Extracellular Transport of VirG Protein in Shigella*

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The ability of Shigella to spread within and between epithelial cells is a prerequisite for causing bacillary dysentery and requires the function encoded by the virG gene on the large plasmid. The outer membrane VirG (IcsA) protein 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 an N-terminal 80-kDa VirG portion is exposed on the bacterial cell surface and released into the external medium, while the following 37-kDa C-terminal portion is embedded in the outer membrane, although little is known about the extracellular transport of the VirG protein. In this study, we attempted to elucidate the export pathway of VirG protein across the outer membrane and found that the C-terminal 37-kDa portion, termed VirG β-core, serves as the self-transporter for the secretion of the preceding 80-kDa portion from the periplasmic side of the outer membrane to the external side. Indeed, foreign polypeptides such as MalE or PhoA covalently linked to the N terminus of VirG β-core were transported to the external side of the outer membrane, and it was further shown that the folding structure of the passenger polypeptide at the periplasmic side of the outer membrane interferes with its translocation. Analysis of the secondary structure of VirG β-core predicted that the critical structural property was a β-barrel channel consisting of amphipathic antiparallel transmembrane β-strands, interspersed by hairpin turns and loops. These results thus strongly suggest that the secretion of VirG protein from Shigella is similar to the export system utilized by the IgA protease of Neisseria.

Shigella are the causative agents of shigellosis, a disease which provokes a severe, bloody diarrhea in humans and primates. Shigellosis is endemic in developing countries and is a major killer of young children. The abilities of Shigella to enter colonic epithelial cells and to subsequently spread within and between the cells is a prerequisite for causing dysentery. The capacity of the bacteria to spread in the cytoplasm and then to move into adjacent epithelial cells is known as intra/intercellular spreading, respectively, and is encoded by the virG gene on the large plasmid (1–3). This gene enables intracellular bacteria to elicit actin polymerization (2).

The outer membrane VirG (IcsA) protein of Shigella, comprising 1102 amino acids, 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 (4, 5). This observed F-actin tail associated with the infecting bacteria apparently provides a motive force, since the accumulation of F-actin filaments results in the formation of extracellular protrusions through which bacteria penetrate adjacent cells (4). Thus, the presentation of a portion of the VirG protein on the cell surface at one pole of the bacterium is crucial for expressing the VirG function within the host cell cytoplasm. Recent studies have shown that an N-terminal 80-kDa VirG portion exposed on the cell surface is cleaved off from the joining C-terminal 37-kDa portion at the Arg<sup>255</sup>–Arg<sup>259</sup> bond, leaving the 37-kDa portion embedded in the outer membrane (6). Sequencing of the N-terminal amino acids of the 80-kDa VirG portion using Edman degradation has indicated that the 52 N-terminal amino acids of the VirG polypeptide preceding the 80-kDa portion are cleaved off and contain 21 amino acids most likely corresponding to an atypical signal sequence (5, 6). These studies thus suggest that the VirG protein undergoes N-terminus processing while crossing the cytoplasmic membranes and that the remaining N-terminal 80-kDa portion is cleaved off after secretion onto the outer membrane, although the mechanisms underlying the export of the 80-kDa portion across the outer membrane remain unknown.

