7. A. W. Murray, *Curr. Opin. Cell Biol.* **6**, 872 (1994); W. A. E. Wells, *Trends Cell Biol.* **6**, 228 (1996); A. D. Rudner and A. W. Murray, *Curr. Opin. Cell Biol* **8**, 773 (1996).
8. X. M. Wang, Y. Zhai, J. E. Ferrell Jr., *J. Cell Biol.* **137**, 433 (1997).
9. H. Tamemoto *et al.*, *J. Biol. Chem.* **267**, 20293 (1992); H. Heider, C. Hug, J. M. Lucocq, *Eur. J. Biochem.* **219**, 513 (1994).
10. K. Takenaka and E. Nishida, unpublished results.
11. J. Rouse *et al.*, *Cell* **78**, 1027 (1994); J. Han, J.-D. Lee, L. Bibbs, R. J. Ulevitch, *Science* **265**, 808 (1994); S. Matsuda, H. Kawasaki, T. Moriguchi, Y. Gotoh, E. Nishida, *J. Biol. Chem.* **270**, 12781 (1995).
12. B. M. Yashar, C. Kelley, K. Yee, B. Errede, L. I. Zon, *Mol. Cell. Biol.* **13**, 5738 (1993); N. Masuyama and E. Nishida, unpublished results.
13. Y. Masui and C. Markert, *J. Exp. Zool.* **177**, 129 (1971); N. Sagata, N. Watanabe, G. F. Vande Woude, Y. Ikawa, *Nature* **342**, 512 (1989).
14. In these experiments, we added or injected activated p38 together with MKK6 to maintain the p38 in an active state. Phosphorylated, activated p38 alone was not sufficient to induce the mitotic arrest. MKK6 did not cause arrest of the cell cycle in M phase (*10*).
15. J. C. Lee *et al.*, *Nature* **372**, 739 (1994); A. Cuenda *et al.*, *FEBS Lett.* **364**, 229 (1995); Y. Jiang *et al.*, *J. Biol. Chem.* **271**, 17920 (1996); M. Goedert, A. Cuenda, M. Craxton, R. Jakes, P. Cohen, *EMBO J.* **16**, 3563 (1997).
16. R.-H. Chen, J. C. Waters, E. D. Salmon, A. W. Murray, *Science* **274**, 242 (1996); Y. Li and R. Benezra, *Ibid.*, p. 246.
17. Recombinant MKK6 and recombinant p38 proteins were expressed in *E. coli* as described [T. Moriguchi *et al.*, *J. Biol. Chem.* **271**, 13675 (1996)]. The p38 protein (1 μg) with or without 0.5 μg of MKK6 protein was incubated for 15 min at 30°C in the presence of 100 μM adenosine triphosphate (ATP) and 15 mM $MgCl_2$. ATF2 (5 μg) and [γ-^{32}P]ATP (1 μCi) were then added to the mixture, which was incubated for 10 min. Phosphorylated ATF2 was detected by electrophoresis and autoradiography.
18. NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing calf serum (CS, 10%). Cells were seeded onto 225-cm^2 flasks and allowed to grow for 3 days. Cells were cultured in DMEM containing CS (0.5%) for 30 hours to synchronize cells in G_0. Fresh DMEM supplemented with 10% CS was then added to induce the cells to reenter the cell cycle. After 18 hours, nocodazole (0.4 μg/ml) was added before cells entered M phase. Mitotic cells were collected 4 hours later by mechanical shake-off, washed with phosphate-buffered saline (PBS), and lysed in 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EGTA, 20 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cells remaining on flasks were scraped and lysed in the same buffer.
19. Extract (5 μl) was incubated with histone H1 (5 μg) in a final volume of 15 μl in the presence of 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 10 min at 30°C. The reactions were terminated by the addition of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. After boiling, the samples were subjected to SDS-PAGE and autoradiography.
20. Samples were incubated with anti-p38 (antibody to a COOH-terminal peptide, 20 μl; Santa Cruz) and 20 μl of protein A–Sepharose in the presence of 0.1% SDS for 2 hours at 4°C. The beads were washed three times with tris-buffered saline [20 mM tris-HCl (pH 7.5), 150 mM NaCl] containing 0.05% Tween 20. For immune-complex kinase assays, immunoprecipitated beads were incubated with recombinant ATF2 protein (5 μg) in a final volume of 20 μl in the presence of 100 μM ATP, 5 μCi [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 30 min at 30°C. After electrophoresis, radioactivity was detected by autoradiography or analyzed with an image analyzer (BAS2000, Fuji Photo Film). Similar results were obtained with an antibody to recombinant p38 that we produced.
21. MAPK was immunoprecipitated with anti-MAPK [Y. Gotoh *et al.*, *EMBO J.* **10**, 2661 (1991)], and the kinase activity was determined with myelin basic protein (MBP; 0.3 mg/ml) as a substrate in the presence of 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 30 min at 30°C. JNK was immunoprecipitated with an antibody to a COOH-terminal peptide of JNK (Santa Cruz) and assayed as described [T. Moriguchi, H. Kawasaki, S. Matsuda, Y. Gotoh, E. Nishida, *J. Biol. Chem.* **270**, 12969 (1995)].
22. *Xenopus* egg extracts were prepared as described [A. W. Murray, *Methods Cell Biol.* **36**, 573 (1991)]. Recombinant GST (gluthathione *S*-transferase)–p38 and histidine-tagged MKK6 proteins were added to the extracts at a final concentration of 0.1 mg/ml each.
23. Recombinant GST-p38 protein was incubated with or without recombinant MKK6 protein (*17*). Ste11ΔN was expressed in *E. coli* as described (*6*). Recombinant proteins were then added to the cell cycle extracts (final concentration, 0.1 mg/ml).
24. Samples (10 nl) were microinjected into one blastomere of the two-cell embryo as described (*4*).
25. We thank Y. Grotoh and M. Fukuda for helpful discussions and N. Masuyama for assistance in the microinjection experiments. K.T. and T.M. are Research Fellows of the Japan Society for the Promotion of Science. Supported by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan (E.N.).

