

Cleavage of *Shigella* Surface Protein VirG Occurs at a Specific Site, but the Secretion Is Not Essential for Intracellular Spreading

ICHIRO FUKUDA,¹ TOSHIHIKO SUZUKI,¹ HIROSHI MUNAKATA,² NORIO HAYASHI,²
EISAKU KATAYAMA,³ MASANOSUKE YOSHIKAWA,¹ AND CHIIHIRO SASAKAWA^{1*}

Departments of Bacteriology¹ and Fine Morphology,³ Institute of Medical Science, University of Tokyo,
4-6-1 Shirokanedai, Minato-ku, Tokyo 108, and Department of Biochemistry, School of Medicine,
Tohoku University, Aoba-ku, Sendai 980,² Japan

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The large plasmid-encoded outer membrane protein VirG (IcsA) of *Shigella flexneri* is essential for bacterial spreading by eliciting polar deposition of filamentous actin (F-actin) in the cytoplasm of epithelial cells. Recent studies have indicated that VirG is located at one pole on the surface of the bacterium and secreted into the culture supernatant and that in host cells it is localized along the length of the F-actin tail. The roles of these VirG phenotypes in bacterial spreading still remain to be elucidated. In this study, we examined the surface-exposed portion of the VirG protein by limited trypsin digestion of *S. flexneri* YSH6000 and determined the sites for VirG processing during secretion into the culture supernatant. Our results indicated that the 85-kDa amino-terminal portion of VirG is located on the external side of the outer membrane, while the 37-kDa carboxy-terminal portion is embedded in it. The VirG cleavage required for release of the 85-kDa protein into the culture supernatant occurred at the Arg-Arg bond at positions 758 to 759. VirG-specific cleavage was observed in *Shigella* species and enteroinvasive *Escherichia coli*, which requires an as yet unidentified protease activity governed by the *virB* gene on the large plasmid. To investigate whether the VirG-specific cleavage occurring in extracellular and intracellular bacteria is essential for VirG function in bacterial spreading, the Arg-Arg cleavage site was modified to an Arg-Asp or Asp-Asp bond. The *virG* mutants thus constructed were capable of unipolar deposition of VirG on the bacterial surface but were unable to cleave VirG under in vitro or in vivo conditions. However, these mutants were still capable of eliciting aggregation of F-actin at one pole, spreading into adjacent cells, and giving rise to a positive Serény test. Therefore, the ability to cleave and secrete VirG in *Shigella* species is not a prerequisite for intracellular spreading.

The ability of *Shigella* species to spread within and between epithelial cells is a prerequisite for causing dysentery. Indeed, bacterial mutants lacking these functions are avirulent and are unable to provoke keratoconjunctivitis in the eyes of guinea pigs in the Serény test (30). The ability of the bacteria to spread in the cytoplasm and then to move into adjacent epithelial cells is known as intra/intercellular spreading and is encoded by the *virG* gene on the large plasmid (6, 16, 17). In this step, the intracellular bacteria elicit actin polymerization (6).

The *virG* gene encodes a surface-exposed 116-kDa outer membrane protein, VirG (IcsA) (6, 16, 17), which was initially identified as one of the plasmid-encoded antigens specifically recognized by convalescent-phase human or monkey sera (22). The VirG protein has been shown to be responsible for the localized deposition of filamentous actin (F-actin) trailing from one pole of invading bacterial cells and extending in a filament through the host epithelial cytoplasm (6, 12, 23, 24). This observed F-actin tail appearing from the infected bacteria apparently provides a motive force, since the accumulation of F-actin fibrils results in the formation of extracellular protrusions through which bacteria penetrate adjacent cells (12, 24). Although the molecular mechanisms underlying actin polymerization by VirG are still largely obscure, it is clear that F-actin tail formation at one pole of the bacterial cell is closely linked to the unipolar deposition of surface VirG protein (10). Inter-

estingly, the motive system used by *Listeria monocytogenes* has been shown to be very similar to that of *Shigella* species. For example, the F-actin tail formed at one pole of the invading bacterium consists of a tight bundle of short filaments of polymerized actin, and the bacteria pass from one cell to another through protrusions extending from the infected-cell surface (7, 25, 32–36). Furthermore, *L. monocytogenes* surface protein ActA is deposited at one pole of the bacterial surface and secreted into the culture supernatant, although the protein is not found within the F-actin tail (9, 14, 15, 21). The *Shigella* VirG protein is secreted into the culture supernatant (10, 40) and is associated with the F-actin tail of the intracellular bacteria (10). Taken together, these studies have indicated that the specific cleavage of VirG on the bacterial surface observed in vitro and the cleavage that probably occurs in intracellular bacteria are prerequisites for F-actin aggregation at one pole of the invading bacterium (10), although no direct evidence is available.

In this context, we examined the surface-exposed VirG portion by limited trypsin digestion and determined the cleavage site during secretion into the culture supernatant. On the basis of these results, we successively modified the amino acid bonds at the cleavage sites required for VirG secretion and tested for the capability of these strains to secrete VirG into the culture supernatant. The *virG* mutants constructed produced VirG which showed unipolar expression on the bacterial surface but failed to cleave the VirG protein under both in vitro and in vivo conditions. Nevertheless, *S. flexneri* strains containing the mutagenized *virG* allele were able to elicit F-actin aggregation

* Corresponding author. Fax: 81-3-5449-5405.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
<i>S. flexneri</i>		
YSH6000	Virulent strain	28
YSH6200	Avirulent strain of YSH6000 cured of the 230-kb plasmid pMYSH6000	29
M94	YSH6000 carrying pMYSH6000 <i>virG</i> ::Tn5	17
M223	YSH6000 carrying pMYSH6000 <i>virB</i> ::Tn5	1
N777	YSH6000 carrying pMYSH6000 <i>ipaB</i> ::Tn5	26
S324	YSH6000 carrying pMYSH6000 <i>spa47</i> ::Tn5	29
S325	YSH6000 carrying pMYSH6000 <i>mxiA</i> ::Tn5	26
CS2522-1	YSH6000 carrying pMYSH6000 <i>virG</i> ::pGP704Km- <i>virG</i>	This study
CS2522-2	YSH6000 carrying pMYSH6000 <i>virG</i> ::pGP704Km- <i>virG202</i>	This study
<i>S. dysenteriae</i> 9		Our collection
<i>S. flexneri</i> 6		Our collection
<i>S. boydii</i> 4		Our collection
<i>S. sonnei</i> form I		Our collection
EIEC O124		Our collection
<i>E. coli</i> K-12		
W3110		Our collection
MC1061	<i>hsd mcr araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi</i>	M. Casadaban
KS272	<i>ΔlacX74 galE thi rpsL ΔphoA</i>	G. Georgiou
SF100	Same as KS272 but <i>ompT</i>	G. Georgiou
UT5600	<i>ΔompT</i>	J. J. Dunn
Plasmids		
pTB101	pBR322Tp, a Tm ^r derivative of pBR322, containing a <i>tac</i> promoter	37
pBN1	pBR322Tp containing a 2.4-kb <i>virB</i> segment	1
pMYSH6601	pBR322Tp containing a 6.1-kb <i>virG</i> segment	17
pD10	An <i>EcoRV</i> deletion derivative of pMYSH6601 containing <i>virG</i>	16
pTSG202	Same as pD10 but containing the <i>virG202</i>	This study
pTSG203	Same as pD10 but containing the <i>virG203</i>	This study
pGP704Km	<i>ori</i> R6K, <i>mob</i> RP4, Km ^r derivative of pGP704	19
pGP704Km- <i>virG</i>	pGP704Km, containing the 3.3-kb <i>XbaI</i> - <i>PvuII</i> fragment of pD10	This study
pGP704Km- <i>virG202</i>	Same as pGP704Km- <i>virG</i> but containing the <i>XbaI</i> - <i>PvuII</i> fragment of pSTG202	This study