In this context, we attempted to elucidate the export pathway of the VirG protein through the outer membrane. The data presented in this study revealed that the C-terminal 37-kDa portion embedded in the outer membrane is involved in the translocation of the preceding VirG portion from the periplasmic space to the external side of the outer membrane. Analysis of the secondary structure of the amino acids of the 37-kDa portion with the AMPHI secondary structure prediction computer program (7) indicated the presence of a number of amphipathic antiparallel β-sheets and an α-helix capable of forming a channel in the outer membrane. Translational fusion of a foreign protein such as MalE or PhoA protein to the N terminus 37-kDa VirG portion revealed that the 37-kDa portion was capable of transporting the passenger polypeptides from the periplasm to the external side of the outer membrane. Based on these results, we propose a model of the VirG export pathway across the outer membrane.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—YSH6200 (cured of the 230-kilobase plasmid) and M94 (the virG::Tn5 mutant), derived from YSH6000 (Shigella flexneri 2a) (1, 8). UT560 was an ompT mutant of Escherichia coli K-12 (9). pCHR404 was a derivative of pBR322 with a part of the ampicillin resistance gene replaced by a 1.2-kilobase trimethoprim resistance gene (10). pD10 was a derivative of pCHR404 (see above) encoding the cloned virG gene (3). pMAL<sup>™</sup>-P was a MalE fusion vector in which the expression of the MalE fusion protein was under the control of the tac promoter (New England Biolabs, Beverly, MA).

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Site-directed Mutagenesis—The nucleotide sequence of the mutagenic oligonucleotide was 5′-GGGCGGTTTACGCTTGCTCT-3′ and was used to replace Ala residues with Arg residues at positions 50 and 52 in the VirG polypeptide. Site-directed mutagenesis was performed with the U.S.E. mutagenesis kit (Pharmacia Biotech, Uppsala, Sweden). Mutagenesis was performed on the 2444-bp EcoRI-HindIII fragments (pD10), which consisted of a 1197-bp DNA sequence upstream of the 5′ end of the virG gene and 1247 bp of the 5′ virG sequence. The 2444-bp fragment cloned into pBR322 was digested with EcoRI and HindIII. Single-stranded DNA was then annealed with the phosphorylated mutagenesis primer and selection primer (5′-GAGCTT- TACCGCGGCTGGCTTCGCGT-3′), which prevented linearization of the double-stranded DNA upon PvuI digestion. The second strand was synthesized by incubation with a reaction mixture containing T4 DNA polymerase, T4 DNA ligase, and 32 protein. The resulting double-stranded circular DNA was digested with PvuI and transformed into a mutS E. coli strain. The parental strand, in linear form due to PvuI digestion, was not efficiently transformed. The plasmid DNA was extracted from a pool of transformants, digested with PvuI again and transformed into the same strain. After recovering plasmid DNA from several transformants, mutated DNA sequences were confirmed by sequencing and used to replace the parental 2444-bp EcoRI-HindIII fragment on pD10. The resulting pD10 derivative containing the mutated gene was named pTSG204.

Cell Fractionation—Bacteria were grown to mid-log phase in BHI broth (Difco) and pelleted, and the proteins contained in the resulting supernatant was precipitated by adding trichloroacetic acid to a final concentration of 8% (w/v). The bacterial pellet was washed in 10 mM HEPES buffer (pH 7.5) (1 mM NaOH, 0.2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupetin) and then disrupted by two passages through a French press at 12,000 kg/cm². After cell debris was eliminated by centrifugation at 3000 × g for 20 min, the cleared lysate thus obtained was pelleted down at 150,000 × g for 2 h at 4°C in a Type 60 Ti rotor (Beckman Instruments). The total membrane pellet was suspended in 1 ml of the same HEPES buffer, and the inner and outer membranes were separated by discontinuous sucrose gradient centrifugation (11). Periplasmic proteins were released from cells by the cold osmotic shock procedure of Neu and Chou (12), while the cytoplasmic fraction was subsequently obtained from the bacterial debris as described by Maagd and Lungenberg (13). The cell fractions were precipitated by trichloroacetic acid, dissolved in SDS sample buffer (2% SDS, 4% 2-ME, 10% glycerol, 0.1 M Tris-HCl (pH 8.0)), and was used to replace Ala residues with Arg residues at positions 50 and 52 (Ref. 5 and 6 and this study). The stretch of 21 hydrophobic amino acid residues just preceding the signal peptide cleavage site is indicated in bold lettering. shaded areas indicate repetitive sequences rich in Gly residues. The β-core is indicated by cross-hatching (6). Filled bars indicate the synthetic oligodeoxyribonucleotides used to raise antibodies VRG-120 and VRG-G (6, 19).