12 September 1997; accepted 24 February 1998

Supramolecular Structure of the *Salmonella typhimurium* Type III Protein Secretion System

Tomoko Kubori,* Yukiyasu Matsushima, Dai Nakamura, Jaimol Uralil, Maria Lara-Tejero, Anand Sukhan, Jorge E. Galán,† Shin-Ichi Aizawa

The type III secretion system of *Salmonella typhimurium* directs the translocation of proteins into host cells. Evolutionarily related to the flagellar assembly machinery, this system is also present in other pathogenic bacteria, but its organization is unknown. Electron microscopy revealed supramolecular structures spanning the inner and outer membranes of flagellated and nonflagellated strains; such structures were not detected in strains carrying null mutations in components of the type III apparatus. Isolated structures were found to contain at least three proteins of this secretion system. Thus, the type III apparatus of *S. typhimurium*, and presumably other bacteria, exists as a supramolecular structure in the bacterial envelope.

Several plant and animal bacterial pathogens have evolved a specialized protein secretion system, termed type III, to interact with host cells [reviewed in (*1*)]. Characteristics of this system are (i) the absence of a typical, cleavable, *sec*-dependent signal sequence in secreted substrates; (ii) the requirement of accessory proteins for secretion; (iii) the export of proteins through both the inner and outer bacterial membranes; and (iv) the requirement of activating signals to initiate secretion. Although most of the putative components of this system have been identified, little is known about their function or their organization in the bacterial envelope. Genetic analyses have established that these systems are both structurally and functionally conserved across bacterial species (*2*).

The human pathogen *Salmonella typhimurium* encodes two type III secretion systems, although only one of them, located at centisome 63 of its chromosome, appears to be expressed in vitro (*3*). This system directs the translocation of several bacterial proteins into the host cell (*4*), which activate host cell signaling pathways, leading to a variety of responses, such as reorganization of the actin cytoskeleton, cytokine production, and the induction of programmed cell death in macrophages (*1*). This system has also been associated with the assembly of invasomes, appendage-like structures that appear on the bacterial surface upon contact with host cells (*5*). Some of the putative components of the secretion apparatus share sequence homology with proteins of the flagellar export machinery, suggesting an evolutionary relation.