as well as to spread from one cell to another. Visualization of intracellular bacteria containing either the wild-type or the mutagenized *virG* allele by double-fluorescence microscopy with fluorescein isothiocyanate-VirG-specific antibody and rhodamine-phalloidin indicated that VirG was restricted to the bacterial surface. The results indicate that VirG cleavage near the surface of bacteria grown in vitro and in vivo is not directly involved in spreading within the cytoplasm or into adjacent epithelial cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. All the bacteria were grown in either LN broth (28), Mueller-Hinton broth (Difco), or brain heart infusion broth (Difco) at 37°C. The Mueller-Hinton broth was used only to grow bacteria encoding a trimethoprim resistance phenotype.

Detection of VirG and its cleaved forms. Protein extracts from culture supernatants and from whole bacterial cells were prepared as follows. Bacterial culture grown up to mid-log phase (1 ml) was centrifuged, and the resulting supernatant was passed through a 0.45-μm-pore-size filter (Amersham). Trichloroacetic acid was added to 0.6 ml of the supernatant to a final concentration of 6%, and the precipitated proteins were dissolved in 60 μl of sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl [pH 6.8]) and boiled for 5 min. The bacterial pellet from the centrifugation step was suspended in 1 ml of cold water, and one-third volume of 24% trichloroacetic acid was added. After the precipitated proteins were centrifuged, the pellet was dissolved in 100 μl of SDS sample buffer and boiled for 5 min. Then, 10-μl samples of each whole bacterial protein extract and the supernatant protein extract were loaded on an SDS-7.5% polyacrylamide gel. The electrophoresed protein bands were transferred to a nitrocellulose membrane (Schleicher & Schuell) by methods described by Towbin et al. (39). VirG and its processed form were detected by immunoblots with the rabbit VirG-specific antibodies VRG-120 and VRG-C (20). Immunostaining was done with horseradish

peroxidase-conjugated protein A (Boehringer Mannheim) and developed with 4-chloronaphthol plus hydrogen peroxide in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.4], 150 mM NaCl). When necessary, a hypersensitive chemiluminescence-based immunoassay (ECL; Amersham) was used.

Trypsin treatment of whole bacterial cells. A 1-ml sample of bacterial culture grown to the mid-log phase was washed twice with TBS and suspended in 1 ml of TBS. Then 250 μl of this cell suspension was incubated with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Washington Biochemical Co.) to a final concentration of 100 μg/ml at 4°C for 1 h. When necessary, the cells were osmotically shocked by being suspended in cold 20% sucrose-5 mM EDTA in TBS prior to the trypsin treatment (13). The trypsin digestion was terminated by adding soybean trypsin inhibitor (Sigma) to a final concentration of 200 μg/ml. After being washed once with TBS, the bacterial pellet obtained was suspended in 1 ml of cold water, 1/3 volume of 24% trichloroacetic acid was added, and the mixture was incubated at 4°C for 30 min. The protein precipitates were dissolved in 50 μl of SDS sample buffer and boiled for 5 min. A 20-μl aliquot was loaded on an SDS-7.5% polyacrylamide gel, and VirG and its processed form were analyzed by the immunoblotting method described above.

Preparation of the 37-kDa VirG portion. Bacterial culture of M94 carrying pD10 (500 ml of culture) grown to the mid-log phase was centrifuged, and the bacterial pellet was washed twice with TBS, suspended in 20 ml of 30 mM Tris-HCl (pH 7.5), and disrupted in a French press at 1,500 kg/cm² twice. After cell debris was eliminated, the membrane fraction was collected by centrifugation at 100,000 × g for 2 h at 4°C. This pellet was suspended in solubilization buffer containing 10 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.1% deoxycholic acid, 0.1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The insoluble material was removed by centrifugation at 100,000 × g for 45 min at 4°C. The clear supernatant was passed through a small column of Sepharose 4B (Pharmacia) which contained covalently coupled VRG-C antibody. The column was successively washed with 50 ml of the following buffers: (i) the solubilization buffer described above; (ii) 10 mM Tris-HCl (pH 7.5)-0.1% Triton X-100-1 mM EDTA-1 mM PMSF; and (iii) 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.5)-150 mM NaCl-1 mM EDTA-1 mM PMSF. The bound proteins were eluted from the affinity column with pH 2.5 elution buffer (10 mM glycine-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). The elute was immediately neutralized by the addition of 1 M Tris, and the proteins thus eluted were applied to an SDS-7.5%

polyacrylamide gel. The electrophoresed protein bands were transferred to a polyvinylidene difluoride membrane with TransBlot transfer medium (Bio-Rad) by previously described methods (18) and stained with Coomassie brilliant blue, and the appropriate proteins were excised. The amino-terminal sequences were determined by Edman degradation with an Applied Biosystems model 470A protein sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer.

Site-directed mutagenesis. The synthetic oligonucleotides synthesized were 5'-ATGAGTAGTCGCGACGCTAGTTCACAG-3' (oligonucleotide 202) and 5'-ATGAGTAGTGACGACGCTAGTTCACAG-3' (oligonucleotide 203). The two 27-mers encompassed residues 755 to 763 of the VirG sequence (16). Oligonucleotide 202 coded for an aspartic acid (Asp) to replace arginine (Arg) at residue 759, while oligonucleotide 203 coded for two aspartic acids (Asp) replacing both arginines (Arg) at residues 758 and 759. Site-directed mutagenesis was performed with the U.S.E. mutagenesis kit (Pharmacia). Mutagenesis was performed on the 1,386-bp *HindIII-EcoRV* fragment of pD10, which contains the putative VirG cleavage site required for secretion into the culture supernatant. This fragment was cloned into pBR322 digested with *HindIII* and *EcoRV*. The single-stranded DNA was annealed with the phosphorylated mutagenesis primer and U.S.E. selection primer, which prevented linearization of the double-stranded DNA upon *PvuII* digestion. The second strand was synthesized by incubation with a reaction mixture containing T4 DNA polymerase, T4 DNA ligase, and gene 32 protein (Pharmacia). The resultant double-stranded circular DNA was digested with *PvuII* and transformed into a *mutS* *Escherichia coli* strain. The parental strain, forming linear DNA, was not efficiently transformed. The plasmid DNAs extracted from a pool of transformants were digested with *PvuII* again and transformed into the same strain. After recovering plasmid DNA from several transformants, we confirmed the mutagenized DNA sequences by nucleotide sequencing and replaced the 1,386-bp *HindIII-EcoRV* fragment, containing mutagenized sequences, with the parental fragment on pD10. The resulting pD10 derivatives containing the mutagenized DNA sequence corresponding to oligonucleotides 202 and 203 were called pTSG202 (*virG202*) and pTSG203 (*virG203*), respectively.