Results

Processing of VirG Polypeptide—To confirm that cleavage occurred after the putative signal sequence of VirG protein was...
employed in crossing the cytoplasmic membrane (5), we utilized antibodies, as indicated. by immunoblotting with VRG-120, anti-IpgC, PotA, PhoA, and OmpC extracts from each fraction were separated by SDS-PAGE and analyzed membrane, and culture supernatant. Equivalent amounts of protein extracts from each fraction were separated by SDS-PAGE and analyzed using the AMPHI secondary structure prediction program of membrane proteins (7, 20). Accordingly, in the 344 amino acids encompassing Arg759 to Phe1102 of the C-terminal 37-kDa VirG portion (see Fig. 1), at least 11 amphipathic transmembrane β-strands were predictable (Fig. 3A). We were also able to predict four additional transmembrane domains (Fig. 3A); three of those, containing lle823 Val929, and Asp986 as the hydrophobic center, respectively, possessed an amphipathic β-strand-like profile (although Hβ1 < 1.6) of hydrophobic center (see ref. 20), while the remaining one seemed to exhibit an amphipathic α-helical transmembrane structure (presented as an open cylinder in Fig. 3A). Furthermore, the prediction of high surface probability regions (21) using GCG software (22) revealed that there were at least six regions found in loops exposed on the outer membrane (see Fig. 3B). Based on the AMPHI prediction together with the high surface probability prediction, we propose a β-barrel structure model for the 37-kDa VirG portion integrated into the outer membrane as shown in Fig. 3C. We thereby referring to the C-terminal 344 amino acid VirG sequence as VirG β-core (see Fig. 1).

Functional Analysis of VirG β-Core—To demonstrate the ability of VirG β-core to present its preceding 80-kDa VirG portion, termed VirG α-domain, on the outer membrane, a MalE polypeptide of E. coli K-12 was covalently linked to the N terminus of VirG β-core and examined for its ability to present on the cell surface. To do this, the 1145-bp DNA sequence encoding VirG β-core was ligated to the EcoRI-XbaI sites in the multicloning site 12 bp upstream from the 3’ end of the malE gene on pMAL™-P. Consequently, the fifth last amino acid of the MalE polypeptide was followed by 17 amino acids, created by insertion of the multicloning sequence used, fused to Leu764 at the N terminus of VirG β-core. The plasmid encoding this fusion protein (MalE-VirG β-core 1) was designated pTSG221 (Fig. 4A). pTSG221 transformed into UT5600 was then induced by 10 mM isopropyl β-D-thiogalactopyranoside to express the fusion protein at a similar level to that of the endogenous MalE expressed in UT5600, and the MalE level present on the cell surface was examined using whole-cell ELISA with a MalE-specific antibody. As the control, pMAL™-P introduced into UT5600 was used, since the MalE protein translationally fused with LacZα has been shown to be presented in the periplasm (Fig. 4A). The results showed that UT5600 carrying pTSG221 (MalE-VirG β-core 1), but not UT5600 carrying pMAL™-P (MalE-LacZα), was able to present MalE on the cell surface (Fig. 5A). To check the topology of MalE-VirG β-core 1 in the outer membrane, intact or shocked cells of UT5600 carrying pTSG221 were treated with proteinase K and analyzed by immunoblots with VRG-C antibody. The data revealed that a 37-kDa protein band corresponding to the β-core appeared in both intact and shocked cells, but the protein band corresponding to the MalE-VirG β-core 1 disappeared (Figs. 5, C and D, lane 1). These results indicated that the core was embedded in the outer membrane, while the fused MalE portion was exposed on the cell surface.