The similarity between type III secretion components and the flagellar export machinery prompted us to investigate whether the *S. typhimurium* cell envelope contains structures similar to those involved in flagellar assembly. A mutant strain with a deletion in the *flh*C gene and therefore lacking all flagellar proteins was osmotically

T. Kubori, Y. Matsushima, D. Nakamura, S.-I. Aizawa, Department of Biosciences, Teikyo University, 1-1 Toyosatodai, Utsunomiya 320, Japan.
J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galán, Department of Molecular Genetics and Microbiology, School of Medicine, State University of New York, Stony Brook, NY 11794, USA.

*Present address: Structural Biology Center, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan.
†To whom correspondence should be addressed. E-mail: galan@asterix.bio.sunysb.edu

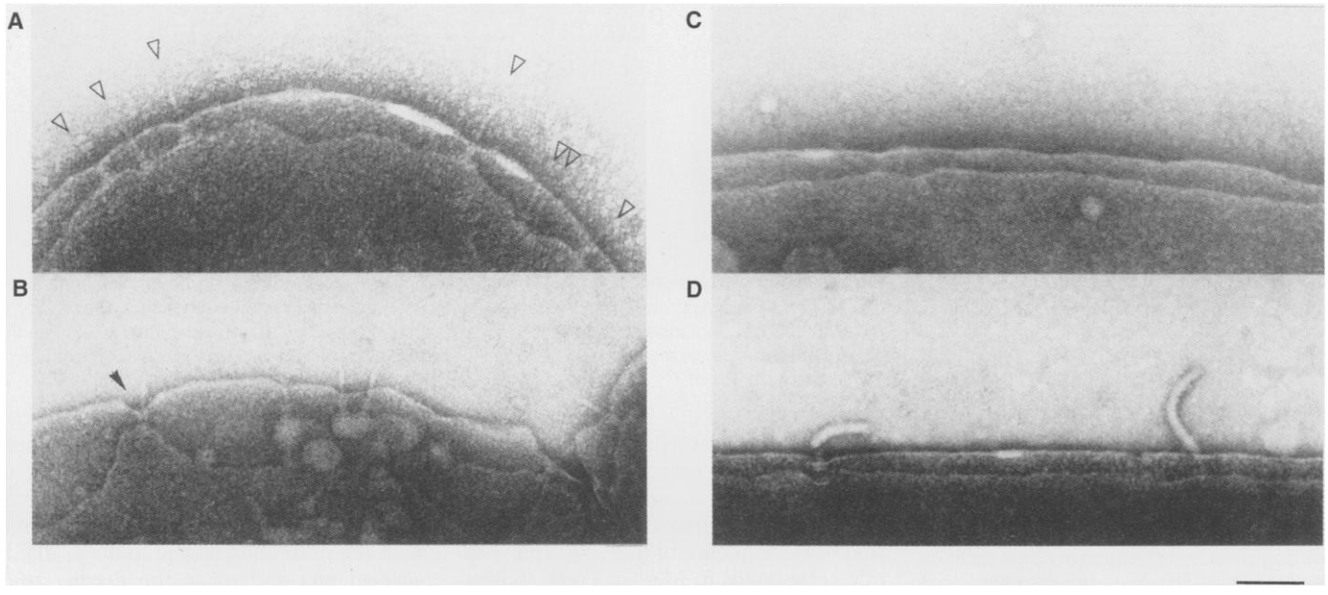

Fig. 1. Electron micrographs of osmotically shocked *S. typhimurium* strains. (**A** and **B**) Nonflagellated Δ*flhC S. typhimurium* exhibits needle complexes on the bacterial envelope (open arrows). Note the depression at the insertion point of the needle complex (closed arrow). (**C**) An invasion-defective strain of *S. typhimurium* carrying a mutation in *invG* shows no evidence of needle complexes. (**D**) An *S. typhimurium fliK* mutant exhibits flagellar polyhook basal bodies that span the inner and outer membranes. TEM samples were prepared as in (*20*). Samples were negatively stained with 2% phosphotungstic acid (pH 7.0) and observed under a JEM-1200EXII transmission electron microscope (JEOL, Tokyo). Micrographs were taken at an accelerating voltage of 80 kV. Scale bar, 100 nm.

