

**Fig. 4.** The Sec machinery is exclusively localized. SecA is localized to a single focus (A and B). Double-labeling experiments demonstrated that only a single cluster was observed (C) that contained antibodies recognizing both SecA (18 nm beads) and SpeB (12 nm beads) (D). Note that the label is often associated with a nascent division septum. Magnification as in Fig. 1.

unlikely that all copies of the plasmid would be restricted to a specific compartment. Concurrently, we examined expression of PhoZ, a secreted protein not native to *S. pyogenes*, to determine whether the targeting pattern was a general phenomenon or one restricted to SpeB. Analysis by immunogold EM using PhoZ antiserum revealed a pattern of gold particles clustered at a discrete focus identical to that observed for SpeB (Fig. 3, A and B). This contrasts with the circumferential distribution of gold particles observed using an antiserum directed against the M protein (Fig. 3, C and D) and the lack of gold particles observed in sections stained in the absence of the primary antibodies (Fig. 3, E and F). After its secretion from the cell, M protein becomes cross-linked to the cell wall (14) and is subsequently uniformly distributed over the cell surface in late exponential phase (15). Taken together, these data indicate that protein secretion in *S. pyogenes* occurs at a distinct microdomain of the cytoplasmic membrane dedicated to protein export. We have named this novel microdomain the ExPortal.

Asymmetric secretion and localization of proteins are essential to many processes in bacteria (16). In some cases, asymmetric secretion is the result of polar localization of a specialized secretion complex (12). For the general secretory pathway, asymmetry does not appear to be due to a restricted distribution of the Sec pathway translocons but rather to various specialized accessory factors (16, 17). Because SpeB and PhoZ are substrates for the Sec pathway, we examined whether secretion was directed by accessory targeting factors or by a restricted distribution of the Sec translocons. For this analysis, streptococci were analyzed by immunogold EM with an antiserum raised against SecA of *Bacillus subtilis*. This component of the Sec pathway

is a highly conserved ATPase that directly interacts with the SecYEG translocase in the membrane and powers translocation of polypeptides through the Sec channel (18). This antiserum specifically recognized SecA of *S. pyogenes*, as determined by a Western blot analysis of cell lysates, and localized SecA exclusively to a single locus as visualized in immunogold electron micrographs (Fig. 4, A and B). Colocalization of large and small gold particles following staining with both rabbit anti-SecA and mouse monoclonal anti-SpeB antibodies confirmed that SecA is exclusively located in the ExPortal (Fig. 4, C and D). These data are consistent with a model in which targeting of secreted proteins results from the specialization of this microdomain to accumulate a high concentration of the Sec translocons.

These data represent a mechanism for asymmetric secretion of proteins from bacteria using the Sec pathway distinct from those previously described. This localization may serve to organize accessory folding factors to coordinate their interaction with nascent unfolded polypeptides. Most examples of asymmetry in bacteria involve localization of proteins and structures to the poles of the cell, possibly by exploiting differences in lipid and cell wall content between the poles and the lateral surfaces (16). However, because the streptococcal translocons do not appear to localize to the poles, a different mechanism may be used to establish their restricted positioning. The contribution of the ExPortal to host-pathogen interactions raises intriguing possibilities, including possible cooperation with the CMT pathway for introduction of streptococcal

effector proteins into the host-cell cytoplasm. Continued analysis will enrich our understanding of how streptococci cause disease and may provide greater insight into the protein secretion pathways of other Gram-positive bacteria.

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19. This work was supported by Public Health Service grant AI46433 from NIH. We thank D. Oliver and A. J. M. Driessen for providing SecA antisera, J. M. Musser for providing the monoclonal SpeB antisera, and W. Beatty for technical assistance with EM imaging.

#### Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5676/1513/DC1  
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2 March 2004; accepted 14 April 2004

## A Process for Controlling Intracellular Bacterial Infections Induced by Membrane Injury

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Strategies for inhibiting phagolysosome fusion are essential for the intracellular survival and replication of many pathogens. We found that the lysosomal synaptotagmin Syt VII is required for a mechanism that promotes phagolysosomal fusion and limits the intracellular growth of pathogenic bacteria. Syt VII was required for a form of Ca<sup>2+</sup>-dependent phagolysosome fusion that is analogous to Ca<sup>2+</sup>-regulated exocytosis of lysosomes, which can be triggered by membrane injury. Bacterial type III secretion systems, which permeabilize membranes and cause Ca<sup>2+</sup> influx in mammalian cells, promote lysosomal exocytosis and inhibit intracellular survival in Syt VII <sup>+/+</sup> but not <sup>-/-</sup> cells. Thus, the lysosomal repair response can also protect cells against pathogens that trigger membrane permeabilization.

