intact *BAX* genes from NSAID-mediated apoptosis. This prediction was confirmed by introducing a Bcl-X_L expression vector into HCT116 cells and assessing colony formation after treatment with NSAIDs (Fig. 4D).

It was not expected that deletion of a single gene could so profoundly affect cell death in a human cancer cell. Previous experiments have shown that NSAIDs induce heterogeneous changes in human tumor cells, including growth arrest, apoptosis, and necrosis (*21*). Despite this heterogeneity, we found that most colorectal cancer cell lines exhibited markedly similar changes in BAX:Bcl-X_L ratios, whereas the others died through mechanisms that were independent of BAX:Bcl-X_L. Furthermore, the importance of *BAX* was rigorously demonstrated in HCT116 cells through three different approaches for generating cells with disrupted *BAX* genes. In addition to their implications for understanding basic determinants of drug responsiveness in human cancer cells, these results may have important clinical implications. It is currently believed that chemoprevention offers the best hope for nonsurgical management of patients with hereditary predispositions to colorectal cancer. The most common form of such predisposition is hereditary nonpolyposis colorectal cancer, which is caused by defects in mismatch repair (*22*). Our results suggest that such tumors may easily develop resistance to NSAIDs through an inherent instability in the mononucleotide tract in *BAX*. By analogy with the successful strategy used to combat the highly mutable retroviruses that cause acquired immunodeficiency syndrome (*23*), it may therefore be important to consider combinations of chemopreventive drugs, rather than single agents, in such patients.

Note added in proof: It has recently been shown that tumor cells without BAX undergo less apoptosis when xenografted in mice (*27*).

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18. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1055633.shl.
19. An HCT116 clone heterozygous for *BAX* was transfected with the targeting construct (Fig. 2A) using Lipofectamine (Life Technologies, Rockville, MD). Twenty-four hours after transfection, cells were plated at clonal density in 96-well plates in media containing hygromycin (0.1 mg/ml). Hygromycin-resistant clones were expanded and screened for targeting events with PCR. Two PCR-positive clones were recovered from ~5000 hygromycin-resistant clones. Homologous recombination at the *BAX* locus was verified by genomic Southern blotting with the probe indicated in Fig. 2A, sequencing of the G_8-tract and surrounding nucleotides of the remaining *BAX* allele, and analysis of BAX protein expression. Details of the targeting construct and the primers used for PCR are available from the authors upon request.
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A PEST-Like Sequence in Listeriolysin O Essential for *Listeria monocytogenes* Pathogenicity

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Establishment and maintenance of an intracellular niche are critical to the success of an intracellular pathogen. Here, the pore-forming protein listeriolysin O (LLO), secreted by *Listeria monocytogenes*, was shown to contain a PEST-like sequence (P, Pro; E, Glu; S, Ser; T, Thr) that is essential for the virulence and intracellular compartmentalization of this pathogen. Mutants lacking the PEST-like sequence entered the host cytosol but subsequently permeabilized and killed the host cell. LLO lacking the PEST-like sequence accumulated in the host-cell cytosol, suggesting that this sequence targets LLO for degradation. Transfer of the sequence to perfringolysin O transformed this toxic cytolysin into a nontoxic derivative that facilitated intracellular growth.

Intracellular pathogens reside in specific cellular compartments, e.g., a modified phagosome (*Mycobacterium tuberculosis*) (*1*), the Golgi apparatus (*Chlamydia trachomatis*) (*2*), or the cytosol (*L. monocytogenes*) (*3*). How pathogens establish and maintain these intracellular niches is the essence of pathogenesis. Although we know little of the molecular mechanisms by which intracellular pathogens achieve compartmentalization, one emerging theme is that pathogens exploit the existing cellular machinery of the host (*4*).

The secreted pore-forming protein LLO of the facultative intracellular bacterial pathogen *L. monocytogenes* is an essential virulence determinant that allows the bacterium to escape from the host vacuole and reach the host cytosol (*5*). Although LLO is produced by bacteria in both the cytosol and the vacuole, LLO activity is restricted to the vacuolar

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compartment (*6, 7*). In contrast, *L. monocytogenes* that are engineered to produce a related pore-forming protein, perfringolysin O (PFO), from the extracellular pathogen *Clostridium perfringens*, escape from the vacuole but subsequently lyse the plasma membrane and kill the host cell (*8*). Thus, LLO is unique, not in its ability to mediate vacuolar escape, but in its lack of host-cell toxicity.

