Commentary

Tyrosine-phosphorylated Bacterial Proteins: Trojan Horses for the Host Cell

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For over a century, since the discovery by Roux and Yersin that sterile culture supernatants of *Corynebacterium diphtheriae* contained a potent toxin able to reproduce the lesions caused by diphtheria (1), most pathogenic bacteria have been considered to be microorganisms able to intoxicate local and distant tissues by secreting toxins in the extracellular medium. Recently, it has been shown that some bacteria inject toxic proteins directly into the cytoplasm of host cells using a specialized, needle-like (2) secretion apparatus (molecular syringe) known as a type III secretion system (3, 4). A report in this issue by Ashai et al. (5) and reports from Haas et al. (Haas, R., personal communication), Segal et al. (6), and our own laboratory (7) now provide definitive evidence for the existence in *Helicobacter pylori* of a second type of molecular syringe (type IV secretion system) that is also able to inject toxic proteins into eukaryotic cells. These papers describe the type IV–mediated delivery into eukaryotic cells and the subsequent tyrosine phosphorylation of CagA, an immunodominant protein of *H. pylori*, encoded by a 40-kb pathogenicity island (*cag*). The need for a functional type IV secretion system for CagA translocation into the membrane fraction of host cells and its tyrosine phosphorylation is shown at several levels using a variety of isogenic mutants in the *cag* region (7), biochemical fractionation of the host cell compartments (7), and confocal microscopy (6). In addition, it has been shown that CagA present in culture supernatants or bacterial cell lysates is unable to enter eukaryotic cells, indicating that this is not a function that the soluble protein can perform on its own (5, 7). Although the correlation between expression of CagA and *H. pylori* virulence was described a long time ago (8, 9), the last seven years have been marked by the frustration of not finding any role for the CagA protein. Finally, the concomitant report by four independent laboratories of a role for CagA sends important messages to the scientific community. They are summarized below.

Type IV and Type III Secretion Systems Are Functionally Equivalent Molecular Syringes. Gram-negative bacteria build and anchor different extracellular organelles, such as flagella and conjugative pili, using specialized supramolecular structures. These molecular engines transport the building blocks of flagella and pili across both the inner and outer membranes and polymerize the external filamentous structures by adding new monomers from the inside. In their evolution, bacteria found it convenient to duplicate the ancestral cluster of genes and use one set for further specialization as secretion apparatuses to translocate proteins or protein complexes into host cells (Fig. 1). In other terms, we may consider the type III and type IV secretion systems as spin-offs of flagella and conjugative pili, respectively. The two secretion systems have been extensively reviewed (4, 10); a list of the bacteria known to contain them is printed in Table I. Here we will just mention that although both systems are functionally equivalent and are used to translocate proteins into mammalian or plant cells, a number of properties differentiate them: (a) Type III apparatus and flagella (4) are encoded by 15–35 genes, at least 8 of which are well conserved in most systems. Type IV system and conjugative pili (10) are encoded by 11–31 genes, at least 6 of which are well conserved in most of them. No homology is present between the genes present in the two systems; however, both encode proteins with predicted ATPase activity. (b) The type III system secretes monomeric proteins with no apparent cleavable *sec*-dependent signal sequence. The type IV system may secrete assembled multimeric proteins such as pertussis toxin that are composed of different monomers, each having a typical *sec*-dependent signal peptide. This suggests that in some cases the proteins may enter the export machinery after being exported across the inner membrane by the general secretion system. Type IV secretion may, however, also export across both the inner and outer membranes nucleoproteins containing proteins and DNA (as in the case of the Ti plasmid T-DNA of *Agrobacterium tumefaciens*). In the case of the CagA protein reported in references 5–7, it is not known whether this is exported as a monomer or a more complex structure; however, the absence of a typical *sec*-dependent signal sequence in this protein suggests that the type IV system assists the translocation of CagA across both bacterial membranes and also across the host cell membrane. Finally, both type III and type IV secretion systems are encoded by genes that are clustered, often present in pathogenicity islands with a GC content different from the rest of the chromosome and that most likely have been acquired by horizontal transfer.

cag Delivers Multiple Signals. The presence of the *cag* pathogenicity island in *H. pylori* correlates with increased virulence and disease severity. The findings reported by