Surface Presentation by N-terminally Truncated Derivatives of VirG β-Core—To determine the minimum region of the VirG β-core required for surface presentation of the N-terminally fused protein on the outer membrane, we made four additional N-terminally truncated β-core constructs and linked them to the MalE polypeptide on pMAL™-P. The four deletion derivations truncated β-core up to positions glu785 (MalE-VirG β-core 2), leu800 (MalE-VirG β-core 3), ser917 (MalE-VirG β-core 4), and asp986 (MalE-VirG β-core 5), respectively, and were connected to thr366 of the MalE polypeptide in the same manner as pTSG221 on pMAL™-P (Fig. 4A). The resulting plasmids encoded the MalE-VirG β-core 2, 3, 4, and 5 versions, and were analyzed using the AMPHI secondary structure prediction program of membrane proteins (7, 20). Accordingly, in the 344 amino acids encompassing Arg759 to Phe1102 of the C-terminal 37-kDa VirG portion (see Fig. 1), at least 11 amphipathic transmembrane β-strands were predictable (Fig. 3A). We were also able to predict four additional transmembrane domains (Fig. 3A); three of those, containing lle823 Val929, and Asp986 as the hydrophobic center, respectively, possessed an amphipathic β-strand-like profile (although Hβ1 < 1.6) of hydrophobic center (see ref. 20), while the remaining one seemed to exhibit an amphipathic α-helical transmembrane structure (presented as an open cylinder in Fig. 3A). Furthermore, the prediction of high surface probability regions (21) using GCG software (22) revealed that there were at least six regions found in loops exposed on the outer membrane (see Fig. 3B). Based on the AMPHI prediction together with the high surface probability prediction, we propose a β-barrel structure model for the 37-kDa VirG portion integrated into the outer membrane as shown in Fig. 3C. We thereby referring to the C-terminal 344 amino acid VirG sequence as VirG β-core (see Fig. 1).

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designated pTSG212, pTSG213, pTSG214 and pTSG215, respectively (Figs. 3 and 4). UT5600 carrying pTSG212, pTSG213, pTSG214, or pTSG215 was then analyzed for the production of fusion proteins by immunoblots with VRG-C antibody. As shown in Fig. 5B, pTSG212, pTSG213, pTSG214, and pTSG215 gave rise to 79-, 77-, 75-, and 73-kDa proteins, respectively. The levels of MalE moiety presented on the cell surface as measured by the whole-cell ELISA with the MalE antibody showed that VirG \( \beta \)-cores 2 and 3, but not VirG \( \beta \)-cores 4 and 5, were able to present the MalE moiety on the cell surface (Fig. 5A).

To investigate the topology of the fusion proteins in the outer membrane, the intact or shocked cells of UT5600 carrying pTSG212, pTSG213, pTSG214, or pTSG215 treated with proteinase K were analyzed for the presence of the respective fusion proteins by immunoblots with VRG-C antibody. The intact cells of UT5600 carrying pTSG212 (MalE-VirG \( \beta \)-core 2) gave rise to 79- and a faint 36-kDa bands that corresponded to the fusion protein and its \( \beta \)-core, respectively (Fig. 5C, lane 2).

Similarly, the intact cells of UT5600 carrying pTSG213 (MalE-VirG \( \beta \)-core 3) gave rise to a 77-kDa band that corresponded to the fusion protein and whose density was slightly stronger than that of the 79 kDa from pTSG212. However, the protein band corresponding to the VirG \( \beta \)-core was hardly detectable (Fig. 5C, lane 3). Intact cells of UT5600 carrying pTSG214 and pTSG215 gave rise to a protein band corresponding to the respective fusion proteins (Fig. 5C, lanes 4 and 5), while the shocked cells of each of the strains did not result in protein bands corresponding to the respective fusion proteins (Fig. 5D, lanes 2-5). These results taken together indicated that although the truncated VirG \( \beta \)-cores 2 and 3 were still capable of translocating the passenger polypeptides through the outer membrane, their targeting to the outer membrane was somehow incomplete as assayed by proteinase K accessibility (see "Discussion").