To identify features of LLO that might mediate its compartment-specific activity, we compared the LLO and PFO primary sequences (*9*). LLO and PFO have 43% sequence identity and 70% sequence similarity. However, LLO contains a 27–amino acid sequence in its NH_2-terminus that is absent from PFO (Fig. 1A). Within this unique region is a 19–amino acid PEST-like sequence (*10*). PEST sequences are thought to target eukaryotic proteins for phosphorylation and/or degradation (*10, 11*) and, more generally, may represent sites of protein-protein interaction. We considered the possibility that the PEST-like sequence of LLO may target this potentially toxic bacterial protein for degradation, and thus inactivation, specifically within the host-cell cytosol.

To determine if the PEST-like sequence of LLO is important for *L. monocytogenes* pathogenicity, we generated a 26–amino acid in-frame deletion that removed the PEST-like sequence (Fig. 1A) and introduced the resulting allele onto the *L. monocytogenes* chromosome in place of the wild-type allele (strain DP-L4042) (*12*). In vitro, the mutant protein (LLOΔ26) exhibited full hemolytic activity (Table 1). However, in a murine model of infection, strain DP-L4042 was four orders of magnitude less virulent than wild-type bacteria (Table 1).

To understand why the mutant bacteria were less virulent in vivo, we examined the interaction of these bacteria with macrophages in vitro. Subsequent to internalization, the mutant bacteria escaped from host phagosomes with an efficiency similar to that of wild-type bacteria (Table 1). However, during the first 6 hours of intracellular growth, wild-type bacteria doubled approximately five times, whereas bacteria producing either LLOΔ26 or PFO showed a 1000-fold decrease in colony forming units (Fig. 1B). In contrast, bacteria that did not produce any cytolysin remained trapped, but viable, in phagosomes of the nonbactericidal J774 cells (Fig. 1B). Because the antibiotic gentamicin was added to the tissue culture media 30 min after bacterial internalization, these results suggest that, like PFO, LLOΔ26 permeabilized the host plasma membrane and consequently allowed gentamicin access to the intracellular bacteria.

Upon visualization of infected macrophages, it was clear that bacteria producing LLOΔ26 were toxic to their host cells in a manner that was inversely correlated to the presence of gentamicin. Thus, in the absence of gentamicin, virtually all macrophages infected with the mutant bacteria were dead by 6 hours after infection, as indicated by their dark condensed nuclei and loss of membrane integrity (Fig. 2C). In contrast, infections carried out in the presence of gentamicin revealed that, although some macrophages infected with bacteria producing LLOΔ26 were dead (~5 to 10%), most macrophages infected with the mutant bacteria appeared viable. However, these viable macrophages contained only 4 to 12 bacteria at time points when macrophages infected with wild-type bacteria contained ~100 bacteria (Fig. 2, B and A, respectively). Given that many mammalian cells are able to patch holes in their plasma membrane (*13*), we speculate that most infected macrophages recovered when gentamicin was present to kill the LLOΔ26-producing bacteria.

To measure cytotoxicity directly, we monitored the release of a host cytosolic enzyme, lactate dehydrogenase (LDH), into the tissue culture medium (*14*). To favor host-cell lysis over rapid bacterial death and host-cell repair, we performed the infections for this assay in the absence of gentamicin. Infection of J774 cells with LLOΔ26-producing bacteria resulted in 90% of the maximal LDH release, compared to only 2% for wild-type bacteria. Thus, LLOΔ26 was inappropriately active within the host-cell cytosol and conse-