Replacement of *virG* on the large plasmid with *virG202*. To mutate the *virG* gene on the large plasmids of *Shigella* and enteroinvasive *E. coli* (EIEC) strains, an internal 3.3-kb *XbaI-PvuII* fragment from pTSG202 was cloned into pGP704Km, a derivative of pGP704 (19) in which the ampicillin resistance gene was replaced with a kanamycin resistance gene (Km) (Pharmacia). The resultant plasmids, pGP704Km-*virG202* and its parental control pGP704Km-*virG*, were introduced into the bacteria, and Km^r transformants were selected. The integration of the plasmid was confirmed by restriction analysis with *SalI*.

Immunofluorescence experiments. Bacterial infection was carried out as previously described with monkey kidney 2 (MK2) cells (27). The infected MK2 cells were fixed in 3% formaldehyde in Dulbecco's phosphate-buffered saline (PBS) at room temperature for 30 min, and the aldehyde residues were inactivated by incubation with 50 mM ammonium chloride for 10 min. After permeabilization by treatment with 0.2% Triton X-100 in PBS for 10 min, the cells were incubated with 8% bovine serum albumin at room temperature for 30 min. For incubation with antibody or rhodamine-phalloidin, coverslips containing cells were inverted on top of a 100- μ l drop of the reagents. Bacterial particles were detected with a rabbit anti-lipopolysaccharide primary antibody (Denka-Seiken) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as a secondary antibody. The F-actin was stained with rhodamine-phalloidin (Wako Pure Chem) as previously described (20). Detection of VirG expression on the surface of intracellular bacteria in MK2 cells was performed by the same method but with VRG-120. Specimens were observed with a Zeiss fluorescence microscope.

Fp and Serény tests. The focus-plaque-forming test (Fp test) and Serény test were performed as previously described (27).

Detection of VirG cleavage in vivo. M94 carrying pD10 and M94 carrying pTSG202 were used to infect a semiconfluent monolayer of MK2 cells in a 25-cm² tissue culture flask (Corning). The MK2 cells were scraped off with a rubber policeman at 2 h postinfection and washed with PBS five times. The cells were dissolved in 200 μ l of 0.5% Triton X-100, and a 10 μ l aliquot of this was plated on LN agar to determine the intracellular bacterial number. Proteins contained in the remaining portion were precipitated by adding trichloroacetic acid to a final concentration of 6%, centrifuged, dissolved in 50 μ l of SDS sample buffer, and then boiled for 5 min. Sample buffer containing approximately 5×10^4 bacteria was run on an SDS-7.5% polyacrylamide gel and analyzed for VirG proteins by using immunoblots with VRG-120 or VRG-C antibody.

RESULTS

Topology of VirG protein in the outer membrane of *S. flexneri*. To determine the surface-exposed VirG domain, whole cells of YSH6000 treated by limited trypsin digestion were analyzed by immunoblotting with VRG-120 and VRG-C antibodies. These antibodies were raised against synthetic VirG peptides of amino acids 120 through 146 from the amino terminus of VirG and the last 20 amino acids at the carboxy terminus, respectively (Fig. 1). Bacteria grown to mid-log

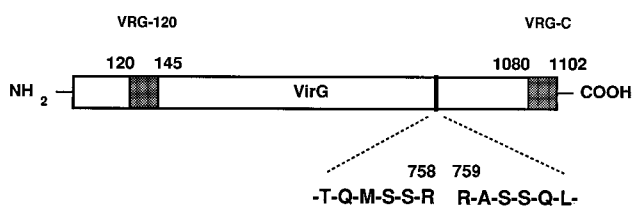


FIG. 1. Schematic representation of the structure of VirG polypeptide. The box indicates the 116-kDa VirG protein (16), in which the vertical bar indicates the cleavage site near the cell surface (see the text). The cross-hatched portions near the amino and carboxy termini show the oligopeptides synthesized for the two antibodies VRG-120 and VRG-C. The letters under the bar indicate the amino acid sequences containing the VirG cleavage sites near the bacterial surface. The amino-terminal cleavage site after the Ala residue at position 52 is not shown (10).

phase with or without osmotic shock were treated with a limited amount of trypsin, and the protected VirG moiety was analyzed by whole-cell immunoblotting. The results of the immunoblots with VRG-120 showed that the 116-kDa VirG protein completely disappeared on trypsin digestion (Fig. 2A), while the immunoblots with VRG-C showed an increase in the level of 37-kDa protein (Fig. 2B). The 37-kDa protein appeared regardless of osmotic shock (Fig. 2B), suggesting that the protein is embedded in the outer membrane.

Cleavage and secretion of VirG from bacteria grown in vitro. It has been shown that a 92- to 98-kDa VirG-derived protein, called VirG*, appears upon incubation of *S. flexneri* in a hypotonic medium (40) or in the culture supernatant of bacteria grown to late log phase (10). These studies indicate that the VirG protein undergoes processing for secretion into the culture medium. Hence, we undertook an investigation of the relationship between the surface-exposed VirG portion revealed by the limited trypsin digestion and the VirG fragment released into the culture supernatant. To do this, supernatant proteins from bacterial cultures at various stages of growth and whole-cell protein extracts were analyzed by immunoblots with VRG-120 and VRG-C antibodies. The protein extracts from culture supernatants yielded an 85-kDa VirG protein, which was recognized in immunoblots with VRG-120 (Fig. 3B) but not with VRG-C (Fig. 3D), while 116- and 37-kDa VirG proteins were recognized in the whole-cell lysate immunoblots with VRG-C (Fig. 3A and C). This suggests that the 85-kDa protein is derived from the amino-terminal portion of VirG.

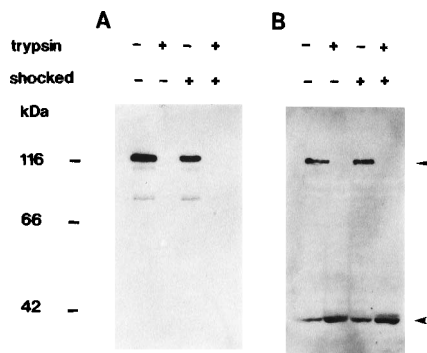


FIG. 2. Immunoblot analysis of VirG protein in YSH6000 in the presence or absence of trypsin treatment. Shocked cells were prepared by treatment with 20% sucrose and 5 mM EDTA prior to limited trypsin digestion. Immunoblots with VRG-120 (A) and VRG-C (B) are shown. The large and small arrowheads indicate the 116-kDa VirG protein and the 37-kDa portion, respectively. The faint bands in panel A under VirG are the 85-kDa amino-terminal VirG portion.