Folded Protein Structure Affects Export by VirG \( \beta \)-Core—
Since the proposed model for the structure of VirG β-core suggested that foreign polypeptides covalently linked to the β-core could pass through the putative β-barrel channel structure, the effect of the folding structure of the passenger protein on its translocation across the outer membrane was investigated using PhoA protein as the passenger. A 1534-bp phoA segment containing its promoter sequence was cloned into the N-terminus of pMAL™-p. The pMAL™-p vector encodes MalE fusion protein. To confirm the surface exposition of the PhoA moiety, UT5600 carrying pTSG220 grown in the phosphate-deprived medium in the presence or absence of 10 mM 2-ME was treated with proteinase K and analyzed for the fusion protein as was done for the MalE-VirG β-core with the VRG-C antibody. The results of the immunoblots showed that intact cells of UT5600 carrying pTSG220 grown without 2-ME gave rise to an 85-kDa protein band corresponding to the fusion protein (Fig. 6B, lane 1) and that the 85-kDa protein band almost disappeared in the shocked cells when treated with proteinase K (Fig. 6B, lane 4). Moreover, proteinase K-treated shocked cells presented a 37-kDa protein band corresponding to the β-core (see lane 4). This thus indicates that the PhoA moiety is presented in the periplasm. As expected, the 85 kDa protein expressed in the intact cells grown in the presence of 10 mM 2-ME became sensitive to the proteinase K treatment, but a 37-kDa protein band corresponding to the β-core appeared in both intact and shocked cells (Fig. 6B, lanes 6 and 8). These data thus indicated that the folded structure of the passenger protein resulting from intact intramolecular disulfide bonds interfered with its export across the outer membrane. Indeed, when the dsbA::Tn5 mutation from E. coli K-12 SK101 (34) was introduced into UT5600 carrying pTSG220, the capacity of the PhoA moiety of the fusion protein to be presented on the cell surface as determined by whole-cell ELISA was increased to 4.9-fold that of UT5600 carrying pTSG220 (Fig. 6A).

**DISCUSSION**

Several studies have indicated that VirG, localized at one pole of the bacterium, is exposed on the external side of the outer membrane and that the surface-exposed amino-proximal 80-kDa portion (VirG α-domain) is released into the external medium (3, 5, 6, 19), although little is known about the export pathway across the outer membrane. In this study, we thus attempted to elucidate the mechanisms underlying VirG secretion, especially focusing upon the step of crossing the outer membrane. According to our data, VirG protein is transported through the cytoplasmic membrane by the N-terminal signal.
sequence. After removal of the signal sequence, the C-terminal 37-kDa portion (VirG β-core) of the remaining VirG polypeptide is then integrated into the outer membrane. The VirG β-core embedded in the outer membrane forms a pore through which the preceding VirG α-domain could be translocated from the periplasmic space to the external side of the outer membrane. The VirG α-domain thus presented onto the cell surface is cleaved off from the VirG β-core at the Arg-Arg bond near the surface of the outer membrane (Fig. 1) (6). The proposed export pathway for VirG secretion in this study therefore resembles the secretion pathway displayed by IgA protease of Neisseria gonorrhoeae (25, 35–37), although the VirG protein and IgA protease share neither an amino acid homology nor a common role in pathogenicity (1–3, 25, 35–37).