Synaptotagmins (Syt) are thought to function as transducers of Ca<sup>2+</sup> signaling in membrane fusion events, through interactions mediated by their cytosolic C<sub>2</sub>A and C<sub>2</sub>B do-

main (1). The Syt VII isoform is localized on lysosomes, where it regulates Ca<sup>2+</sup>-triggered lysosomal exocytosis (2). Phagolysosome fusion is also a Ca<sup>2+</sup>-dependent process (3, 4),

so we investigated whether modulating Syt VII function affected the intracellular survival of bacteria that have to evade lysosomes in order to replicate. We found that expression in CHO cells of the soluble Syt VII C<sub>2</sub>A domain, which inhibits Ca<sup>2+</sup>-triggered lysosomal exocytosis (2), did not affect invasion by *Salmonella enterica* serovar Typhimurium, but enhanced its intracellular growth between 1 and 2 hours after it entered the host cell (Fig. 1, A to C).

The involvement of Syt VII in a mechanism that inhibits *Salmonella* growth was confirmed using primary cells isolated from Syt VII-deficient mice (5). Intracellular growth in the first 6 hours after invasion was enhanced in Syt VII <sup>-/-</sup> murine embryonic fibroblasts (MEFs) when compared to cells isolated from Syt VII <sup>+/+</sup> embryos (Fig. 1D). As previously reported (6, 7), in both Syt VII <sup>+/+</sup> and <sup>-/-</sup> MEFs, *Salmonella* replicated in vacuoles that did not fuse with

lysosomes (Fig. 1E), in spite of containing the lysosomal glycoprotein Lamp-1 (fig. S1) (8). In macrophages from Syt VII <sup>+/+</sup> mice, intracellular replication of *Salmonella* could only be detected approximately 4 hours after infection, which is consistent with previous reports (9). In Syt VII <sup>-/-</sup> macrophages, however, bacterial growth started shortly after entry and proceeded vigorously with no lag period (Fig. 1F). This increase in intracellular growth was not related to *Salmonella*-induced macrophage cytotoxicity. Under the growth conditions used, *Salmonella* induced similar low levels of programmed cell death on both Syt VII <sup>+/+</sup> and <sup>-/-</sup> macrophages and did not cause differential cell loss between 2 and 6 hours after infection (fig. S2). The bacteria replicating intracellularly, as previously reported (7), were in Lamp-1-positive vacuoles devoid of dextran pre-loaded into lysosomes. Thus, *Salmonella* evades phagolysosome fusion more efficiently in Syt VII-deficient cells.

We also investigated whether Syt VII deficiency affected the intracellular replication of *Listeria monocytogenes*. Shortly after invasion, this bacterium escapes from the phagosome and replicates free in the cytosol, where it sequesters host actin as a propelling mechanism (10). Nearly identical intracellular growth curves were observed with *Listeria* in Syt VII <sup>+/+</sup> or <sup>-/-</sup> macrophages (fig. S3A). In addition, similar numbers of bacteria were found associated with actin tails 2 hours after infection, in both

Syt VII <sup>+/+</sup> and <sup>-/-</sup> macrophages (80 and 84%, respectively). Thus, Syt VII deficiency does not affect the process by which *Listeria* escapes from the phagosome before lysosomal fusion (11) and also does not cause major alterations in components of the cytosol required for actin-based motility.

