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.

References and Notes

1. D. L. Clemens, *Trends Microbiol.* **4**, 113 (1996).
2. T. Hackstadt, M. A. Scidmore, D. D. Rockey, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4877 (1995).
3. L. G. Tilney, D. A. Portnoy, *J. Cell Biol.* **109**, 1597 (1989).
4. B. B. Finlay, P. Cossart, *Science* **276**, 718 (1997).
5. D. A. Portnoy, T. Chakraborty, W. Goebel, P. Cossart, *Infect. Immun.* **60**, 1263 (1992).
6. M. S. Villanueva, A. J. Sijts, E. G. Pamer, *J. Immunol.* **155**, 5227 (1995).
7. M. A. Moors, B. Levitt, P. Youngman, D. A. Portnoy, *Infect. Immun.* **67**, 131 (1999).
8. S. Jones, D. A. Portnoy, *Infect. Immun.* **62**, 5608 (1994).
9. The two sequences can be found by SWISS-PROT accession numbers P13128 (LLO) and P19995 (PFO).
10. S. Rogers, R. Wells, M. Rechsteiner, *Science* **234**, 364 (1986).
11. M. Rechsteiner, S. W. Rogers, *Trends Biochem. Sci.* **21**, 267 (1996).
12. Mutant alleles of the LLO gene (*hly*) were generated by oligonucleotide-directed mutagenesis using splice overlap extension (SOE) polymerase chain reaction (PCR) (*22*) and introduced onto the *L. monocytogenes* chromosome by allelic exchange (*23*). In all cases, the starting template for PCR was chromosomal DNA isolated from wild-type *L. monocytogenes* (strain 10403S). For each resulting strain, DP-L4042 (LLOΔ26), DP-L4044 (LLO_{G486D}), DP-L4045 ($LLO\Delta26_{G486D}$), and DP-L4048 ($LLO_{S44A,S48A,T51A}$), the LLO allele was confirmed by sequencing.
13. P. L. McNeil, R. A. Steinhardt, *J. Cell Biol.* **137**, 1 (1997).
14. J774 macrophages were grown in a 96-well plate and infected (or mock infected) with the appropriate bacterial strain at a multiplicity of infection (MOI) resulting in approximately one to two bacteria/macrophage. Five hours after infection, supernatant from each well was removed and assayed for LDH activity by using the CytoTox 96 kit (Promega, Madison, WI). Percent cytotoxicity = 100 × [(experimental LDH release) − (spontaneous LDH release)]/[(maximum LDH release − (spontaneous LDH release)]. The numbers reported were calculated with the mean LDH release from eight wells of a single experiment and are representative of two independent experiments.
15. S. Jones, K. Preiter, D. A. Portnoy, *Mol. Microbiol.* **21**, 1219 (1996).
16. For technical reasons, we were unable to measure half-lives for wild-type LLO or LLO_{G486D}.
17. LLO has an acidic pH optimum that has been hypothesized to compartmentalize intracellular hemolytic activity by exploiting the difference in pH between the vacuole (acidic) and cytosol (neutral) of the host (*8*). In vitro, LLOΔ26 was two times as active at neutral pH as the wild-type protein (Table 1). Thus, it is possible that increased activity at neutral pH was responsible for the toxic phenotype of bacteria producing LLOΔ26. However, bacteria producing a mutant LLO protein exhibiting hemolytic activity similar to that of wild-type LLO at pH 5.5, but 9 to 10 times that at pH 7.4, were not toxic to macrophages (*24*). Moreover, a second protein mutant in the PEST-like region ($LLO_{S44A,S48A,T51A}$) did not exhibit increased hemolytic activity at pH 7.4 (Table 1), yet bacteria synthesizing this mutant protein were also toxic to host cells. Thus, we think it unlikely that altered pH optimum is the cause of toxicity by LLOΔ26.
18. F. A. Gonzalez, D. L. Raden, R. J. Davis, *J. Biol. Chem.* **266**, 22159 (1991).
19. A. L. Decatur, D. A. Portnoy, data not shown.
20. SOE PCR (*22*) was used to join the LLO and PFO coding sequences to generate gene fusions *hly*1-59::*pfo*37-500 (PFO+PEST) and *hly*1-24::*pfo*29-500 (PFO–No PEST) using either wild-type *L. monocytogenes* chromosomal DNA (strain 10403S) or pDP2169 plasmid DNA (*8*) as template. For the PFO+PEST fusion, we chose to fuse the PEST-like sequence of LLO to the 37th residue of PFO because it is at this residue (isoleucine) that PFO resumes strong sequence similarity to LLO (Fig. 1A). For the PFO–No PEST fusion, LLO's signal sequence was fused to full-length mature PFO. The gene fusions were introduced onto the *L. monocytogenes* chromosome by allelic exchange (*23*), yielding strains DP-L4052 (PFO+PEST) and DP-L4055 (PFO–No PEST). In each case, the genotype was confirmed by sequencing.
21. J. E. Alouf, C. Geoffroy, in *Sourcebook of Bacterial Protein Toxins*, J. E. Alouf, J. H. Freer, Eds. (Academic Press, London, 1991), pp. 147–186.
22. S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989).
23. N. E. Freitag, in *Gram-Positive Pathogens*, V. A. Fischetti *et al.*, Eds. (ASM Press, Washington, DC, 2000), chap. 51.
24. M. Gedde, D. A. Portnoy, unpublished data.
25. LD_{50} values for DP-L4042 and DP-L4048 were determined as described (*8*), by using groups of four BALB/c mice for each bacterial dilution.
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.
27. S. Jones, D. A. Portnoy, *Methods Enzymol.* **236**, 463 (1994).