Since analysis of the secondary structure of 344 amino acids of the VirG β-core using the method of Kyte and Doolittle (38) revealed that the predicted membrane-spanning domains lacked a high overall hydrophobicity, but rather exhibited hydrophilic residues in the core can face toward the hydrophobic center of the membrane, while the hydrophilic residues can face toward the hydrophilic center of the membrane (7, 39). This "β-barrel" structure has been confirmed by electron and X-ray diffraction analysis of extracted membranes and crystals of detergent-extracted outer membrane proteins (40, 41). We therefore undertook analysis of the secondary structure of the 344 amino acids of the C-terminal VirG portion using the AMPHI secondary prediction method developed by Vogel and Jähning (7). The results predicted that this VirG portion possessed at least 11 amphipathic transmembrane β-strands and additional four transmembrane domains, encompassing residues Ile108 through to Phe3102, and that the integral portion was capable of forming a β-barrel channel structure. These results, taken together with the fact that the VirG α-domain portion is surface-exposed, led us to propose a model for the export pathway across the outer membrane with the aid of the β-core (Fig. 7), most likely resembling the secretion pathway of IgA protease of N. gonorrhoeae (35).

As predicted above, VirG β-core was capable of translocating translationally fused foreign polypeptides from the periplasmic side of the outer membrane to the external side. Indeed, MalE protein, a typical periplasmic protein of E. coli, covalently linked to the VirG β-core was presented on the cell surface when pTSG211 (a cloned malE-virG β-core) was introduced into YSH6200 (the large virulence plasmid-free derivative of S. flexneri YSH6000) or UT5600 (an ompT mutant of E. coli K-12), also suggesting that the secretion onto the cell surface occurred without any accessory proteins encoded separately from the VirG gene on the large virulence plasmid. Experiments using N-terminally truncated VirG β-core derivatives translationally fused with the MalE protein revealed that the minimum β-core essential for surface presentation could be limited to the amino acids encompassing Tyr801 through to Phe1102, since further truncated VirG β-core (β-core 4) failed to expose the MalE portion as determined by the whole-cell ELISA with MalE antibody (Fig. 5A) or by the immunostaining of the bacteria with fluorescein isothiocyanate-labeled MalE-antibody. Interestingly, the topology of the fusion proteins of MalE-VirG β-cores 2 and 3 in the outer membrane as determined by proteinase K accessibility seemed to be somehow different to that of MalE-VirG β-core 1. VirG β-core 1 presented the MalE moiety on the cell surface as revealed by its full sensitivity to proteinase K digestion. However, under the same condition, MalE-VirG β-core 2 and 3 transported MalE were less sensitive to the proteinase K attack on the cell surface, and the integration of the β-core into the outer membrane also appeared to be incomplete as compared with β-core 1. These results suggested that although VirG β-cores 2 and 3 were capable of translocating the passenger protein through the outer membrane, their targeting to the outer membrane were partly impaired. Removal of the N-terminal short stretch from the Igα-domain of IgA protease of Neisseria has also been shown to cause a change in the placement of the β-core in the outer membrane (25). We therefore assume that a stretch of amino acid residues absent from VirG β-cores 2 and 3, but present in β-core 1, could somehow contribute to β-core function, such as facilitating the interaction with the outer membrane or tightening up the β-barrel structure in the membrane.

Folding of the passenger polypeptides fused with VirG β-core seemed to interfere with their own export across the outer membrane, as has also been shown with the Igα-domain of Neisseria, in that the export of CtxB moiety (the cholera toxin B subunit) of CtxB-Igα hybrid protein across the outer membrane was interfered with the folding structure of the passenger protein at the periplasmic side of the outer membrane (36, 37). Similarly, the surface presentation of PhoA protein covalently linked to VirG β-core 1 in UT5600 was significantly higher when this strain was grown in the presence of 2-ME. The level of the surface-exposed PhoA portion was elevated after introduction of a dsbA::Tn5 mutation into UT5600, indicating that the intramolecular Cys bridge formed in PhoA in the periplasm (34, 42) was incompatible with the subsequent translocation step.