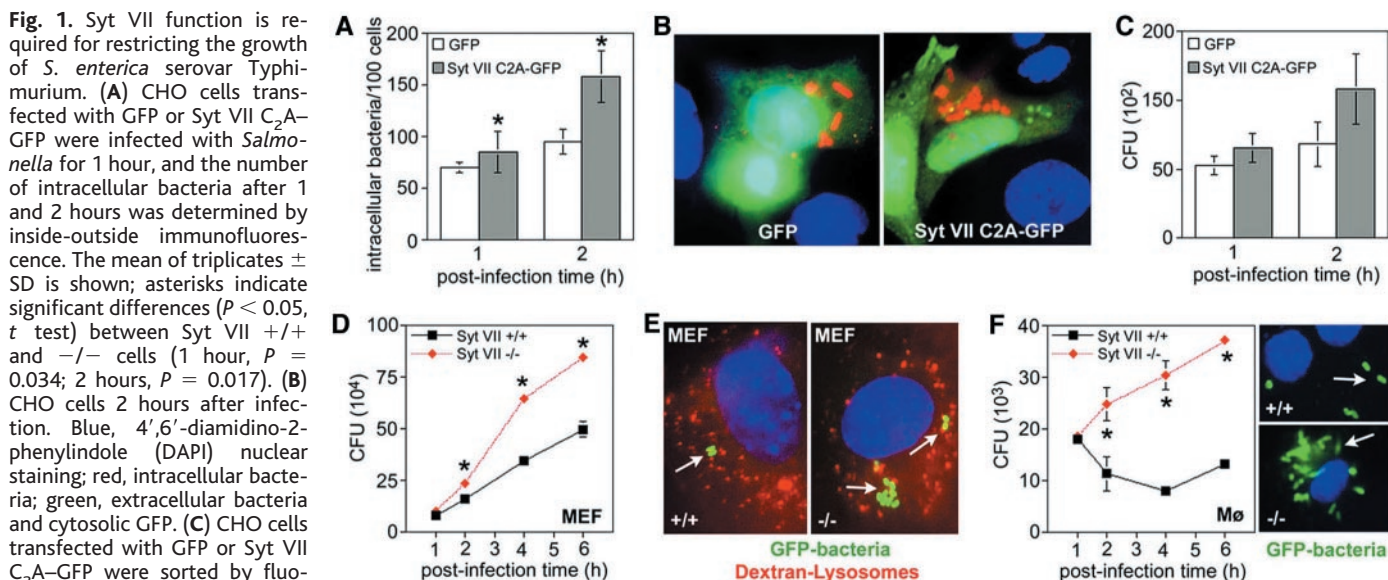
Lysosomal degradation of endocytosed epidermal growth factor occurred with identical kinetics in Syt VII <sup>+/+</sup> and <sup>-/-</sup> MEFs (fig. S3B). Furthermore, nonpathogenic *Escherichia coli* was degraded at a similar rate after uptake by Syt VII <sup>+/+</sup> or <sup>-/-</sup> macrophages (fig. S3C). Thus, Syt VII deficiency did not cause any blockage of the normal endocytic traffic into degradative lysosomes. Defects in nitric oxide (NO) production or NADPH oxidase activity also did not provide an explanation for the growth advantage of *Salmonella* in Syt VII <sup>-/-</sup> cells: Similar levels of NO were produced in response to lipopolysaccharide and interferon- $\gamma$  (fig. S3D), and robust superoxide production was visualized in zymozan-containing phagosomes in both Syt VII <sup>+/+</sup> and <sup>-/-</sup> macrophages (fig. S3, G and J).

Syt VII functions in the regulation of rapid Ca<sup>2+</sup>-dependent fusion of lysosomes with the plasma membrane (2, 5, 12). We investigated whether Syt VII <sup>-/-</sup> macrophages had a defect in the fusion of lysosomes with recently formed phagosomes, which might still retain plasma membrane properties. Lysosomes were loaded with fluorescent dex-

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**Fig. 1.** Syt VII function is required for restricting the growth of *S. enterica* serovar Typhimurium. (A) CHO cells transfected with GFP or Syt VII C<sub>2</sub>A-GFP were infected with *Salmonella* for 1 hour, and the number of intracellular bacteria after 1 and 2 hours was determined by inside-outside immunofluorescence. The mean of triplicates  $\pm$  SD is shown; asterisks indicate significant differences ( $P < 0.05$ ,  $t$  test) between Syt VII <sup>+/+</sup> and <sup>-/-</sup> cells (1 hour,  $P = 0.034$ ; 2 hours,  $P = 0.017$ ). (B) CHO cells 2 hours after infection. Blue, 4',6'-diamidino-2-phenylindole (DAPI) nuclear staining; red, intracellular bacteria; green, extracellular bacteria and cytosolic GFP. (C) CHO cells transfected with GFP or Syt VII C<sub>2</sub>A-GFP were sorted by fluorescence-activated cell sorting, replated, and infected with *Salmonella* for 1 hour; the number of intracellular bacteria after 1 and 2 hours was determined by a gentamicin protection assay (GPA). The mean of triplicates  $\pm$  SD is shown ( $P = 0.233$ ; 2 hours,  $P = 0.077$ ). (D) MEFs from Syt VII <sup>+/+</sup> or deficient <sup>-/-</sup> embryos were infected with *Salmonella* for 1 hour, and the number of intracellular bacteria was determined by GPA. The mean of triplicates  $\pm$  SD is shown; asterisks indicate significant differences between Syt VII <sup>+/+</sup> and <sup>-/-</sup> cells (1 hour,  $P = 0.253$ ; 2 hours,  $P = 0.010$ ; 4 hours,  $P = 0.0001$ ; 6 hours,  $P = 0.0001$ ). (E) MEFs infected with