shocked and examined by transmission electron microscopy (TEM). Complex structures resembling a needle (needle complex) were visualized on the cell surface (Fig. 1, A and B). The base of the structure is on the plane of the cytoplasmic membrane and extends to the outer membrane, where it is connected to a thinner structure, or needle, that projects outward. The dense layer around the proximal end of the needle suggests that this complex is attached to the outer membrane through specialized structures. A depression on the outer membrane was often seen in association with the insertion point of the needle complex (Fig. 1B). There were 10 to 100 needle complexes per cell, and these complexes were distinguishable from flagellar basal bodies of osmotically shocked wild-type *S. typhimurium* cells (Fig. 1D). Although the size of the base is similar to that of the flagellar basal body, the needle itself is much thinner than the flagellar filament. *Salmonella typhimurium* strains carrying mutations in any of 24 different flagellar genes (*6*) exhibited needle complexes in their envelopes, further demonstrating that these complexes are independent of flagella (*7*). In contrast, needle structures were absent from *S. typhimurium* strains carrying mutations in *invG*, *prgH*, or *prgK* (Fig. 1C) (*7*), which encode essential components of the invasion-associated type III secretion system (*8*, *9*).

Needle complexes isolated by a CsCl density gradient (*10*) appear to have cylindrical symmetry because every particle lying on the TEM grid exhibited a similar shape (Fig. 2). The base structure resembles the flagellar basal body (*11*) because it contains two upper (or outer) and two lower (or inner) rings. The lower rings, which interact with the cytoplasmic membrane, are 40 nm in diameter and 20 nm wide and appear to be close together. The upper rings are 20 nm in diameter and 18 nm wide and interact with the outer membrane and the peptidoglycan layer. The two upper rings are more widely separated than the two lower rings. The outermost ring was sometimes observed associated with fragments of the outer membrane, a phenomenon often seen in the L ring of the flagellar basal body (*12*) (Fig. 2C).

The needle structure itself is a stiff, straight tube, 80 nm long and 13 nm wide. The line across its length indicates that the stain solution penetrated into a hollow space in the center of the structure. Occasionally, needle structures were missing from the bases (Fig. 2C), in which case the bases tended to form aggregates through their rings, a phenomenon often observed

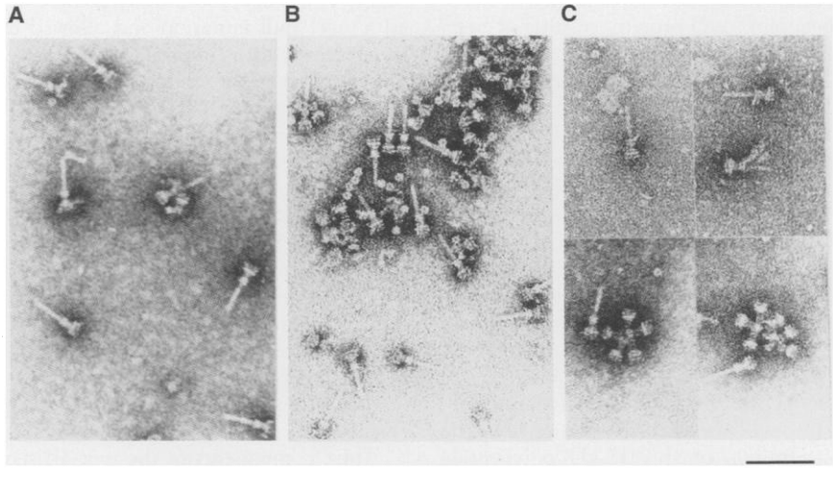

Fig. 2. Needle complexes isolated from *S. typhimurium* Δ*flhC*. (**A** and **B**) Complexes obtained from an enriched fraction of the CsCl density gradient (*10*). (**C**) (Top) Needle complexes associated with the bacterial outer membrane through their outer rings. (Bottom) Needle complexes lacking the needle structure aggregating through their outer rings. Scale bar, 100 nm.