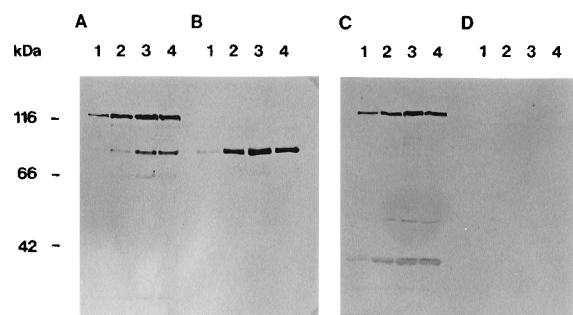


FIG. 3. Secretion of VirG from YSH6000 into the culture supernatants over time. (A and C) Whole-cell protein extracts; (B and D) supernatant protein extracts corresponding to panels A and C, respectively. Panels A and B show immunoblots with VRG-120 antibody, while VRG-C antibody was used in panels C and D. Lane numbers indicate the time (in hours) after inoculation of overnight culture into a 50-fold volume of fresh BHI broth. Bacterial cell lysate (10^7 cells) and 200 μ l of culture supernatant were subjected to SDS-polyacrylamide gel electrophoresis.

To characterize the 37-kDa protein present in the whole-cell lysate, the 37-kDa protein obtained from the outer membrane of M94 (*virG*::Tn5) carrying pD10 (a cloned *virG* gene) was analyzed by Edman degradation for the amino-terminal sequences. It yielded a sequence of Arg-Ala-Ser-Ser-Gln-Leu-Val-Ser, which corresponded to 8 amino acid residues from positions 759 through 766 of the VirG sequence (16). These results, taken together, suggested that release of the 85-kDa portion occurred by cleaving the Arg-Arg bond between positions 758 and 759 in the VirG protein (Fig. 1).

VirG secretion by *Shigella* species is dependent on the presence of the large plasmid of *S. flexneri*. To test if VirG secretion into the culture supernatant was unique to *Shigella* species, we examined *S. dysenteriae* 9, *S. flexneri* 6, *S. boydii* 4, *S. sonnei* form I, and EIEC O124 strains for the production of the 85-kDa VirG portion by immunoblots with VRG-120. All the strains tested were shown to secrete the 85-kDa VirG portion into the culture supernatant (Fig. 4A). We introduced pD10 into various *E. coli* K-12 strains such as W3110, MC1061, and KS272, and all these strains were able to secrete the 85-kDa VirG portion in the supernatant as determined by immunoblot analysis. We have recently found that the disappearance of VirG from the surface of *E. coli* K-12 strains was a result of its cleavage with OmpT, an outer membrane trypsin-like protease (5, 11, 31). Plasmid pD10 was introduced into *E. coli* K-12 strains UT5600 and SF100, which lack the *ompT* region, and VirG secretion was examined. The results showed that these *ompT* mutant strains did not give rise to the 85-kDa protein in the culture supernatant but instead possessed the intact 116-kDa VirG protein, as determined by immunoblots with whole bacterial lysates (Fig. 4B). These results, together with our findings that *Shigella* and EIEC strains lack an *ompT* region (20), indicated that VirG secretion in *Shigella* and EIEC strains required a large-plasmid-encoded function(s). To further investigate this, M94 carrying pD10 and YSH6200 carrying pD10 were examined for the ability to secrete VirG. The results showed that M94 carrying pD10, but not YSH6200 carrying pD10, yielded the 85-kDa protein in the culture supernatant (Fig. 4B). To try to find the relevant genetic region on the large plasmid, various transposon insertion mutants such as M223 (*virB*::Tn5), N777 (*ipaB*::Tn5), S325 (*mxlA*::Tn5), and S334 (*spa47*::Tn5) were examined for the production of the 85-kDa protein in the culture supernatant. We found that all of them, except for M223, produced the 85-kDa protein (Fig. 4B). The VirG secretion abolished in M223 was, however, restored

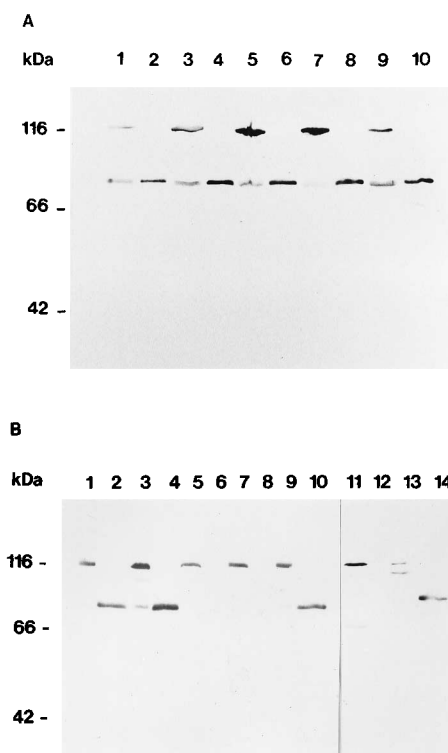


FIG. 4. Secretion of VirG from *Shigella* and EIEC strains. (A) Lanes: 1 and 2, *S. dysenteriae* 9; 3 and 4, *S. flexneri* 6; 5 and 6, *S. boydii* 4; 7 and 8, *S. sonnei* form I; 9 and 10, EIEC O124. The odd and even numbers in each lane show immunoblots of the whole-cell protein extracts and supernatant protein extracts, respectively. (B) Lanes: 1 and 2, YSH6000; 3 and 4, M94 carrying pD10; 5 and 6, YSH6200 carrying pD10; 7 and 8, M223; 9 and 10, M223 carrying pBN1; 11 and 12, UT5600 carrying pD10; 13 and 14, W3110 carrying pD10. The immunoblots with VRG-120 of N777, S325, and S334 strains and that of SF100 carrying pD10 are similar to that of YSH6000 and UT5600 carrying pD10, respectively, while those of MC1061, C600, or KS272 carrying pD10 are similar to that of W3110 carrying pD10 (data not shown).

when the strain was transformed with pBN1 (cloned *virB*), indicating that the *virB* function is involved in expression of the ability to cleave VirG.

Construction of *virG* mutants unable to secrete the 85-kDa fragment into the culture supernatant. Site-directed mutagenesis of the *virG* sequence encoding the Arg-Arg bond in the VirG polypeptide was undertaken to confirm the cleavage site on the VirG sequence responsible for release of the 85-kDa fragment into the culture supernatant and to test whether the in vitro VirG release plays some role in eliciting F-actin aggregation on the intracellular bacteria or in intercellular spreading. Two different mutations, one resulting in the alteration of Arg-758 to Asp (*virG202*) and another changing Arg-Arg at positions 758 and 759 to Asp-Asp (*virG203*), were created with pD10. The resulting two *virG* mutant plasmids, designated pTSG202 and pTSG203, respectively, together with pD10, were introduced into M94 to estimate the ability of transformants to produce the 85-kDa VirG portion in the culture supernatants (Fig. 5B). The results showed that although VirG production and its unipolar deposition on the bacterial surface in vitro were identical in all three strains (data not shown), M94 carrying pTSG202 or pTSG203 failed to release the 85-kDa protein into the culture medium (Fig. 5B, lanes 4 and 5), confirming that the Arg-Arg peptide bond at positions 758 and 759 is the VirG cleavage site.