GFP-expressing *Salmonella* 6 hours after infection: Bacteria (arrows) replicate in compartments that do not fuse with dextran-Texas red-loaded lysosomes. (F) Bone marrow macrophages from Syt VII <sup>+/+</sup> or <sup>-/-</sup> mice were infected with *Salmonella* for 1 hour, and the number of intracellular bacteria was determined by GPA. The mean of triplicates  $\pm$  SD is shown; asterisks indicate significant differences between Syt VII <sup>+/+</sup> and <sup>-/-</sup> cells (1 hour,  $P = 0.507$ ; 2 hours,  $P = 0.007$ ; 4 hours,  $P = 0.0001$ ; 6 hours,  $P = 0.00001$ ). Fluorescent images on the right show intracellular GFP-expressing *Salmonella* (arrows) 6 hours after infection.

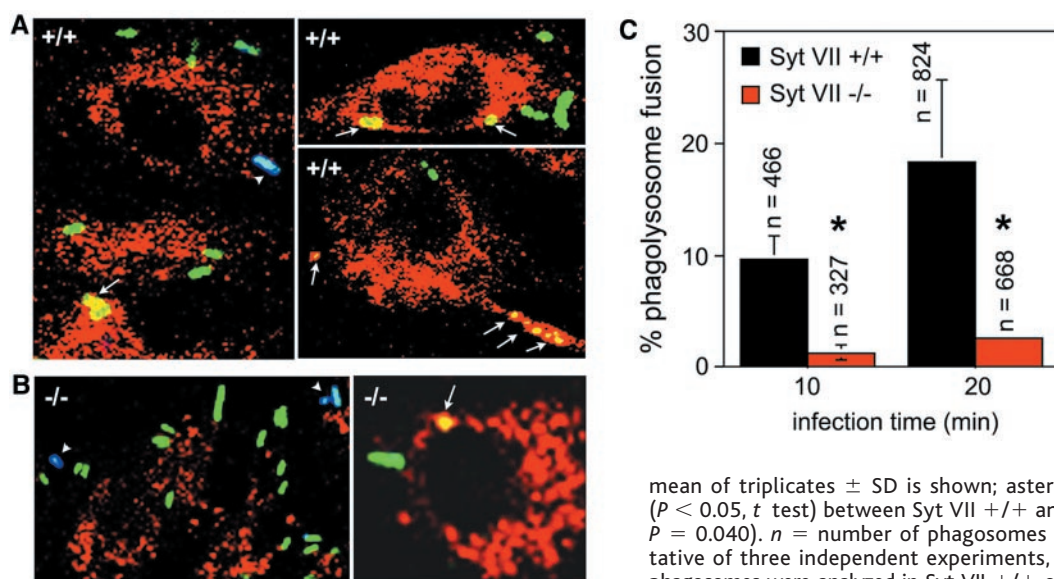
tran, and green fluorescent protein (GFP)-expressing *Salmonella* was incubated with the macrophages for 10 or 20 min, followed by antibody staining to distinguish extracellular bacteria and analysis by confocal microscopy (Fig. 2, A and B). The extent to which *Salmonella*-containing phagosomes colocalized with dextran preloaded into lysosomes was significantly reduced in Syt VII  $-/-$  when compared to Syt VII  $+/+$  macrophages (Fig. 2C). These results reconcile previous reports describing early phagolysosome fusion (13) and killing of *Salmonella* in mouse macrophages (14), with the widely demonstrated capacity of *Salmonella* to block lysosomal fusion through translocated type III secretion system effectors (7). Syt VII-dependent phagolysosome fusion appears to play an important role in the initial inhibition

of intracellular growth observed in wild-type macrophages (Fig. 1F). The fact that intracellular growth is only detected several hours after infection (9) suggests that a fraction of the surviving bacteria, which succeeded in modifying the phagosome to block lysosomal fusion, gradually increase in number.