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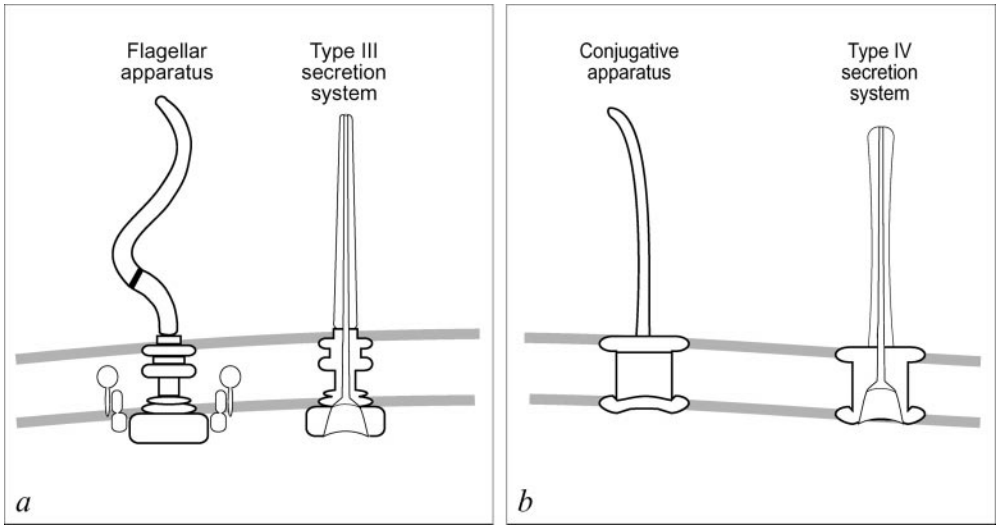

Figure 1. Schematic representation of a bacterial flagellum (*a*) and a conjugative pilus (*b*) showing their similarity to type III and type IV secretion systems, respectively. In *a*, the structural similarities between the core structures of the flagellar apparatus and the type III secretion system are sketched. In *b*, a hypothetical conjugative apparatus and a type IV system are reduced to an artistic impression; the proteic subunits forming the structure are not represented.

Asahi et al. (5), Haas et al. (personal communication), Segal et al. (6), and Stein et al. (7) begin to unravel the molecular mechanisms that may link the presence of *cag* to disease. Two events are known to happen after *cag*-mediated bacterium–cell contact: induction of the proinflammatory lymphokine IL-8 and CagA tyrosine phosphorylation. The latter triggers host cell morphological changes such as cell elongation and spreading, a phenotype similar to that induced in AGS cells by hepatocyte growth factor or bacterial toxins such as cytotoxin necrotizing factor 1, which activate the small GTP-binding proteins Rho, Rac, and Cdc42 (6). Data obtained by mutagenesis of the genes in the *cag* pathogenicity island (Table II) show that although both signals require an intact type IV secretion system, they are delivered by independent effectors. In fact, one of the mutants (*cag*F) is able to activate nuclear factor (NF)-κB but unable to induce IL-8 and CagA tyrosine phosphorylation. A schematic representation of the possible signaling mechanisms is shown in Fig. 2. Here the *cag* type IV secretion system is shown to translocate into the cell an unknown factor (in the figure it is named "?" for convenience) that activates the transcription factor NF-κB to induce IL-8 mRNA. As proposed by Naumann et al. (11), this mechanism is likely to involve activation of mitogen-activated protein (MAP) kinases and the transcription factor activator protein (AP)-1. It is not yet certain whether this pathway needs a yet unknown effector or whether it is simply activated by the type IV system itself, which perturbs the membrane. The second pathway activated by the *cag* system involves the protein CagA. This protein, as reported in references 5–7, is translocated into the eukaryotic cells, where it is tyrosine phosphorylated by a eukaryotic cell kinase (in vitro c-src and epidermal growth factor receptor protein kinases have been shown to be able to phosphorylate CagA (5); however, we do not yet know which kinase is precisely involved in vivo). Once phosphorylated, CagA is likely to bind an Src homology (SH)2-containing protein (SHC, phosphatidylinositol 3 kinase, and Nck are possible candidates). This complex can activate multiple pathways: (a) it may bind directly to N-WASP (neural Wiskott-Aldrich syndrome protein) and activate it to bind the Arp2/3 actin nucleator, thus stimulating actin polymerization and pedestal formation, as is the case of the *Shigella flexneri* IcsA (12).

