temperature of 68°C. DNA fragments of approximately 260 bp were isolated by electrophoresis in 15% polyacrylamide gels (37). (B and C) PCR amplification of DNA fragments of approximately 92 bp at annealing temperatures of 68 and 55°C, respectively, by using the primers TAF5 (dGGCGGCGGCAATA[G/A]TTCCGGCGGC) and TAF6 (dCGGGCATCG[C/G]TTTGCAGAGCAAGCGC). Lanes: 1, no DNA control; 2, *S. enteritidis* 27655-3b; 3, *S. typhimurium* SU453; 4, *S. infantis* S41-16; 5, *S. hadar* F9-1; 6, *S. agona*; 7, *S. newport*; 8, *E. coli* C600; 9, *E. coli* HB101; 10, *E. coli* NG7c; 11, *E. coli* Gambia 3; 12, *E. coli* Vietnam I/1; 13, *E. aerogenes*; 14, *C. freundii* 8090; 15, *S. sonnei*; 16, *S. choleraesuis*; 17, *S. typhi*; 18, *S. paratyphi* A subsp. *durazzo* ATCC 11511; 19, DNA fragment size markers prepared by *Msp*I digestion of pBR322.

DISCUSSION

The results of the survey of 95 serovars from 24 serogroups of *Salmonella* demonstrate that *agfA*, the structural gene for GVVPQ fimbriae, is widely distributed and is probably common among serovars of this genus. The survey included the serovars most frequently responsible for gastroenteritis worldwide, *S. enteritidis* and *S. typhimurium*; other prominent causes of salmonellosis in North America and Europe, *S. heidelberg*, *S. infantis*, *S. hadar*, *S. newport*, and *S. agona*; and the serovars consistently responsible for enteric fever and septicemia, *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, and *S. choleraesuis*. Of 604 *Salmonella* isolates tested, only a single isolate of *S. saint-paul* was not detected by HGMF colony hybridization to the *agfA* probe, although 6 other *S. saint-paul* isolates hybridized strongly. This single negative result may have been due to the loss of *agfA* analogous to the loss of the K88 fimbrin gene by enterotoxigenic *E. coli* (24) or the spontaneous deletion of genes encoding P fimbriae and P-related fimbriae by extraintestinal *E. coli* (17).

The utilization of an *agfA* nucleotide probe in combination with automatable HGMF technology (40) provides a valuable new diagnostic format for *Salmonella* spp. In comparison with other *Salmonella* DNA probes (5, 6, 14, 16, 34, 43, 48), the *agfA* probe demonstrated excellent inclusivity and exclusivity properties representative of the high degree of conservation of *agfA* among *Salmonella* serovars. Under appropriately stringent conditions, we detected no hybridization of a 333-bp region of *agfA* to the vast majority (81%) of non-*Salmonella* members of the family *Enterobacteriaceae* represented on the HGMF panels, including almost all strains of *E. coli*, *Citrobacter*, and *Enterobacter* and all strains of *Shigella*, *Serratia*, and *Yersinia*. The relatively negligible levels of hybridization to 9% of *E. coli* strains, two *Citrobacter* strains, and a single isolate of *E. cloacae* in no way interfered with recognition of strongly hybridizing *Salmonella* DNA. Therefore, this diagnostic format is very competitive with existing *Salmonella* DNA hybridization assays for use in the prevention of food-borne or waterborne infection. Although fimbrial gene probes have been developed for strain- or serovar-specific diagnosis for certain strains of enterotoxigenic *E. coli* (24), some pyelonephritic *E. coli* isolates (39), and *Pseudomonas aeruginosa* isolates obtained from patients with cystic fibrosis (42), application of the *agfA* probe is the first instance in which a fimbrial gene probe has been used as a genus-specific diagnostic tool.