Cleavage of VirG in vivo. To investigate if the VirG cleavage

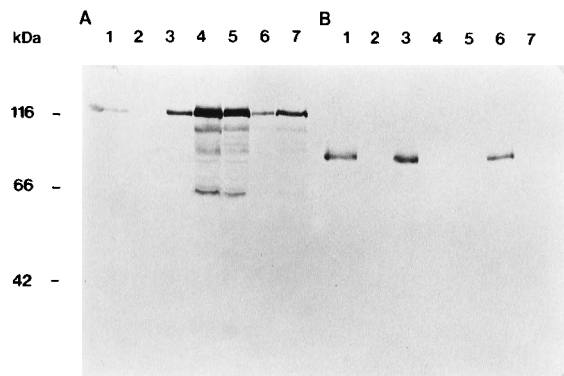


FIG. 5. VirG production and secretion from *virG* mutants. Immunoblots of whole-cell protein extracts (A) and supernatant protein extracts (B) were analyzed with VRG-120 antibody. Lanes: 1, YSH6000; 2, M94; 3, M94 carrying pD10; 4, M94 carrying pTSG202; 5, M94 carrying pTSG203; 6, CS2522-1; 7, CS2522-2. The heavy bands of the 116-kDa VirG protein seen in M94 carrying pTSG202 (lane 4), M94 carrying pTSG203 (lane 5), and CS2522-2 (lane 7) are due to accumulation of VirG.

seen in bacteria grown in vitro also took place in vivo, proteins extracted from epithelial cells infected with M94 carrying pD10 or pTSG202 were analyzed by immunoblotting with the VRG-120 and VRG-C antibodies. The results of VRG-120 immunoblots indicated that epithelial cells infected with M94 carrying pD10 contained both the 116- and 85-kDa VirG proteins, while those infected with M94 carrying pTSG202 showed only the 116-kDa VirG protein (Fig. 6A). Similarly, when the same protein extracts were analyzed with VRG-C, those infected with M94 carrying pD10 contained 116- and 37-kDa VirG proteins while those with M94 carrying pTSG202 contained only the 116-kDa VirG protein (Fig. 6B, lane 5). These results thus demonstrated that VirG protein expressed in intracellular bacteria was also subject to similar processing to that observed in vitro.

VirG cleavage is not essential for bacterial spreading in vivo. We examined if *S. flexneri* VirG cleavage in vitro or in vivo is an essential phenotype for intra- and intercellular

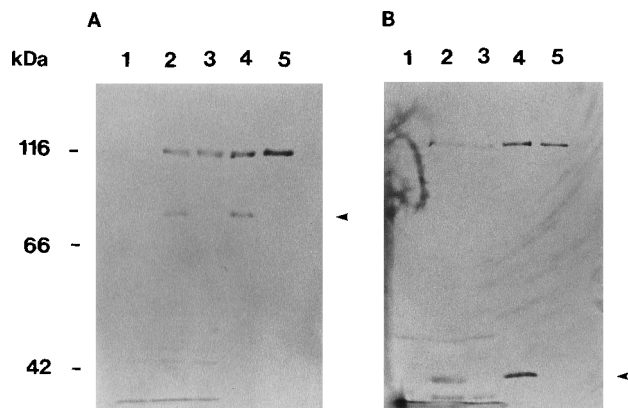


FIG. 6. VirG cleavage in vivo. Immunoblots of whole epithelial cell lysates obtained from MK2 cells infected with M94 carrying pD10 or M94 carrying pTSG202 analyzed with VRG-120 (A) and VRG-C (B). The immunostain was done with the ECL assay kit. Lanes: 1, noninfected MK2 cells; 2, MK2 cells with M94 carrying pD10; 3, MK2 cells with M94 carrying pTSG202; 4 and 5, bacterial lysates from the same number of M94 bacteria carrying pD10 (lane 4) and M94 carrying pTSG202 (lane 5). Arrowheads in panels A and B indicate the 85- and 37-kDa proteins.

spreading. The pGP704Km suicide vector was used to replace the wild-type *virG* allele on the large plasmids of YSH6000, *S. dysenteriae* 9, *S. flexneri* 6, *S. boydii* 4, *S. sonnei* form I, and EIEC O124 strains with the *virG* mutation *virG202*. The resulting *Shigella* and EIEC strains possessing *virG202* were checked for the inability to secrete VirG into the culture supernatants (data not shown) and tested for the ability to elicit formation of an F-actin tail on the intracellular bacteria. An F-actin tail double-fluorescence labeling technique with fluorescein isothiocyanate-anti-LPS and rhodamine-phalloidin revealed that the formation of F-actin tail at 2 h postinfection on each of the *virG202* mutants and their respective wild-type strains was essentially the same (data not shown). When the intercellular bacteria of the YSH6000 *virG202* mutant (CS2522-2) and the wild type (CS2522-1) were immunostained with rhodamine-phalloidin (Fig. 7A and C) and fluorescein isothiocyanate-VRG-120 (Fig. 7B and D), the bacterial particles were visible with VRG-120 (Fig. 7B and D), in which the unipolar deposition of surface VirG protein observed was always restricted to the surface of bacteria.

The YSH6000 *virG202* mutant was subsequently tested for intercellular spreading by the Fp test. The results showed that the *virG202* mutant formed a similar-sized plaque to that of the wild type at 3 days postinfection (Fig. 8). To further confirm this, the YSH6000 *virG202* mutant and the wild type were tested by the Serény test for the ability to provoke keratoconjunctivitis in the eyes of guinea pigs. The results showed that the *virG202* mutant and the wild type gave rise to a positive reaction, although the mutant caused an earlier reaction. These data, taken together, indicate that the in vitro and in vivo VirG cleavage phenotypes of *Shigella* species are not prerequisites for intercellular spreading.

DISCUSSION

VirG is one of the major virulence proteins of *Shigella* species (6, 16, 17) and is required for eliciting unipolar deposition of F-actin on the surface of intracellular bacteria, enabling movement within the host cytoplasm and spread into adjacent epithelial cells (6). Several studies have indicated that VirG that is localized at one pole of the bacterium is exposed on the external side of the outer membrane (10, 16, 20). Its cleavage results in its release into the culture supernatant (10, 40). However, neither the precise cleavage site of in vitro VirG secretion nor its requirement for intracellular bacterial spreading has been characterized. In this context, we attempted to clarify which portion of VirG is exposed on the external side of the outer membrane and whether the VirG cleavage observed in vitro is a prerequisite for utilization of the host cytoskeleton that promotes movement of bacteria from one cell to another.