Table I. *Bacterial Species with a Fully Annotated Type III or Type IV Secretion System*

Bacterial species	Type III	Type IV
Actinobacillus actinomycetemcomitans	—	+
Agrobacterium tumefaciens	—	+
Bordetella bronchiseptica	+	+
Bordetella pertussis	+	+
Brucella suis	—	+
Chlamydia spp.	+	—
Citrobacter rodentium	+	—
E. coli	+	+
Enteropathogenic E. coli	+	—
Enterohemorrhagic E. coli	+	—
Erwinia amylovora	+	—
Erwinia chrysanthemi	+	—
Erwinia herbicola pv. *gysophila*	+	—
Erwinia stewartii	+	—
Hafnia alveii	+	—
H. pylori	—	+
Legionella pneumophila	—	+
Pseudomonas aeruginosa	+	—
Pseudomonas syringae	+	—
Ralstonia solanacearum	+	—
Rickettsia prowazekii	—	+
Rhizobium spp.	+	—
Salmonella enterica	+	—
Shigella spp.	+	—
Xantomonas spp.	+	—
Yersinia spp.	+	—

Table II. *List of Genes and Crossed Nomenclature of the cag Pathogenicity Island of H. pylori*

Nomenclature of *cag* genes according to			Properties of single gene inactivation in the *cag* region		
Tomb et al.*	Akopyants et al.‡	Censini et al.§	IL-8 secretion	NF-κB activation	CagA–Ptyr translocation
ND	ND	G27wt,*cag*+	+	+	+
ND	ND	G27Δ*cag*	−	−	−
HP0520 *cag*1	ORF6	*cag*ζ	ND	ND	ND
HP0521 *cag*2	ORF7	*cag*ϵ	−	ND	ND
HP0522 *cag*3	ORF8	*cag*δ	ND	ND	ND
HP0523 *cag*4	ORF9	*cag*γ	ND	ND	ND
HP0524 *cag*5	ORF10	*cag*β(**virD4**)	++	ND	−
HP0525	ORF11	*cag*α(**virB11**)	−	ND	−
HP0526 *cag*6	ORF12	*cag*Z	ND	ND	ND
HP0527 *cag*7	ORF13	*cag*Y(**virB10**)	−	ND	−
HP0527 *cag*7	ORF14	ND	−	ND	ND
HP0528 *cag*8	ORF15	*cag*X(**virB9**)	−	ND	−
HP0529 *cag*9	ORF16	*cag*W(**virB8**)	−	ND	ND
HP0530 *cag*10	ORF17	*cag*V	ND	ND	ND
HP0531 *cag*11	ORF18	*cag*U	−	ND	ND
HP0532 *cag*12	ORF19	*cag*T(**virB7**)	−	ND	ND
HP0533	ND	ND	ND	ND	ND
HP0534 *cag*13	ORF20	*cag*S	−	ND	ND
ND	ORF21	*tnp*A	ND	ND	ND
ND	ORF22	*tnp*B	ND	ND	ND
HP0535 *cag*14	ND	*cag*Q	ND	ND	ND
ND	ND	*cag*R	ND	ND	ND
HP0536 *cag*15	ND	*cag*P	ND	ND	ND
ND	ND	*cag*O	ND	ND	ND
HP0537 *cag*16	ND	*cag*M	−	−	−
HP0538 *cag*17	ND	*cag*N	+	+	+
HP0539 *cag*18	ND	*cag*L	−	−	−
HP0540 *cag*19	ND	*cag*I	−	−	−
HP0541 *cag*20	ND	*cag*H	−	−	−
HP0542 *cag*21	ND	*cag*G	−	−	−
HP0543 *cag*22	ND	*cag*F	−	+	−
HP0544 *cag*23	ND	*cag*E(**virB4**), *pic*B	−	−	−
HP0545 *cag*24	ND	*cag*D	−‖	ND	−
HP0546 *cag*25	ND	*cag*C	−‖	ND	−
ND	ND	*cag*B	−‖	ND	−
HP0547 *cag*26	ND	*cag*A	+	+	−
HP0548	ND	*cag*Ω	ND	ND	ND
HP0549 *glr*	ND	*glr*	ND	ND	ND

List of genes and crossed nomenclature of the *cag* pathogenicity island of *H. pylori* and of the effects on IL-8 secretion, NF-κB activation, and CagA translocation and tyrosine phosphorylation after single gene inactivation. Type IV homologues are indicated in bold type in parentheses. The CagE gene was also independently identified by Tummuru et al. as *pic*B (reference 20). The wild-type G27 strain and its mutant derivative Δ*cag* are positive and negative controls.