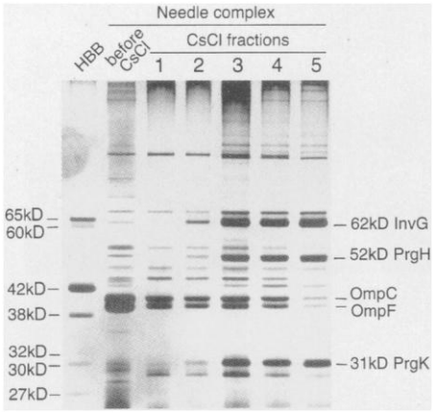

Fig. 3. Components of the needle structure. Needle complexes from *S. typhimurium* Δ*flhC* were purified on CsCl density gradients (*10*), and the proteins were visualized by silver staining of a 12% SDS-polyacrylamide gel. HBB, purified flagellar hook and basal body [isolated from the wild-type strain SJW1103 and included for comparison purposes (*20*)].

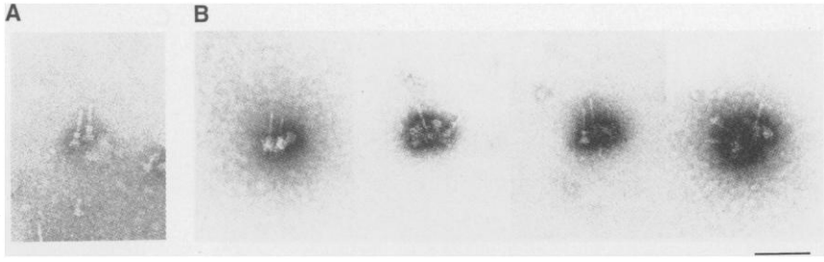

Fig. 4. Immunoelectron microscopy of needle complexes isolated from an *S. typhimurium* strain expressing (**A**) wild-type or (**B**) M45 epitope–tagged PrgH after staining with a monoclonal antibody to the M45 epitope. Immunoelectron microscopy was carried out as previously described (*17*). Note the cloud of electron-dense material representing the antibody molecules bound to the base of the structure (B). Scale bar, 100 nm.

with flagellar basal body preparations (*13*).

To identify the components of this supramolecular structure, we fractionated purified needle complexes on SDS-polyacrylamide gels and visualized the proteins by silver staining. Although the needle complex was present in several fractions of the CsCl gradient, it was consistently enriched in higher density regions that contained three major protein species of 62, 52, and 31 kD (Fig. 3). Minor amounts of other proteins were also present but not consistently. To identify the proteins in the needle complexes, we blotted samples onto membranes, visualized the proteins by Coomassie brilliant blue staining, and determined their NH_2-terminal sequence (*14*).

Ten amino acids of the 62-kD polypeptide exactly matched the sequence of the *S. typhimurium* InvG protein, starting at Ser^{25} of the predicted sequence (*15*). InvG is an essential component of the centisome-63 type III secretion system (*8*) and shares sequence homology with secretins, proteins that organize into homomultimeric structures (*16*). InvG is also required for the assembly of the appendage-like invasomes (*5*). The predicted InvG protein contains 564 amino acids, a molecular mass of 62,275 daltons, and a cleavable *sec*-dependent signal sequence with a signal peptidase recognition sequence located next to Ser^{25}, in close agreement with the observed size and sequence of the 62-kD polypeptide.

The sequence of nine residues from the NH_2-terminus of the 52-kD polypeptide (*15*) was identical to the deduced sequence of PrgH, another component of the type III secretion machinery (*9*). PrgH has a deduced sequence of 392 amino acids with a predicted molecular mass of 44,459 daltons, and it contains a stretch of nonpolar residues, followed by a canonical lipoprotein-processing site. However, the first amino acid in the sequence of the 52-kD polypeptide corresponds to Met^1 of PrgH, indicating that PrgH is not processed as predicted.