Analysis of the VirG portion exposed on the bacterial surface by using limited trypsin digests of whole bacterial cells indicated that an 85-kDa amino-terminal portion of VirG exists on the external side of the outer membrane while a 37-kDa carboxy-terminal portion is bound to the outer membrane. Determination of the amino-terminal sequence of the 37-kDa protein left in the outer membrane after natural VirG cleavage showed that cleavage occurred at the Arg-Arg bond at positions 758 and 759, which corresponded to the preferential trypsin cleavage site. Results of recent studies are in agreement with our observations on VirG cleavage; Venkatesan et al. (40) found that a 92- to 98-kDa VirG form, called VirG*, was secreted when *S. flexneri* M90T was resuspended in water and incubated for 1 h, and Goldberg et al. (10) showed that VirG (IcsA) was secreted as a 95-kDa protein from bacteria grown to late exponential phase. The latter study showed that the

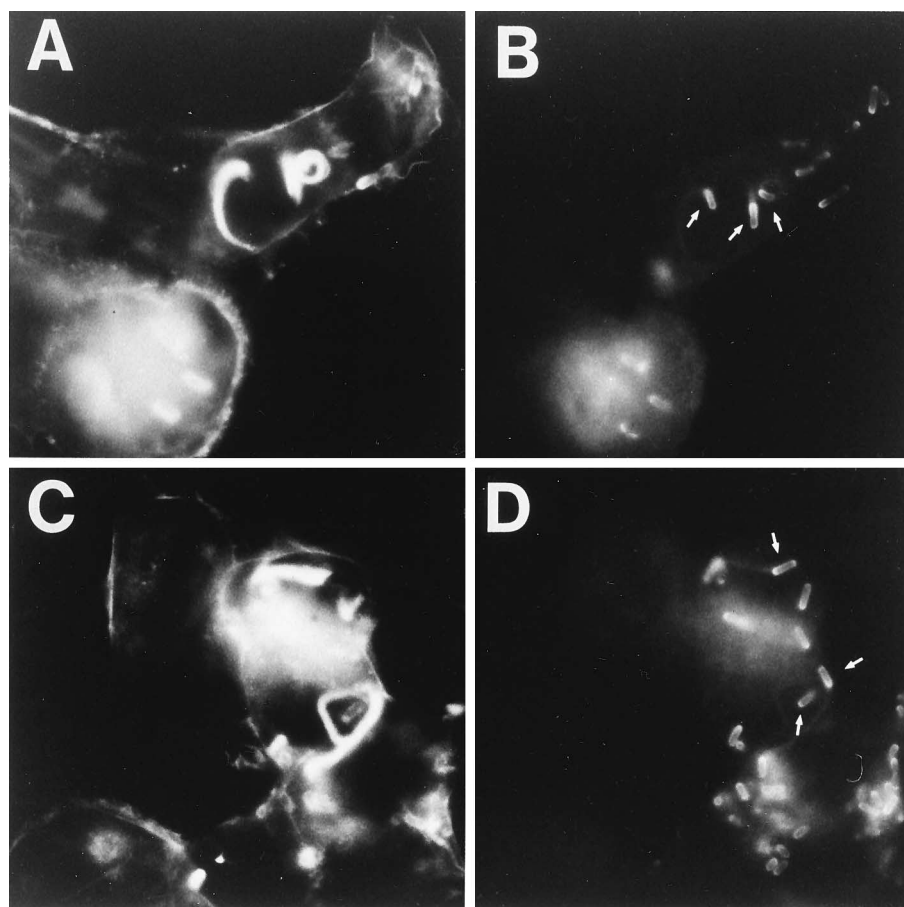


FIG. 7. F-actin aggregation and localization of VirG on the intracellular bacteria. Immunofluorescent staining of infecting bacteria of CS2522-1 (A and B) and CS2522-2 (C and D) was performed with phalloidin (A and C) and VRG-120 (B and D). Bacteria forming the F-actin tail and possessing polar depositions of VirG protein are indicated by arrows (B and D).

secreted IcsA form lacked a 52-residue amino-terminal sequence which was predicted to contain a potential internal signal sequence (10).

The secretion of VirG into the culture medium was not observed in YSH6200 carrying pD10, even though these bacteria could express VirG on their surface (Fig. 4B). However, as indicated by Venkatesan et al. (40) and Goldberg et al. (10), this was not because of the absence of functions encoded by *mxi* and *spa* loci essential for the secretion of the invasion proteins, IpaB, IpaC, and IpaD, into the culture supernatant (2–4, 29, 40). Indeed, various Tn5 insertion mutants with mutations of the *mxi* and *spa* loci, such as S325 (*mxiA*::Tn5) and S343 (*spa47*::Tn5), still allowed the bacteria to secrete VirG in the same manner as the wild-type strain. Interestingly, one of the Tn5 insertion mutants, M223 (*virB*::Tn5), failed to secrete any VirG into the culture supernatant. The introduction of pBN1, a plasmid containing a cloned *virB* gene (1), into M223 restored the presence of the 85-kDa VirG portion in the culture supernatant, indicating that *Shigella* VirG undergoes specific cleavage by an as yet unidentified protease, whose expression is probably governed by *virB*, the large plasmid-encoded positive regulator gene (1, 37, 38).

The observations of *in vitro* VirG secretion from the bacteria into the culture supernatant and the localization of VirG within the F-actin tail at a distance from bacteria in the infected epithelial cells (10) raised the possibility that VirG secretion from bacteria *in vitro* as well as *in vivo* involved the

assembly or stability of F-actin aggregation at one pole of the intracellular bacterium (10). To address this issue and confirm that the Arg-Arg bond at positions 758 and 759 in the VirG sequence was the cleavage site on the bacteria grown *in vitro*, we attempted to modify the Arg-Arg bond to either Arg-Asp or Asp-Asp by site-directed mutagenesis of the *virG* sequence on pD10. The resulting two mutagenized *virG* alleles, *virG202* (Arg-Asp) and *virG203* (Asp-Asp) on pTSG202 and pTSG203, respectively, were introduced into M94 and tested for the ability to secrete the VirG protein. As expected, the mutants failed to secrete VirG into the culture supernatant (Fig. 5), although they were still capable of expressing VirG protein and eliciting F-actin aggregation at one pole under intracellular conditions (Fig. 7).

Immunoblot analysis with VRG-120 antibody on protein extracts from lysates of epithelial cells infected with M94 carrying pD10 revealed the presence of 85- and 116-kDa VirG proteins, but those infected with M94 carrying pTSG202 contained only the 116-kDa protein. Similarly, when infection was carried out with M94 carrying pD10 and analyzed with VRG-C, the cell lysates contained 37- and 116-kDa VirG proteins, but those infected with M94 carrying pTSG202 contained only the 116-kDa VirG protein (Fig. 6). These results indicate that VirG cleavage takes place in intracellular bacteria similarly to that observed in bacteria grown *in vitro*. It remains, however, to be elucidated if the site of VirG cleavage *in vivo* is the same as *in vitro*.