*Reference 17.

‡Reference 18.

§Reference 19.

‖Additional mutant with a *cag*B–E deletion.

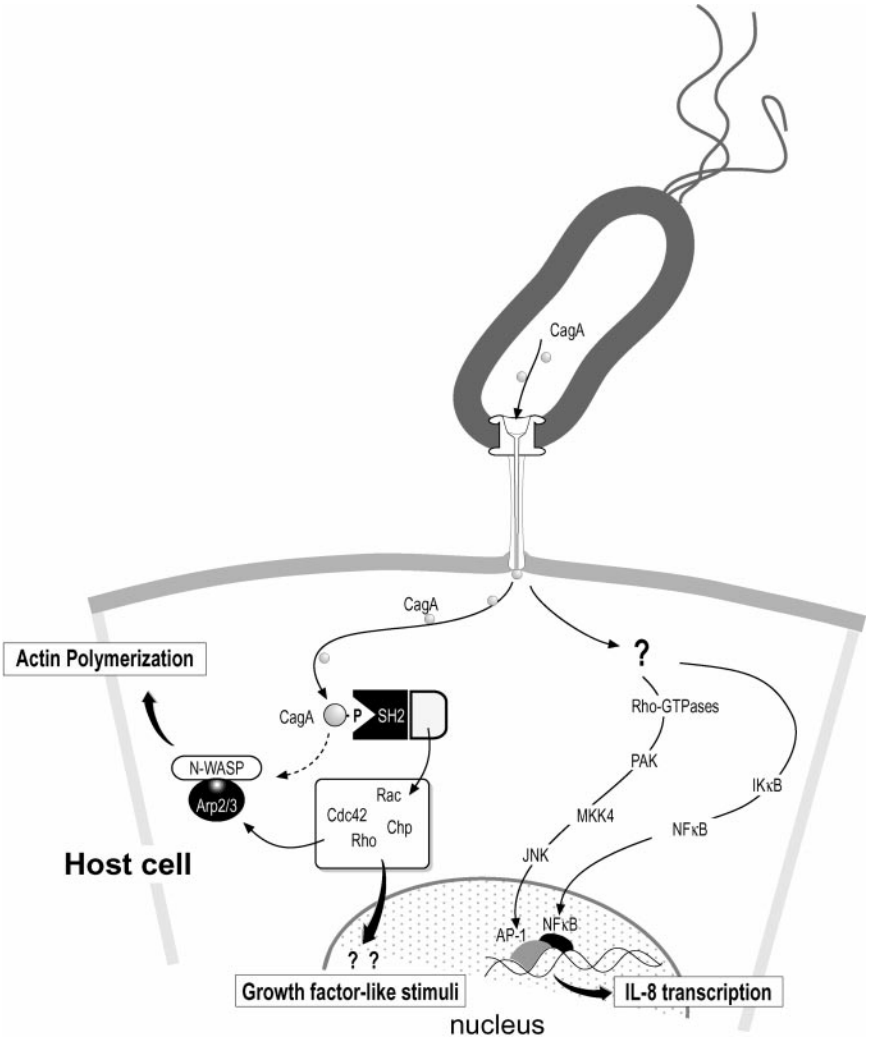

Figure 2. After type IV contact and CagA translocation, two independent signaling pathways are induced in the host cell.

(b) Alternatively, the CagA–P-SH2 protein complex may activate the Rho family of small, GTP-binding proteins (Cdc42, Rac, or Chp), which control the organization of the actin cytoskeleton. This pathway could also cause actin polymerization and pedestal formation by activating N-WASP (13). (c) The CagA–P-SH2 protein complex may trigger a signaling cascade, possibly via the MAP pathway, which may induce transcription of nuclear genes. Although there is plenty of evidence that actin polymerization occurs and therefore one or both of the mechanisms mentioned in (a) or (b) must be activated by CagA–P, there is not yet any evidence for the pathway mentioned in (c). However, the increased frequency of gastric cancer in patients infected by *cag*$^+$ *H. pylori* strains and the *H. pylori*–dependent MALT (mucosal-associated lymphoid tissue) lymphoma suggest that nuclear signaling is a possible mechanism of pathogenesis. Whether the delivery of CagA into host cells facilitates antigen presentation, thus explaining why CagA is the immunodominant antigen of *H. pylori*, is an intriguing possibility that deserves further investigation.