The *agfA*-based PCR assay is potentially sensitive to the presence of a single cell and might most effectively serve as a highly sensitive, inexpensive, and rapid (≤24 h) means of screening for *Salmonella* spp. since the cells present in aquatic samples and processed foods are typically present in low numbers and are often viable but unculturable (2, 36). Although the PCR assay is not quantitative, there is zero tolerance for *Salmonella* isolates in foodstuffs. However, there are serious concerns for the potential interference of food which could obviate application of the PCR assay described here.

The development of a diagnostic test based on anti-AgfA polyclonal antibodies is improbable given the lack of the requisite exclusivity and, as a result of variable phenotypic expression of GVVPQ fimbriae in *Salmonella* isolates, the inclusivity required of a diagnostic test. Whether GVVPQ fimbriae occur in all *Salmonella* strains during infection or whether levels of production correlate with virulence is not known. The relatively low proficiency of GVVPQ fimbriae production by many *Salmonella* strains obtained from existing culture collections reflected biased sampling and strain deposition resulting from the strongly autoaggregative nature of high-level GVVPQ fimbria producers. Indeed, abundant production of GVVPQ fimbriae may partially account for the frequent reporting of untypeable *Salmonella* isolates since autoaggregation could mask the serotype profile when using standard agglutination tests to identify flagellar or lipopolysaccharide O antigens.

Congo red binding, a property associated with enteroinvasive *E. coli* and virulent strains of *Yersinia* and *Shigella* (4, 33, 44, 47) and potentially indicative of porphyrin-binding ability (20), is also associated with GVVPQ fimbria production (8, 10). However, the Congo red plate assay reliably detected only abundant producers of GVVPQ fimbriae within 24 h. Other evidence indicates that GVVPQ fimbriae are produced by members of several genera of the family *Enterobacteriaceae*. A 19,000-MW protein that is cross-reactive with AgfA was purified from *E. aerogenes* by using the unique, rigorous protocol developed for the isolation of GVVPQ fimbriae (10). Weak hybridization of the *agfA* probe to isolates of *S. sonnei*, *E. cloacae*, *C. freundii*, and a *Citrobacter* sp. were indicative of related GVVPQ fimbrin

genes. Further evidence of a GVVPQ fimbrin gene in *S. sonnei* was obtained by PCR amplification of a 92-bp fragment from *S. sonnei* and both *Salmonella* and *E. coli* strains by using DNA primers derived from *agfA* of *S. enteritidis*. The lower efficiency of amplification of the 92-bp fragment from *S. sonnei* is consistent with the degree of sequence dissimilarity indicated by weak hybridization of the *agfA* probe. Similarly, the relatively inefficient PCR amplification of *csgA* fragments from *E. coli* by using primers derived from *agfA* and the weak level of hybridization of the *agfA* DNA probe to DNAs from several *E. coli* strains demonstrate that the *csgA* and *agfA* sequences are less conserved than the respective N-terminal protein sequences (Fig. 4) (3, 10). The development of a PCR assay for the detection of related GVVPQ fimbrin genes provides a reliable, rapid technique for further screening of a broad cross-section of members of the family *Enterobacteriaceae* for the potential to produce GVVPQ fimbriae. In turn, this work will facilitate efforts to understand the structure and antigenic relationships of GVVPQ fimbriae and the potential roles of members of this class of thin, aggregative fimbriae in enterobacterial pathogenesis.

ACKNOWLEDGMENTS

We thank Trevor J. Trust (University of Victoria) for several stimulating discussions.

This work was supported by operating grants provided to W.W.K. by the British Columbia Health Foundation and the NRC/NCE Canadian Bacterial Diseases Network and by an operating grant provided to J.L.D. by the Science Council of British Columbia. J.L.D. was supported by an NSERC industrial fellowship. S.K.C. was supported by an NSERC postdoctoral fellowship.

We are very grateful to Janet MacKenzie (Division of Microbial Hazards, Health Protection Branch) for the preparation of HGMF panels of bacterial DNA samples and to Sandy Kielland (Protein Microsequencing Laboratory, University of Victoria) for N-terminal protein sequence analyses.

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