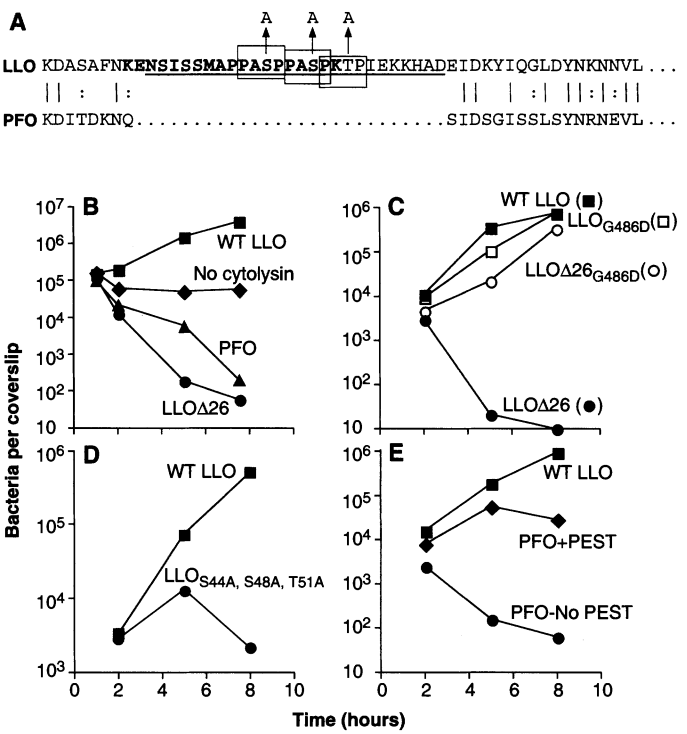

Fig. 1. (**A**) LLO, but not PFO, contains a PEST-like sequence in its NH_2-terminus. In (A), a partial sequence comparison between LLO (top) and PFO (bottom) is shown, starting at the mature NH_2-terminus of each protein (*26*). Identical residues are indicated by vertical lines; similar residues are indicated by two dots. The PEST-like sequence of LLO scored a 4.72 in the PESTFind algorithm (*10*) and is shown in bold. The 26 amino acids removed in LLOΔ26 are underlined. The three consensus MAPK sites are boxed. (**B** to **E**) Growth of *L. monocytogenes* strains in J774 macrophages. Monolayers of J774 cells were grown on glass coverslips (*27*) and infected with bacteria producing the indicated cytolysin at an effective MOI of 1 (B) or 1:10 (C to E). At the specified times after infection, monolayers were lysed, and the number of bacteria per coverslip was determined in triplicate. In each case, gentamicin (50 μg/ml) was added 30 min after bacterial internalization.

Table 1. Properties of bacteria producing either wild-type (WT) or mutant LLO proteins. Hemolytic units are expressed as the reciprocal of the dilution of bacterial culture supernatant required for 50% lysis of a 0.5% sheep red blood cell suspension as described (*8*). Escape from primary phagosomes of murine bone marrow–derived macrophages was determined by using indirect immunofluorescence to count the percentage of bacteria decorated with actin filaments 90 min after infection (*8*). Association with host actin indicates that the bacterium has reached the cytosol. Strain DP-L2161 contains a complete deletion of the LLO gene (*8*). LD_{50}, 50% lethal doses (*25*).

Strain	Cytolysin synthesized	Hemolytic units		Escape from phagosome (%)	LD_{50}
		pH 5.5	pH 7.4		
10403S	WT LLO	1061	114	59 ± 6	2×10^4
DP-L4042	LLOΔ26	1514	243	55 ± 4	2×10^8
DP-L4048	LLO$_{S44A,S48A,T51A}$	1330	151	54 ± 4	3×10^6
DP-L2161	No cytolysin	0	0	0	2×10^9

quently acted on the cytoplasmic membrane.

The observed toxicity of LLOΔ26 may reflect the fact that the 26–amino acid sequence missing from this mutant protein targets wild-type LLO for degradation and, thus, inactivation within the host cytosol. If so, removal of this signal should lead to the accumulation of LLO within the host cytosol. Detection of intracellular LLOΔ26, however, was technically difficult because bacteria synthesizing this protein caused host-cell lysis as early as 2 hours after infection, a time at which there were too few bacteria for recovery and detection of most bacterial proteins. Thus, we introduced a second site mutation in the COOH-terminal domain of LLO [$Gly^{486} \rightarrow Asp^{486}$ (G486D)] based on an analogous substitution in PFO (*15*) that rendered LLO much less active (>100-fold), yet still allowed bacteria to escape, albeit less efficiently, from host phagosomes. Bacteria synthesizing $LLO\Delta26_{G486D}$ were much less toxic to their host cells and were able to replicate intracellularly (Fig. 1C). As a control, we also introduced the G486D substitution into an otherwise wild-type LLO molecule. We immunoprecipitated LLO from infected J774 macrophages that had been metabolically labeled at 5 hours after infection. Two species of intracellular wild-type LLO were detected: full-length mature LLO of 58 kD (as confirmed by comigration with in vitro labeled LLO) and a truncated form of 55 kD, which may represent a degradation product (*7*). We observed the same two species with LLO_{G486D}. In contrast, $LLO\Delta26_{G486D}$ was present inside host cells at substantially higher levels than either wild-type LLO or LLO_{G486D} (Fig. 3B). In addition, we did not detect any degradation products of $LLO\Delta26_{G486D}$. These data do not distinguish between increased intracellular synthesis or decreased intracellular degradation (*16*). However, we did not observe an increased accumulation of LLOΔ26 when the bacteria were grown in broth culture (Fig. 3A), suggesting that the mutant protein was synthesized and secreted normally outside of the host environment (*17*).