Tyrosine Phosphorylation of Injected Bacterial Proteins Is an Emerging Signaling Mechanism. Before CagA, another protein was described as injected into mammalian cells and tyrosine phosphorylated by eukaryotic cell kinases. This is the translocated intimin receptor (Tir) of enteropathogenic *Escherichia coli* that, after injection and tyrosine phosphorylation, serves as a receptor for "intimin" adhesin (Fig. 3; reference 14). CagA is the second example of a bacterial protein that is shown to be injected into eukaryotic cells and then tyrosine phosphorylated by eukaryotic cell kinases. During *Chlamydia* infection, it has been found that eukaryotic cells can also phosphorylate a bacterial protein at serine/threonine residues (15). The discovery in different bacteria of this new type of cell intoxication mechanism suggests that many more examples will emerge in the near future.

The use of phosphotyrosines by bacterial pathogens is not surprising; pathogenic bacteria are known to target most crucial regulatory circuits of the eukaryotic cell. Large and small GTP-binding proteins that act on most of the cellular signal transduction pathways are targets of many classical toxins and toxins injected by the type III secretion system. Now that bacterial virulence factors have been shown to also target tyrosine phosphorylation, the other

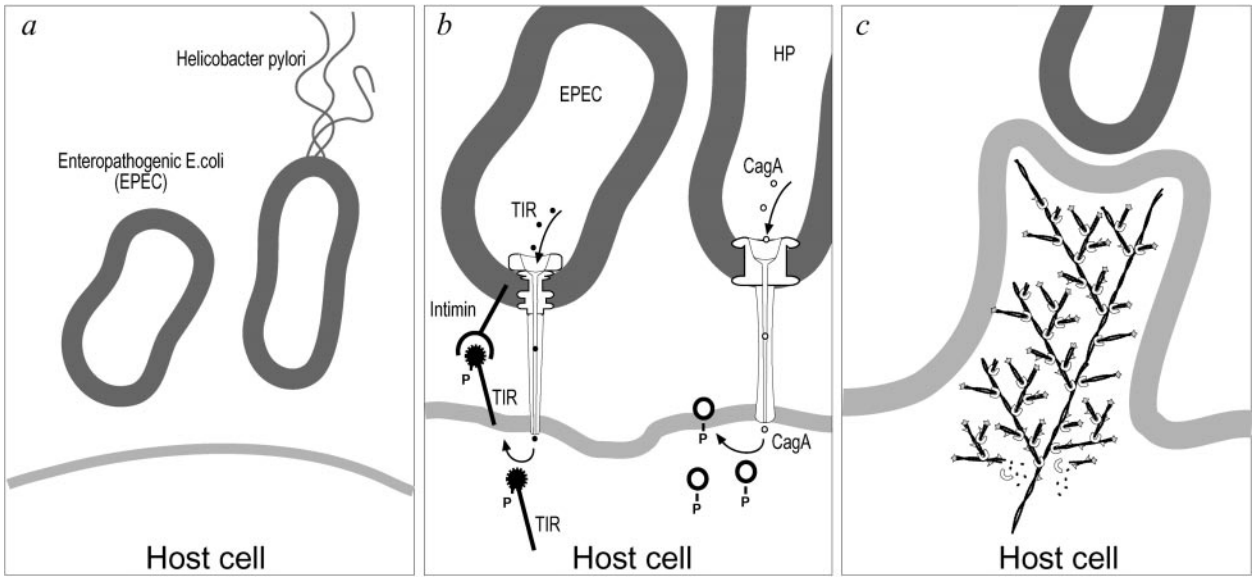

Figure 3. Type III and type IV secretion systems functionally converge. Enteropathogenic *E. coli* and *H. pylori* (*a*) inject tyrosine-phosphorylated effector proteins by type III or type IV engines, respectively. (*b*) Tir is the EPEC receptor for intimin (binary products of the LEE [locus of enterocyte effacement] pathogenicity island). CagA is a *Helicobacter*-translocated molecule. (*c*) After phosphorylation on a tyrosine residue by a host cell kinase, cortical actin polymerization and pedestal protrusion are induced. Both microorganisms promote similar cellular responses.