Five amino acids from the NH_2-terminus of the 31-kD protein (*15*) exactly matched the predicted amino acid sequence of PrgK, starting at Cys^{18}. This protein, also a component of the type III system, has a predicted molecular mass of 28,210 daltons and a canonical lipoprotein-processing site at position 18, consistent with the size and sequence of the 31-kD polypeptide (*9*). Proteins of 40 and 39 kD present in low amounts were identified by NH_2-terminal sequence analysis as OmpC and OmpF, respectively; these proteins are major components of the outer membrane and therefore likely contaminants of the needle complex preparation.

To confirm that proteins from the type III system are components of the needle complex, we constructed an *S. typhimurium* strain expressing an epitope-tagged form of PrgH (*17*). This protein fully complemented a *prgH* null mutation and allowed bacterial entry into cultured epithelial cells, indicating that the epitope-tagged protein functions similarly to wild-type PrgH. The needle complex was isolated from this strain, labeled with a monoclonal antibody to the epitope tag, and examined by TEM. The antibody specifically decorated the base of the needle complex isolated from the strain expressing the epitope-tagged PrgH, further demonstrating that this protein is a component of this structure (Fig. 4B). In contrast, the antibody did not label needle complex structures from *S. typhimurium* strains expressing wild-type PrgH (Fig. 4A).

Thus, components of the type III secretion machinery are organized in a supramolecular structure that appears to span both the inner and outer membranes of the *S. typhimurium* envelope. This hypothesis is supported by the following: (i) the structure was observed in the wild type but not in *invG*, *prgH*, or *prgK* mutant strains of *S. typhimurium*; (ii) InvG, PrgH, and PrgK were the most abundant proteins in highly purified preparations of the needle structure; and (iii) PrgH was identified as one of the components of the needle structure by immunoelectron microscopy. The architecture of the needle complex and the similarity between components of the flagellar export machinery and the type III secretion system provide strong support for the postulated common ancestry of these structures. In this context, the needle complex may be viewed as the functional equivalent of the flagellar basal body, serving as a channel through which the substrate proteins of the secretion apparatus cross the two bacterial membranes. Furthermore, the needle itself may be directly involved in the delivery of effector molecules into the host cell. Contact of *S. typhimurium* with cultured epithelial cells results in the transient assembly of invasomes on the surface of the bacteria (*5*), a process that requires the function of the invasion-associated type III secretion machinery. Although the components of the invasomes have not been defined, the needle complex may constitute the base of such structures. Proteins homologous to components of the needle complex are widely distributed among type III secretion systems in plant and animal pathogenic bacteria (*1*), and appendage-like structures whose assembly requires the function of this secretion system have also been observed in other bacterial pathogens (*18*). Therefore, it is likely that similar supramolecular structures are a common feature of all type III secretion systems.

REFERENCES AND NOTES

1. J. E. Galán and J. B. Bliska, *Annu. Rev. Cell Dev. Biol.* **12**, 219 (1996).
2. C. Ginocchio and J. E. Galán, *Infect. Immun.* **63**, 729 (1995); R. Rosqvist, S. Hakansson, A. Forsberg, H. Wolf-Watz, *EMBO J.* **14**, 4187 (1995).
3. J. E. Galán, *Mol. Microbiol.* **20**, 263 (1996); J. E. Shea, M. Hensel, C. Gleeson, D. W. Holden, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2593 (1996); H. Ochman,