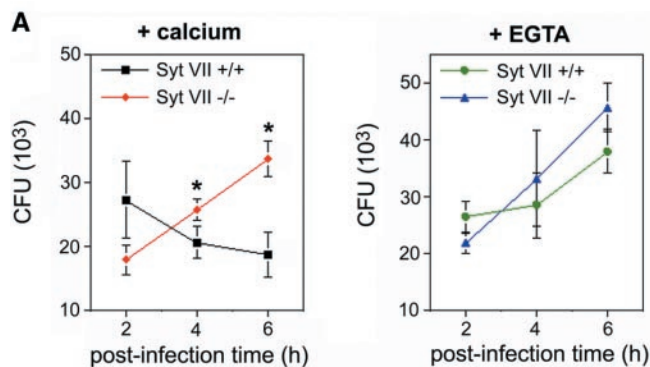
Syt VII-dependent lysosomal exocytosis promotes membrane resealing, a process triggered by  $Ca^{2+}$  influx through membrane wounds (12). We investigated whether a lysosomal response to membrane injury was involved in the survival advantage of *Salmonella* in Syt VII-deficient cells. If a Syt VII-dependent form of phagolysosome fusion occurred in response to membrane injury, one prediction is that it should be dependent on extracellular  $Ca^{2+}$ . This was confirmed: When wild-type macrophages were infected with *Salmonella* in medium containing

EGTA [a condition that does not inhibit actin-mediated particle uptake (15)], the initial decline in bacterial numbers observed in wild-type macrophages was markedly altered, resembling instead the rapid growth pattern typically observed in Syt VII  $-/-$  macrophages (Fig. 3A). These findings are consistent with a requirement for  $Ca^{2+}$  influx from the extracellular medium for the Syt VII-dependent growth inhibition response.

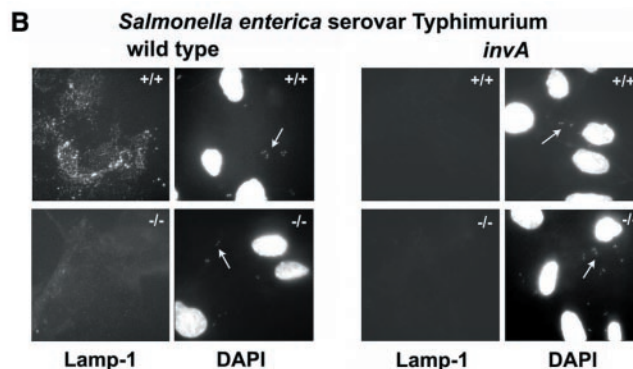
Next, we investigated the possible source of membrane injury responsible for  $Ca^{2+}$  influx during *Salmonella* infection. Several pathogenic bacteria express specialized secretion systems that mediate direct transport of effector proteins into the cytosol of host cells (16). The type III secretion systems of Gram-negative bacteria have been extensively characterized and are known to transiently permeabilize the plasma membrane of target cells, through assembly of a