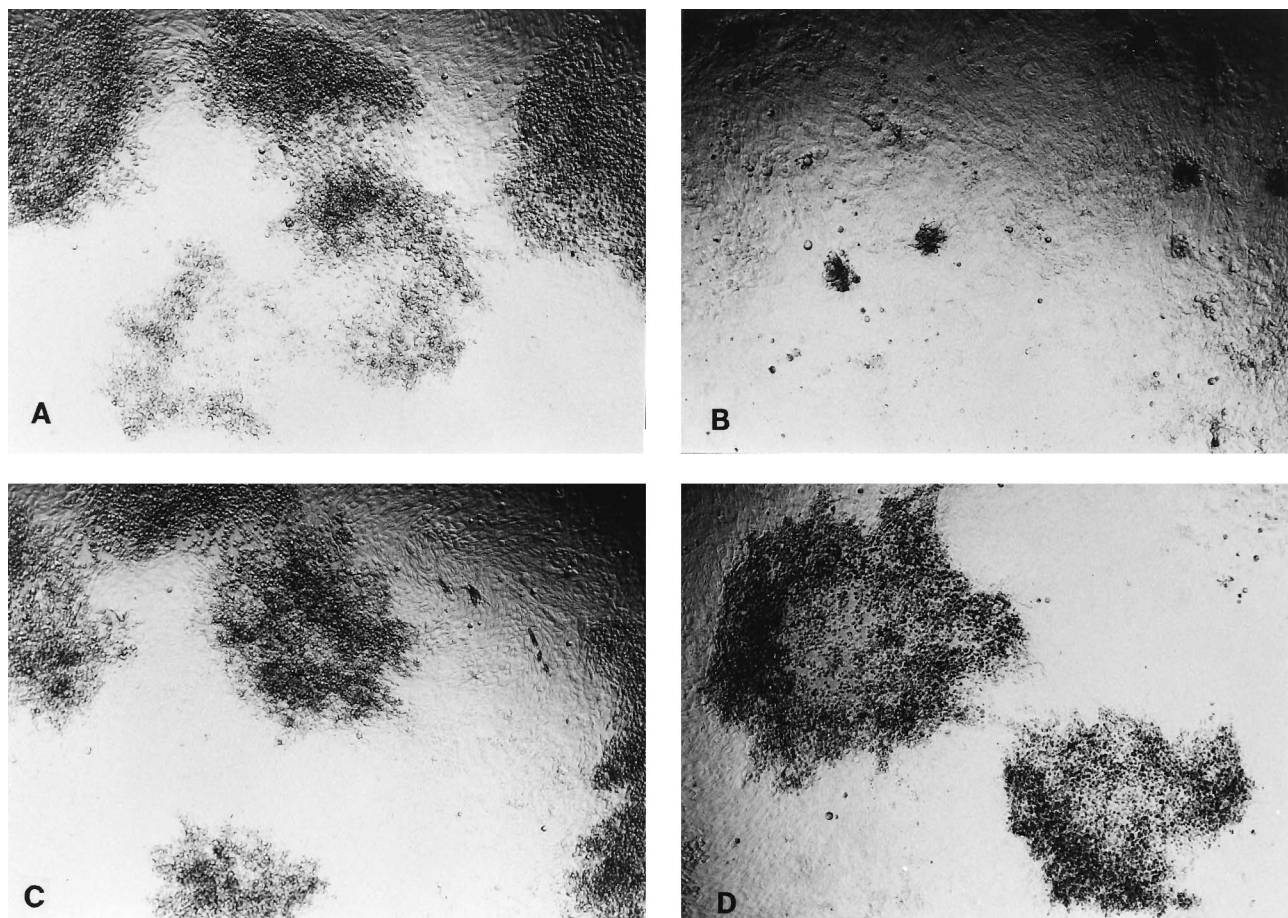


FIG. 8. Phase-contrast micrographs of focus-plaque formation in infected MK2 cells (Fp test) at day 3 postinfection. (A) YSH6000; (B) M94; (C) CS2522-1; (D) CS2522-2.

One of the mutated *virG* alleles, *virG202*, was used to replace the wild-type *virG* allele on the large plasmids of YSH6000 and other *Shigella* and EIEC strains. These strains were examined for their capacity to elicit F-actin tail within cells and to spread from one cell to another. The mutants showed no substantial difference from the wild type in their ability to form F-actin tails as judged by fluorescence labeling of infected epithelial cells with rhodamine-phalloidin, in their ability to deposit VirG at one pole of an intracellular bacterium (Fig. 7), or in their intercellular spreading ability as seen with the Fp test (Fig. 8). It would be worth noting that the distribution of VirG on the intracellular bacteria possessing either the wild-type *virG* allele or the mutagenized *virG* allele seems to be restricted to the bacterial surface but not associated with the F-actin tail (Fig. 7). A notable change in virulence phenotypes was the earlier positive reaction of the YSH6000 *virG202* mutant (CS2522-2) in the Serény test compared with that of the wild type (CS2522-1). Therefore, we can conclude that the VirG cleavage phenomenon of *Shigella* species is not a prerequisite for eliciting aggregation of F-actin on the intracellular bacteria or for eliciting bacterial cell-to-cell spread. In this respect, it would be interesting to compare the VirG phenotype observed in *Shigella* species with the ActA proteins of *L. monocytogenes*, since both proteins are localized at one pole of the bacterium and elicit aggregation of F-actin there (9, 10, 14, 15) and are secreted into the culture super-

natant (10, 21, 40). Nevertheless, ActA and VirG have no significant similarity in their amino acid sequences (14, 16).

It should be mentioned that the Arg-Arg site of in vitro VirG cleavage determined in this study coincided with a sequence encoding a phosphorylation consensus motif, Ser-Ser-Arg-Arg-Ala-Ser-Ser, located at residues 756 through 762 (8). Indeed, d'Hauteville and Sansonetti (8) showed that phosphorylation of IcsA (VirG) by cyclic AMP-dependent protein kinase was abolished when either or both of the Arg residues located at positions 758 and 759 were altered. These mutants expressed a "super Ics" phenotype, characterized by an increased capacity to spread from one cell to another during the first 3 h of HeLa cell infection, as well as an increased rate of spreading in the plaque assay or the Serény test (8). Although we did not quantitatively assay the initial intracellular spreading rate of YSH6000 *virG202* mutant, the earlier reaction in the Serény test would be indicative of the super Ics phenotype as described by d'Hauteville and Sansonetti (8). Because the phosphorylation consensus motif found in the VirG sequence contains the specific cleavage site for VirG secretion, and because alteration of the Arg-Arg bond results in blockage of both phosphorylation (8) and secretion of VirG (this study), the super Ics phenotype might result from the inability to cleave VirG protein.

The *virG* secretion mutants constructed were shown to have the ability to elicit F-actin aggregation and spread from one

cell to another, and the VirG-specific cleavage and secretion phenotype was present in all *Shigella* and EIEC strains tested. Therefore, we assume that the ability of *Shigella* species to cleave and secrete VirG protein is beneficial to the bacteria at some stages of infection not tested in this study. In any case, we must await the results of further studies to determine whether VirG-specific cleavage plays some roles in pathogenicity.