major cellular signaling mechanism, we can probably conclude that bacteria manage to target virtually all key regulatory circuits of eukaryotic cells. It is interesting that in this case the targets are not the key enzymes such as kinases or phosphatases, which would have a more pleiotropic effect. Rather, the action is very precise: bacteria inject molecules that appear as host proteins but that act as Trojan horses containing a bacterial hidden core message that allows the microorganism to take control over the host cell. Surprisingly, vaccinia virus exploits similar mechanisms of forced actin polymerization—mediated by tyrosine phosphorylation of the viral protein A36R—to spread from cell to cell (16).

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References

1. Roux, E., and A. Yersin. 1888. Contribution a l'etude de la diphterie. *Ann. Inst. Pasteur.* 2:629–661.
2. Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J.F. Galan, and S.I. Aizwawa. 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science.* 280:602–605.
3. Cornelis, G.R. 1998. The Yersinia deadly kiss. *J. Bacteriol.* 180:5495–5504.
4. Hueck, C.J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62:379–433.
5. Asahi, M., T. Azuma, S. Ito, Y. Ito, H. Suto, Y. Nagai, M. Tsubokawa, Y. Tohyama, S. Maeda, M. Omata, et al. 2000. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J. Exp. Med.* 191:593–602.
6. Segal, E.D., J. Cha, J. Lo, S. Falkow, and L.S. Tompkins. 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA.* 96:14559–14564.
7. Stein, M., R. Rappuoli, and A. Covacci. 2000. Tyrosine phosphorylation of *Helicobacter pylori* CagA after cag-driven host translocation. *Proc. Natl. Acad. Sci. USA.* In press.
8. Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z.Y. Xiang, N. Figura, et al. 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc. Natl. Acad. Sci. USA.* 90:5791–5795.
9. Tummuru, M.K., T.L. Cover, and M.J. Blaser. 1993. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infect. Immun.* 61:1799–1809.
10. Covacci, A., J.L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli. 1999. *Helicobacter pylori*, virulence and genetic geography. *Science.* 284:1328–1333.
11. Naumann, M., S. Wessler, C. Bartsch, B. Wieland, A. Covacci, R. Haas, and T.F. Meyer. 1999. Activation of activator protein 1 and stress response kinases in epithelial cells colonized by *Helicobacter pylori* encoding the *cag* pathogenicity island. *J. Biol. Chem.* 274:31655–31662.
12. Egile, C., T.P. Loisel, V. Laurent, R. Li, D. Pantaloni, P.J. Sansonetti, and M.F. Carlier. 1999. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol.* 146:1319–1332.
13. Kalman, D., O.D. Weiner, D.L. Goosney, J.W. Sedat, B.B. Finlay, A. Abo, and J.M. Bishop. 1999. Enteropathogenic *E. coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nat. Cell Biol.* 1:389–391.
14. Kenny, B., R. DeVinney, M. Stein, D.J. Reinscheid, E.A. Frey, and B.B. Finlay. 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell.* 91:511–520.

15. Rockey, D.D., D. Grosenbach, D.E. Hruby, M.G. Peacock, R.A. Heinzen, and T. Hackstadt. 1997. *Chlamydia psittaci* IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion. *Mol. Microbiol.* 24:217–228.
16. Frischknecht, F., V. Moreau, S. Rottger, S. Gonfloni, I. Reckmann, G. Superti-Furga, and M. Way. 1999. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature*. 401:926–929.
17. Tomb, J.F., O. White, A.R. Kerlavage, R.A. Clayton, G.C. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, et al. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*. 388: 539–547.
18. Akopyants, N.S., S.W. Clifton, D. Kersulyte, J.E. Crabtree, B.E. Youree, C.A. Reece, N.O. Bukanov, E.S. Drazek, B.A. Roe, and D.E. Berg. 1998. Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Mol. Microbiol.* 28:37–53.
19. Censini, S., C. Lange, Z. Xiang, J.E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci. 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA*. 93:14648–14653.
20. Tummuru, M.K., S.A. Sharma, and M.J. Blaser. 1995. *Helicobacter pylori picB*, a homologue of the *Bordella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. *Mol. Microbiol.* 18:867–876.