It becomes evident that, as mentioned above, the export system for VirG secretion falls into a class of protein export systems represented by the IgA protease of N. gonorrhoeae. This class includes the IgA cognate such as the IgA protease and the IgA protease-like protein (Hap) of Haemophilus influenzae (43–45), the temperature-sensitive hemagglutinin (Tsh) of avian pathogenic E. coli (46), and the vacuolating cytotoxin (VacA) of Helicobacter pylori (47–49). In addition, the serine protease of Serratia marcescens (50), the 120-kDa surface-exposed protein of Rickettsia rickettsii (51, 52), and the 132-kDa AIDA-1 protein, an adhesin for the diffuse adherence phenotype of enteropathogenic E. coli (O126:H27) (53–55) could also be included as class members that utilize an export pathway similar to the IgA protease-like type exoproteins. Those exoproteins seem to share characteristic features in their export pathway across the outer membrane and their autoproteolytic processing of the precursors, leading to the release and maturation of the mature protein.
the exoproteins (56). It should also be noted that although processing of VirG after exposition on the cell surface occurs, cleavage is not required for expressing VirG function, since Shigella expressing a VirG mutant unable to cleave off the α-domain is still fully capable of inducing actin polymerization and spreading intra- and intercellularly (6). These studies thus indicate that the IgA protease-type secretion pathway is utilized by a variety of types of exoproteins important in the pathogenicity of Gram-negative bacteria.