PEST sequences often contain internal phosphorylation sites, and phosphorylation at these sites often precedes protein degradation (*11*). LLO contains several potential phosphorylation sites within the 26–amino acid region described above, including three consensus mitogen-activated protein kinase (MAPK) sites (PXS/TP) (*18*) (Fig. 1). To determine whether these sites play a role in inactivating cytosolic LLO, we generated a mutant LLO protein in which all three of the potential phosphate acceptor residues were changed to a residue (Ala) that cannot accept phosphate. In vitro, $LLO_{S44A,S48A,T51A}$ [S44A, $Ser^{44} \rightarrow Ala^{44}$; S48A, $Ser^{48} \rightarrow Ala^{48}$; T51A, $Thr^{51} \rightarrow Ala^{51}$] exhibited hemolytic activity similar to that of the wild-type protein (Table 1), and bacteria synthesizing $LLO_{S44A,S48A,T51A}$ escaped from the primary vacuole with an efficiency similar to that of wild-type bacteria (Table 1). However, in support of our hypothesis, bacteria producing $LLO_{S44A,S48A,T51A}$ were toxic to macrophages in vitro (Fig. 1D) and were 100-fold less virulent in vivo (Table 1). Moreover, like $LLO\Delta26_{G486D}$, LLO_{G486D} lacking the three putative MAPK sites ($LLO_{S44A,S48A,T51A,G486D}$) accumulated to higher levels within infected macrophages than either wild-type LLO or LLO_{G486D} (*19*).

If the PEST-like sequence inactivates LLO within the host cytosol, transfer of this sequence to the related cytolysin PFO should render PFO less toxic to host cells. To test this idea, we generated a protein chimera in which the leader peptide and the first 35 residues of mature LLO (which contain the PEST-like sequence) were fused in-frame to the PFO protein (PFO+PEST) (*20*). To control for possible differences in secretion efficiency between the leader peptides of LLO and PFO, we generated a second protein chimera in which the LLO leader peptide alone was fused in-frame to PFO (PFO–No PEST) (*20*). We tested *L. monocytogenes* strains carrying either the PFO+PEST or the PFO–No PEST allele in place of the wild-type LLO allele for the ability to grow inside macrophages. Bacteria producing the PFO+PEST protein were able to replicate intracellularly and were much less toxic to their host macrophages than bacteria producing the PFO–No PEST protein (Fig. 2, D through F). This rescue was only partial, however, in that bacteria producing PFO+PEST did not grow as well as wild-type bacteria and were still toxic at later time points in the infection (Fig. 1E). Nonetheless, when we quantitated cytotoxicity by monitoring the release of host LDH (*14*), bacteria producing PFO+PEST were much less toxic than those producing the PFO–No PEST control protein (9 versus 97% maximal LDH release, respectively) and only slightly more toxic than wild-type bacteria (2% maximal LDH release).

The PEST-like sequence of LLO is essential for *L. monocytogenes* to establish a pro-

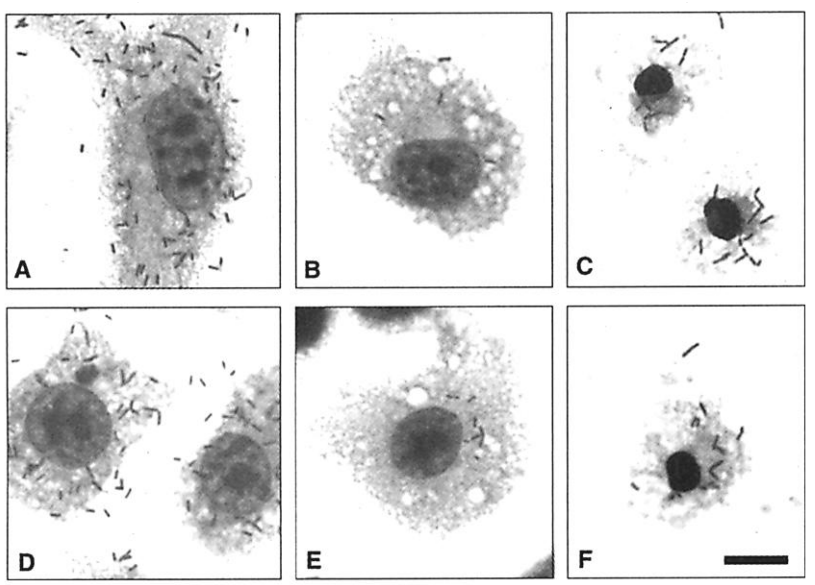

Fig. 2. Light micrographs of J774 macrophages at 6 hours after infection with *L. monocytogenes*. Each panel depicts a macrophage(s) infected with bacteria producing either (**A**) wild-type LLO, (**B** and **C**) LLOΔ26, (**D**) PFO+PEST, or (**E** and **F**) PFO–No PEST at an effective MOI of 1:10. In (C) and (F), macrophages were infected in the absence of gentamicin; in all other panels, gentamicin (50 μg/ml) was added 30 min after bacterial internalization. Scale bar, 10 μm.