F. C. Soncini, F. Solomon, E. A. Groisman, *ibid.*, p. 7800.
4. C. Collazo and J. E. Galán, *Mol. Microbiol.* **24**, 747 (1997); Y. Fu and J. E. Galán, *ibid.* **27**, 359 (1998); W.-D. Hardt and J. E. Galán, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9887 (1997).
5. C. Ginocchio, S. B. Olmsted, C. L. Wells, J. E. Galán, *Cell* **76**, 717 (1994).
6. *Salmonella typhimurium* strains were derivatives of SJW1103 [S. Yamaguchi, H. Fujita, A. Ishihara, S. Aizawa, R. M. Macnab, *J. Bacteriol.* **166**, 187 (1986)] or SL1344 [S. K. Hoiseth and B. A. Stocker, *Nature* **291**, 238 (1981)]. The *S. typhimurium* flagellar mutants examined were Δ*flhC*, *flgA*, *flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgG*, *flhA*, *flhB*, *flhC*, *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliM*, *fliN*, Δ*flio-fliR*, *fliP*, Δ*fliP-fliR*, *fliR*, and *fliQ*.
7. T. Kubori *et al.*, unpublished results.
8. K. Kaniga, J. C. Bossio, J. E. Galán, *Mol. Microbiol.* **13**, 555 (1994).
9. D. A. Pegues, M. J. Hantman, I. Behlau, S. I. Miller, *ibid.* **17**, 169 (1995).
10. Bacteria were grown in L broth to an absorbance at 600 nm of 0.8 to 1.0, pelleted, and resuspended in 1/10 of the original volume in a solution of 0.5 M sucrose and 0.15 M Trisma base. Lysozyme and EDTA were added to a final concentration of 1 mg/ml and 10 mM, respectively, and cells were incubated at 4°C for 1 hour. Spheroplasts were lysed by the addition of lauryldimethylamine oxide (LDAO) to a final concentration of 1%, and the samples were further incubated at 4°C for 2 hours. $MgSO_4$ was added to a final concentration of 10 mM, and samples were centrifuged at 5000*g* for 20 min. The clarified sample was adjusted to pH 11 with NaOH, incubated for 1 hour at 4°C, and centrifuged at 60,000*g* for 1 hour. The pellet was resuspended in a solution containing 0.1 M KCl-KOH (pH 11), 0.5 M sucrose, and 0.1% LDAO and centrifuged at 60,000*g* for 1 hour. The pellet was resuspended in TET buffer [10 mM tris-HCl (pH 8.0), 5 mM EDTA, and 0.1% LDAO] and loaded onto a 30% (w/v) CsCl density gradient. Gradient fractions were centrifuged at 60,000*g* for 1 hour, and the pellets were washed with TET buffer.
11. S. I. Aizawa, *Mol. Microbiol.* **19**, 1 (1996).
12. _____, G. E. Dean, C. J. Jones, R. M. Macnab, S. Yamaguchi, *J. Bacteriol.* **161**, 836 (1985).
13. T. Ueno, K. Oosawa, S. Aizawa, *J. Mol. Biol.* **236**, 546 (1994).
14. Polypeptides separated on standard SDS-polyacrylamide gels were electroblotted onto polyvinylidene difluoride membranes, and the bands of interest were applied to a Beckman LF3000 protein sequencer.
15. The amino acid sequences obtained from the different polypeptide species were as follows: 62 kD, SEKIPVTGSG; 52 kD, METSKEKTI; and 31 kD, CKDKD (*19*).
16. N. A. Linderoth, M. N. Simon, M. Russel, *Science* **278**, 1635 (1997); M. Koster *et al.*, *Mol. Microbiol.* **26**, 789 (1997).
17. The *prgH* and *prgK* genes were cloned from the wild-type strain of *S. typhimurium* SL1344 by polymerase change reaction, and derivative strains carrying nonpolar mutations in these genes were constructed by allele replacement (*8*). *Salmonella typhimurium* strains expressing a functional M45 epitope–tagged PrgH protein were constructed as described elsewhere [C. Collazo and J. E. Galán, *Infect. Immun.* **64**, 3524 (1996)]. The M45 epitope tag consisted of 18 residues from the E4-6/7 protein of adenovirus (MDRSRDRLPPFETETRIL) (*19*) [S. Obert, R. J. O'Connor, S. Schmid, P. Hearing, *Mol. Cell. Biol.* **14**, 1333 (1994)].
18. E. Roine *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3459 (1997).
19. Single-letter abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; T, Thr; and V, Val.
20. T. Kubori, N. Shimamoto, S. Yamaguchi, K. Namba, S.-I. Aizawa, *J. Mol. Biol.* **226**, 433 (1992).
21. We thank S. Yamaguchi for *Salmonella* flagellar mutant strains; M. Iwakura for amino acid sequence analysis; S. Makishima, T. Kubo, and N. Kobayashi for assistance in the needle preparation; and J. Bliska, R. Donis, and members of the Galán laboratory for critical reading of this manuscript. Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture (S.-I.A.), the American Heart Association, and Public Health Service Grants from the NIH (J.E.G.).