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REFERENCES

1. Makino, S., Sasakawa, C., Kamata, T., and Yoshikawa, M. (1986) Cell 46, 551–555.
2. Bernardini, M. L., Mourier, J., D’Hauteville, H., Coquis-Rondon, M., and Sansonetti, P. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3867–3871.
3. Lett, M.-C., Sasakawa, C., Okada, N., Sakai, T., Makino, S., Yamada, M., Komatsu, K., and Yoshikawa, M. (1989) J. Bacteriol. 171, 353–359.
4. Prevost, M.-C., Lesourd, M., Arpin, F., Vernel, F., Mourier, J., Heillo, R., and Sansonetti, P. J. (1992) Infect. Immun. 60, 4088–4099.
5. Goldberg, M. B., Barzu, O., Parsot, C., and Sansonetti, P. J. (1993) J. Bacteriol. 175, 2189–2196.
6. Fukuda, I., Suzuki, T., Munakata, H., Hayashi, N., Katayama, E., Yoshikawa, M., and Sasakawa, C. (1995) J. Bacteriol. 177, 1719–1726.
7. Vogel, H., and Jähnig, F. (1986) J. Mol. Biol. 190, 191–199.
8. Sasakawa, C., Kamata, K., Sakai, T., Murayama, Y., Makino, S., and Yoshikawa, M. (1986) Infect. Immun. 53, 470–475.
9. Earhart, C. F., Lundrigan, M., Pickett, C. F., and Pierce, R. P. (1979) FEMS Microbiol. Lett. 6, 277–280.
10. Tobe, T., Sasakawa, C., Okada, N., Honma, Y., and Yoshikawa, M. (1992) J. Bacteriol. 174, 6359–6367.
11. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972.
12. Neu, H. C., and Chou, J. (1967) J. Bacteriol. 94, 1934–1945.
13. Maeg, R. A., and Lungsberg, B. (1986) J. Bacteriol. 167, 1083–1085.
14. Ménard, R., Sansonetti, P. J., Parsot, C., and Vasselon, T. (1994) Cell 79, 515–525.
15. Watarai, M., Tobe, T., Yoshikawa, M., and Sasakawa, C. (1995) EMBO J. 14, 2461–2470.
16. Kashiwagi, K., Miyamoto, S., Nukui, E., Kobayashi, H., and Igarashi, K. (1993) J. Biol. Chem. 268, 19338–19363.
17. Michaelis, S., Guérante, I., and Beckwith, J. (1983) J. Bacteriol. 154, 356–365.
18. Nikaido, H. (1979) in Bacterial Outer Membranes (Inouye, M., ed) pp. 319–361, J. B. S. Biosci. New York.
19. Nakata, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K., Yoshikawa, M., and Sasakawa, C. (1993) Mol. Microbiol. 9, 459–468.
20. Jähnig, F. (1990) Trends Biochem. Sci. 15, 93–95.
21. Emrini, E. A., Hughes, J. V., Perlow, D. S., and Boër, J. (1985) J. Virol. 55, 836–839.
22. DePuy, J. H., Haebeler, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
23. Chang, C. N., Kuang, W.-J., and Chen, E. Y. (1986) Gene (Amst.) 44, 121–125.
24. Andrews, G. P., Hromocký, A. E., Coker, M., and Maurelli, A. T. (1991) Infect. Immun. 59, 1997–2005.
25. Klauser, T., Krämer, J., Otzelberger, K., Pohler, J., and Meyer, T. F. (1993) J. Med. Biol. 234, 579–593.
26. Fikes, J. D., Bankaitis, V. A., Ryan, J. P., and Bassford, P. J., Jr. (1987) J. Bacteriol. 169, 2345–2351.
27. Fikes, J. D., and Bassford, P. J., Jr. (1987) J. Bacteriol. 169, 2352–2359.
28. Shien, L. L., Lee, J. L., Cheng, S. J., Jutte, H., Kuhn, A., and Dalbey, R. E. (1991) Biochemistry 30, 11775–11781.
29. Pugsley, A. P. (1993) Microbiol. Rev. 57, 50–108.
30. Palkić, T., Le, Q.-Q., Wong, A., and Botstein, D. (1994) J. Bacteriol. 176, 553–569.
31. Bassford, P., and Beckwith, J. (1979) Nature 277, 538–541.
32. Bankaitis, V. A., and Bassford, P. J., Jr. (1984) Cell 37, 243–252.
33. Inouye, H., Michaelis, S., Wright, A., and Beckwith, J. (1983) J. Bacteriol. 146, 668–675.
34. Kamitani, S., Akiyama, T., and Ita, K. (1992) EMBO J. 11, 57–62.
35. Pohler, J., Halter, R., Beyreuther, K., and Meyer, T. F. (1987) Nature 325, 458–462.
36. Klauser, T., Pohler, J., and Meyer, T. F. (1990) EMBO J. 9, 1991–1999.
37. Klauser, T., Pohler, J., and Meyer, T. F. (1992) EMBO J. 11, 2327–2335.
38. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132.
39. Kieffel, B., Garavito, R. M., and Baumeister, W. (1985) EMBO J. 4, 1589–1592.
40. Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., and Schiltz, G. E. (1991) Science 254, 1627–1630.
41. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauw, R. T., and Jänne, K., J.-N., and Rosenbusch, J. P. (1992) Nature 358, 727–733.
42. Bardwell, J. C. A., McCivan, K., and Beckwith, J. (1991) Cell 67, 581–589.
43. Poulsen, K., Brandt, J., Hjorth, J. P., Thyersen, H. C., and Killian, M. (1989) Infect. Immun. 57, 3097–3105.
44. Poulsen, K., Reinholdt, J., and Kristian, M. (1992) J. Bacteriol. 174, 2913–2921.
45. Saupe, J. W., III, de la Morena, M. L., and Fialkow, S. (1994) Mol. Microbiol. 14, 217–233.
46. Providence, D. L., and Curtiss, R. III, (1994) Infect. Immun. 62, 1369–1380.
47. Cover, T. L., Tummuru, M. K. R., Cao, P., Thompson, S. A., and Blaser, M. J. (1994) J. Biol. Chem. 269, 10566–10573.
48. Pohler, J., and Meyer, T. F. (1992) EMBO J. 11, 2327–2335.
49. Phadnis, S. H., Iver, D., Janzon, L., Normark, S., and Westblom, T. U. (1994) Infect. Immun. 62, 1557–1565.
50. Nakata, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K., and Yoshikawa, M. (1993) Mol. Microbiol. 9, 459–468.
51. Klauser, T., Krämer, J., Otzelberger, K., Pohler, J., and Meyer, T. F. (1993) J. Bacteriol. 175, 555–559.
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