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REFERENCES

- Adler, B., C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa. 1989. A dual transcriptional activation system for the 230 kilobase plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. *Mol. Microbiol.* **3**:627–635.
- Allaoui, A., P. J. Sansonetti, and C. Parsot. 1992. MxiJ, a lipoprotein involved in secretion of *Shigella* Ipa invasin, is homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. *J. Bacteriol.* **174**:7661–7669.
- Allaoui, A., P. J. Sansonetti, and C. Parsot. 1993. MxiD: an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasin. *Mol. Microbiol.* **7**:59–68.
- Andrews, G. P., A. E. Hormockyji, C. Coker, and A. T. Maurelli. 1991. Two novel virulence loci, *mxiA* and *mxiB*, in *Shigella flexneri* 2a facilitate excretion of invasion plasmid antigens. *Infect. Immun.* **59**:1997–2005.
- Baneyx, F., and G. Georgiou. 1990. In vivo degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **172**:491–494.
- Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Conquis-Rondon, and P. J. Sansonetti. 1989. Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. USA* **86**:3867–3871.
- Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick. 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. *Proc. Natl. Acad. Sci. USA* **87**:6068–6072.
- d'Hauteville, H., and P. J. Sansonetti. 1992. Phosphorylation of IcsA by cAMP-dependent protein kinase and its effect on intracellular spread of *Shigella flexneri*. *Mol. Microbiol.* **6**:833–841.
- Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wachter, M. Wuenscher, and T. Chakraborty. 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J.* **11**:1981–1990.
- Goldberg, M. B., O. Barzu, C. Parsot, and P. J. Sansonetti. 1993. Unipolar localization and ATPase activity of IcsA, a *Shigella flexneri* protein involved in intracellular movement. *J. Bacteriol.* **175**:2189–2196.
- Grodborg, J., and J. J. Dunn. 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170**:1245–1253.
- Kadurugamuwa, J. L., M. Rohde, J. Wehland, and K. N. Timmis. 1991. Intercellular spreading of *Shigella flexneri* through a monolayer mediated by membranous protrusions and associated with reorganization of the cytoskeletal protein vinculin. *Infect. Immun.* **59**:3463–3471.
- Klauser, T., J. Pohlner, and T. F. Meyer. 1990. Extracellular transport of cholera toxin B subunit using *Neisseria* IgA protease β -domain: conformation-dependent outer membrane translocation. *EMBO J.* **9**:1991–1999.
- Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart. 1992. *L. monocytogenes*-induced actin assembly required the *actA* gene product, a surface protein. *Cell* **68**:521–531.
- Kocks, C., R. Hellio, P. Gounon, H. Ohayon, and P. Cossart. 1993. Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. *J. Cell Sci.* **105**:699–710.
- Lett, M.-C., C. Sasakawa, N. Okada, T. Sakai, S. Makino, M. Yamada, K. Komatsu, and M. Yoshikawa. 1989. *virG*, a plasmid-encoded virulence gene of *Shigella flexneri*: identification of the *virG* protein and determination of the complete coding sequence. *J. Bacteriol.* **171**:353–359.
- Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa. 1986. A genetic determinant required for continuous reinfection of adjacent cells on a large plasmid in *Shigella flexneri* 2a. *Cell* **46**:551–555.
- Matsudaira, P. 1987. Sequence from picomole quantities of protein electro-
- blotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**:10035–10038.
- Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutation: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
- Nakata, N., T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu, M. Yoshikawa, and C. Sasakawa. 1993. The absence of a surface, OmpT, determines the intercellular spreading ability of *Shigella*: the relationship between the *ompT* and *kcpA* loci. *Mol. Microbiol.* **9**:459–468.
- Niebuhr, K., T. Chakraborty, M. Rohde, T. Gazlig, B. Jansen, P. Kollner, and J. Wehland. 1993. Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin "comets". *Infect. Immun.* **61**:2793–2802.
- Oaks, E. V., T. L. Hale, and S. B. Formal. 1986. Serum immune response to *Shigella* protein antigens in rhesus monkeys and humans infected with *Shigella* spp. *Infect. Immun.* **48**:124–129.
- Pal, T., J. W. Newland, S. B. Formal, and T. L. Hale. 1989. Intracellular spread of *Shigella flexneri* associated with the *kcpA* locus and a 140-kilodalton protein. *Infect. Immun.* **57**:477–486.
- Prevost, M.-C., M. Lesourd, M. Arpin, F. Vernel, J. Mounier, R. Hellio, and P. J. Sansonetti. 1992. Unipolar reorganization of F-actin layer at bacterial division and bundling of actin filaments by plactin correlate with movement of *Shigella flexneri* within HeLa cells. *Infect. Immun.* **60**:4088–4099.
- Sanger, J. M., J. W. Sanger, and F. S. Southwick. 1992. Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. *Infect. Immun.* **60**:3609–3619.
- Sasakawa, C., B. Adler, T. Tobe, N. Okada, S. Nagai, K. Komatsu, and M. Yoshikawa. 1989. Functional organization and nucleotide sequence of virulence region-2 on the large virulence plasmid of *Shigella flexneri* 2a. *Mol. Microbiol.* **3**:1191–1201.
- Sasakawa, C., K. Kamata, T. Sakai, S. Makino, M. Yamada, N. Okada, and M. Yoshikawa. 1988. Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. *J. Bacteriol.* **170**:2480–2484.
- Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa. 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infect. Immun.* **51**:470–475.
- Sasakawa, C., K. Komatsu, T. Tobe, T. Suzuki, and M. Yoshikawa. 1993. Eight genes in region-5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a. *J. Bacteriol.* **175**:2334–2346.
- Serény, B. 1957. Experimental keratoconjunctivitis shigellosa. *Acta Microbiol. Hung.* **4**:367–376.
- Sugimura, K., and T. Nishihara. 1988. Purification, characterization, and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: identity of protease VII and OmpT. *J. Bacteriol.* **170**:5625–5632.
- Theriot, J. A., T. J. Mitchson, L. G. Tilney, and D. A. Portnoy. 1992. The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. *Nature (London)* **375**:257–260.
- Tilney, L., and D. A. Portnoy. 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J. Cell Biol.* **109**:1597–1608.
- Tilney, L. G., P. S. Connelly, and D. A. Portnoy. 1990. Actin filament nucleation by the bacterial pathogen, *Listeria monocytogenes*. *J. Cell Biol.* **111**:2979–2988.
- Tilney, L. G., D. J. DeRosier, and M. S. Tilney. 1992. How *Listeria* exploits host cell actin to form its own cytoskeleton. I. Formation of a tail and how that tail might be involved in movement. *J. Cell Biol.* **118**:71–81.
- Tilney, L. G., D. J. DeRosier, A. Weber, and M. S. Tilney. 1992. How *Listeria* exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. *J. Cell Biol.* **118**:83–93.
- Tobe, T., S. Nagai, N. Okada, B. Adler, M. Yoshikawa, and C. Sasakawa. 1991. Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. *Mol. Microbiol.* **5**:887–893.
- Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa. 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. *J. Bacteriol.* **175**:6142–6149.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
- Venkatesan, M. M., J. M. Buysse, and E. V. Oaks. 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. *J. Bacteriol.* **174**:1990–2001.