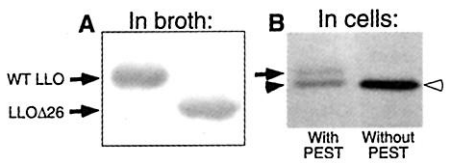

Fig. 3. LLO lacking the PEST–like sequence accumulates to high levels inside, but not outside, of host cells. (**A**) Western blot showing the relative levels of wild-type and LLOΔ26 protein found in the supernatant of bacterial cultures after 5 hours of growth in Luria-Bertani broth. Secreted proteins were isolated as described (*8*) and subjected to Western blot analysis with polyclonal antibodies raised against LLO. Each lane represents supernatant from equivalent numbers of bacteria. (**B**) Autoradiograph showing the relative levels of LLO_{G486D} and $LLO\Delta26_{G486D}$ recovered from infected macrophages. Bacterial proteins were metabolically labeled with [^{35}S]methionine during growth in J774 cells (*7*) at 5 hours after infection. LLO was immunoprecipitated as described (*7*) and subjected to SDS–polyacrylamide gel electrophoresis. Each lane represents a single J774 monolayer (~4×10^6 cells) that contained 7×10^7 DP-L4044 (LLO_{G486D}) or 7.7×10^7 DP-L4045 ($LLO\Delta26_{G486D}$) bacteria. The arrow indicates full-length LLO_{G486D}, the solid arrowhead indicates truncated LLO_{G486D}, and the open arrowhead indicates full-length $LLO\Delta26_{G486D}$.

ductive infection in vivo. Our data suggest that this sequence restricts LLO activity to the host-cell vacuole, thereby preserving the intracellular niche of *L. monocytogenes*. Perhaps rapid host-cell lysis by LLOΔ26 exposes the normally intracellular bacteria to extracellular host defenses such as humoral immunity and bactericidal phagocytes.

LLO and PFO are members of a large family of pore-forming proteins (*21*), but LLO is the only one to be produced by an intracellular pathogen. Our data suggest that the addition of a simple sequence tag to a toxic pore-forming protein can convert it into a molecule specialized for intracellular use. Moreover, because intracellular pathogens often use host-cell machinery for their own purposes, *L. monocytogenes* may achieve the critical balance between efficient escape from a vacuole and avoidance of host-cell damage by incorporating a eukaryotic protein degradation signal into a potentially toxic bacterial virulence factor.

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26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.
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Role of *Bacillus subtilis* SpoIIIE in DNA Transport Across the Mother Cell–Prespore Division Septum

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The SpoIIIE protein of *Bacillus subtilis* is required for chromosome segregation during spore formation. The COOH-terminal cytoplasmic part of SpoIIIE was shown to be a DNA-dependent adenosine triphosphatase (ATPase) capable of tracking along DNA in the presence of ATP, and the NH_2-terminal part of the protein was found to mediate its localization to the division septum. Thus, during sporulation, SpoIIIE appears to act as a DNA pump that actively moves one of the replicated pair of chromosomes into the prespore. The presence of SpoIIIE homologs in a broad range of bacteria suggests that this mechanism for active transport of DNA may be widespread.

Despite decades of studies, few proteins directly involved in chromosome segregation in bacteria have been identified, and mechanistic information about these proteins is meager. In *Bacillus subtilis*, the newly replicated regions of the chromosome are actively and rapidly separated, but how this movement is achieved is unclear (*1–3*). Cell division at the onset of spore formation in *B. subtilis* provides an interesting system for studying chromosome movement. Here, an asymmetrically positioned division septum is formed before the completion of chromosome segregation and closes around one of the pair of replicated chromosomes, pinching it into a larger and a smaller lobe. The larger chromosome lobe is then transported from the mother cell into the prespore, presumably through a small pore in the septum. In the absence of a functional SpoIIIE protein, DNA transfer is blocked (*4*).

During spore formation, SpoIIIE is targeted to the leading edge of the septum. The putative transmembrane domain at the NH_2-terminal part of the protein appears to play an essential role in this specific localization (*5*). The strategic location of the protein suggests

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