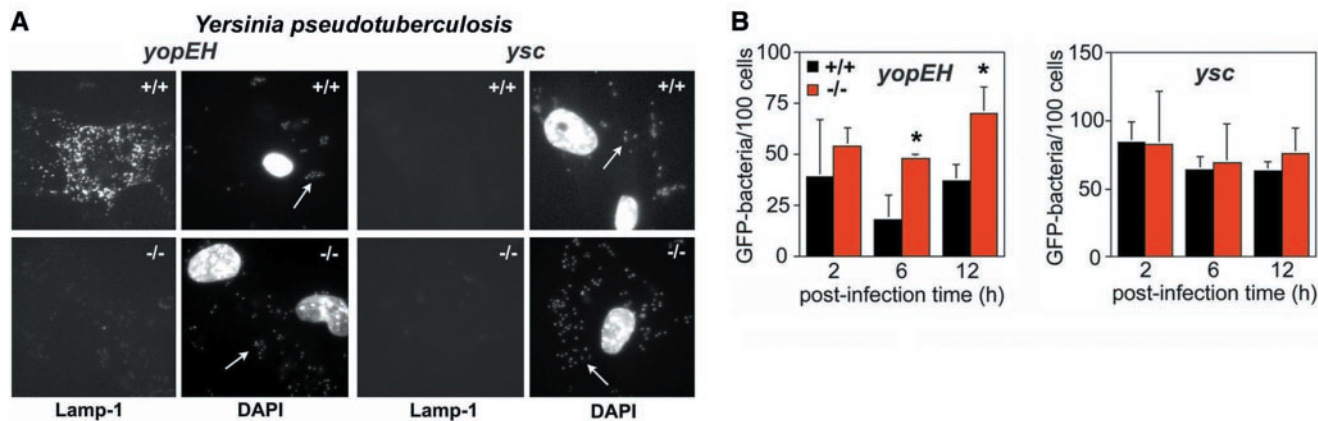
**Fig. 2.** Syt VII promotes phagolysosome fusion. Bone marrow macrophages derived from (A) Syt VII  $+/+$  or (B)  $-/-$  mice were incubated with dextran-Texas red to label lysosomes and incubated with GFP-expressing *Salmonella* for 10 or 20 min before confocal microscopy analysis. Extracellular bacteria were stained with antibodies to *Salmonella* (blue, arrowheads). Arrows point to phagosomes containing *Salmonella* that fused with dextran-Texas red-containing lysosomes. (C) The number of phagosomes containing dextran-Texas red was determined visually on confocal Z-stack images. The mean of triplicates  $\pm$  SD is shown; asterisks indicate significant differences ( $P < 0.05$ ,  $t$  test) between Syt VII  $+/+$  and  $-/-$  (10 min,  $P = 0.005$ ; 20 min,  $P = 0.040$ ).  $n$  = number of phagosomes analyzed. The results are representative of three independent experiments, in which a total of 3167 and 3784 phagosomes were analyzed in Syt VII  $+/+$  or  $-/-$  macrophages, respectively.



**Fig. 3.**  $Ca^{2+}$  influx is required for the Syt VII-dependent killing of intracellular *Salmonella*, under conditions that induce type III secretion system-dependent lysosomal exocytosis. (A) Syt VII  $+/+$  and  $-/-$  macrophages were preincubated or not with 2 mM EGTA and infected with *Salmonella* for 30 min in the absence (+ calcium) or presence (+ EGTA) of 2 mM EGTA, and the number of intracellular bacteria at various times after infection was determined by GPA. The mean of triplicates  $\pm$  SD is shown; asterisks indicate significant



differences ( $P < 0.05$ ,  $t$  test) between Syt VII  $+/+$  and  $-/-$  (+ calcium: 2 hours,  $P = 0.067$ ; 4 hours,  $P = 0.040$ ; 6 hours,  $P = 0.004$ ; + EGTA: 2 hours,  $P = 0.592$ ; 4 hours,  $P = 0.472$ ; 6 hours,  $P = 0.060$ ). (B) Syt VII  $+/+$  and  $-/-$  MEFs were exposed to wild-type *Salmonella* or the type III secretion mutant *invA* for 20 min at 37°C, followed by live surface immunofluorescent labeling of Lamp-1. Images were acquired under identical exposure conditions; the DAPI stain shows cell nuclei and cell-associated bacteria (arrows) on the same field.



**Fig. 4.** The *Yersinia* type III secretion system induces Syt VII–dependent lysosomal exocytosis and killing of intracellular bacteria. **(A)** Syt VII *+/+* and *-/-* MEFs were exposed to *Yersinia yopEH* or *ysc* (type III secretion mutant) for 1 hour at 37°C, followed by live surface labeling with a monoclonal antibody against the luminal domain of Lamp-1. Images were acquired under identical exposure conditions; the DAPI image shows cell nuclei and cell-associated bacteria (arrows) on the same field. **(B)** Syt VII *+/+* and *-/-* macrophages were

infected with the *Yersinia yopEH* or *ysc* type III secretion mutant for 30 min, followed by treatment with gentamicin and isopropyl-β-D-thiogalactopyranoside to induce GFP expression. Numbers of viable intracellular bacteria were determined at different time points; the mean of triplicates ± SD is shown. Asterisks indicate significant differences ( $P < 0.05$ ,  $t$  test) between Syt VII *+/+* and *-/-* (*yopEH*: 2 hours,  $P = 0.399$ ; 6 hours,  $P = 0.015$ ; 12 hours,  $P = 0.016$ ; *ysc*: 2 hours,  $P = 0.975$ ; 6 hours,  $P = 0.765$ ; 12 hours,  $P = 0.338$ ).