30 December 1997; accepted 2 March 1998

A Distinctive Class of Integron in the *Vibrio cholerae* Genome

Didier Mazel,* Broderick Dychinco, Vera A. Webb, Julian Davies†

The ability of bacteria to acquire and disseminate heterologous genes has been a major factor in the development of multiple drug resistance. A gene, *intI4*, was identified that encodes a previously unknown integrase that is associated with a "gene-VCR" organization (VCRs are *Vibrio cholerae* repeated sequences), similar to that of the well-characterized antibiotic resistance integrons. The similarity was confirmed by IntI1-mediated recombination of a gene-VCR cassette into a class 1 integron. VCR cassettes are found in a number of *Vibrio* species including a strain of *V. metschnikovii* isolated in 1888, suggesting that this mechanism of heterologous gene acquisition predated the antibiotic era.

Integrons are gene expression elements that acquire open reading frames (gene cassettes) and convert them to functional genes. More than 40 different antibiotic resistance cassettes have been characterized in these structures, permitting their bacterial hosts to become resistant to a broad spectrum of antimicrobial compounds (*1*). The insertion of a gene cassette takes place by site-specific recombination between the circularized cassette and the recipient integron; the essential components are an integrase gene (*intI*) and a linked attachment site (*attI*) required for the efficient site-specific integration of the gene cassettes into the integron structure (*2*). To date, three classes of integron have been defined on the basis of the sequence of their associated integrase (*1*); the integrases have 43 to 58% amino acid sequence identity and are related to the integrases of temperate bacteriophages. The similarity between the three integrases suggests that their evolutionary divergence extended beyond the half century of the antibiotic era, and probably much longer according to the substitution rates calculated by Ochman and Wilson (*3*).

All integron-inserted cassettes identified share specific structural characteristics (Fig. 1A); the boundaries of each integrated cassette are defined by two GTTRRRY (core-site) (*4*) sequences in the same orientation, which are the targets of the recombination process. The integrated cassettes include a gene and an imperfect inverted repeat located at the 3′ end of the gene called a 59-base element, a diverse family of sequences that function as recognition sites for the site-specific integrase. The organization of these elements starts with an inverse core-site, which is complementary to the core-site located upstream from the gene cassette, followed by a sequence of imperfect dyad symmetry, and ending with the downstream core site sequence (Fig. 1). The 59-base elements vary in size from 60 to 141 base pairs (bp); their nucleotide sequence similarities are primarily restricted to the inverse core-site and the core-site. Both the source of gene cassettes and the mechanism of their genesis are unknown, although they are presumed to be bacterial in provenance.

A number of species of the *Vibrio* genus, a widely distributed bacterial group found primarily in aqueous environments, are pathogenic for humans and animals. *Vibrio cholerae* has been responsible for major epidemics of human disease, especially since the early 19th century. It is known to have at least two pathogenicity islands (*5*): the locus encoding the cholera toxin, which is carried by a M13-like phage inserted into the chromosome (*6*), and a large cluster of genes encoding the accessory colonization factor and genes for the biogenesis of the toxin–co-regulated pilus, which is harbored in a prophage-like structure (*7*). The *V. cholerae* genome has also been found to contain repeated sequences (VCRs) in clusters that have a similar organization to the integron–gene cassette structures (*8*). They were first identified surrounding

Department of Microbiology and Immunology, University of British Columbia, 6174 University Boulevard, Vancouver, B.C., V6T 1Z3, Canada.

*Present address: Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris Cedex 15, France.
†To whom correspondence should be addressed.