macromolecular structure referred to as the translocon (17, 18). In *Salmonella*, the SPI-1 type III secretion system responsible for translocating invasion effectors is known to promote  $Ca^{2+}$  influx (19). We found that wild-type *Salmonella* exhibits extensive pore-forming activity when exposed to mammalian cells, whereas no activity is seen with the SPI-1 type III secretion mutant *invA* (19). Pore formation (20) was also detected in cells exposed to the noninvasive, effectorless SB1304 *Salmonella* mutant (21), confirming that permeabilization is dependent on a functional type III secretion apparatus but not on the introduction of effectors that stimulate membrane ruffling and bacterial entry (fig. S4). In order to determine whether the cell permeabilization induced by the *Salmonella* SPI-1 secretion system triggered Syt VII–dependent lysosomal exocytosis, we incubated Syt VII *+/+* or *-/-* MEFs with wild-type or *invA* *Salmonella*, and stained the cells for detection of surface-exposed Lamp-1 (12). Wild-type *Salmonella* induced extensive surface exposure of Lamp-1 in Syt VII *+/+* MEFs (Fig. 3B). In contrast, a markedly fainter staining was observed under the same conditions in Syt VII *-/-* MEFs. The *invA* mutant, which does not express a functional SPI-1 type III secretion system, did not trigger detectable lysosomal exocytosis in either cell type (Fig. 3B).

Recently formed phagosomes are expected to contain high concentrations of  $Ca^{2+}$ , similar to those in the extracellular medium. It was therefore of interest to determine whether type III secretion-mediated permeabilization of phagosomes and  $Ca^{2+}$  release would result in bacterial killing, as a consequence of lysosome fusion. A *Yersinia pseudotuberculosis* strain lacking the YopE and YopH effectors (*yopEH* mutant), previously shown to promote extensive pore formation in mammalian cells through its well-

characterized type III secretion system (17) (fig. S4), was compared with a strain lacking a functional type III secretion apparatus (*ysc* mutant). Assays for surface exposure of Lamp-1 confirmed that *Yersinia* behaves similarly to *Salmonella*, inducing exocytosis of lysosomes in a Syt VII– and type III secretion system–dependent manner (Fig. 4A). Given that these strains of *Y. pseudotuberculosis* are able to survive and slowly replicate in macrophages (22), we investigated the fate of the *yopEH* and *ysc* mutants in Syt VII *+/+* or *-/-* macrophages. The strain expressing a functional type III secretion system, the *yopEH* mutant, showed an initial decrease in intracellular survival in Syt VII *+/+* macrophages (Fig. 4B), similar to what was observed with *Salmonella* (Fig. 1F). Also similar to what was observed with wild-type *Salmonella*, intracellular survival of the *Yersinia yopEH* mutant was enhanced in Syt VII *-/-* macrophages (Fig. 4B). In contrast, when infections were performed with the *ysc* mutant lacking a functional type III secretion system, no significant differences were observed in the numbers of viable intracellular bacteria between Syt VII *+/+* and *-/-* macrophages, from 2 to 12 hours after infection (Fig. 4B, lower panel).

Thus, membrane injury inflicted by type III secretion systems triggers a Syt VII–dependent process that reduces the number of viable intracellular bacteria. We envision that shortly after invasion, recently formed phagosomes are permeabilized by bacterial type III secretion translocos, triggering  $Ca^{2+}$  influx from the intraphagosomal space into the cytosol, Syt VII–dependent phagolysosome fusion, and bacterial killing (fig. S5). In this scenario, it becomes clear how selection of lysosomes as the intracellular vesi-

cles responsible for membrane repair (12) would have provided an additional evolutionary advantage, by protecting eukaryotic cells from pathogen attack.

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23. We are very grateful to E. Caler, J. Galán, J. Kagan, D. Portnoy, C. Roy, and D. Zamboni for help and advice, and to H. Tan for the artwork shown in fig. S5. This work was supported by NIH grants GM64625 and AI34867 and a Burroughs Wellcome Scholar award to N.W.A., NIH grants AI43389 and AI48507 to J.B.B., and by Brown-Coxe (D.R.) and National Research Service Award (D.R.L.) postdoctoral fellowships.

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24 December 2003; accepted 22 April 2004