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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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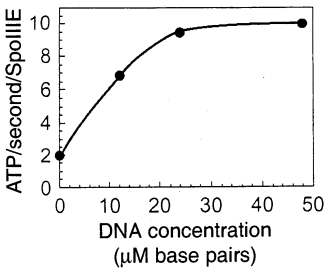

Fig. 1. The rate of ATP hydrolysis in the presence of various concentrations of supercoiled plasmid DNA. The hexahistidine-tagged SpoIIIE protein (0.7 μg/ml or 9.9 nM final concentration) was used in these measurements. Replacing this affinity tag by a streptavidin affinity tag (using recombinant protein expressed from pSG4909) did not significantly alter the ATPase activity. See supplementary material (*6*) for experimental details of the ATPase assay.

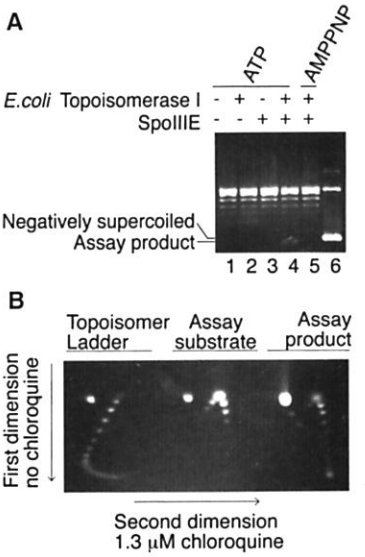

Fig. 2. Generation of positive and negative DNA supercoils by SpoIIIE. (**A**) Relaxed plasmid DNA (lane 1) was incubated with various combinations of SpoIIIE, *E. coli* DNA topoisomerase I, and ATP, as indicated above lanes 2 to 5. Reactions were terminated by phenol extraction, and the products were separated on a 0.7% agarose gel. Lane 6 contained untreated negatively supercoiled plasmid. The position of a product generated by incubation of the relaxed plasmid with SpoIIIE, *E. coli* DNA topoisomerase I, and ATP (lane 4) is indicated in the left margin. This product was not detected if any component of the reaction was omitted, or when ATP in the complete assay mixture was replaced with the nonhydrolyzable β,γ-imido analog AMPPNP. (**B**) Two-dimensional gel electrophoresis of mixtures of DNA topoisomers. (Left) The reference topoisomer mixture, generated by treating negatively supercoiled plasmid with *E. coli* DNA topoisomerase I in the presence of varying amounts of ethidium bromide, was resolved into an arc of topoisomers with their linking numbers increasing in the clockwise direction along the arc. The bright spot to the left of the arc in this and the other panels contained nicked DNA. (Middle) Distribution of topoisomers in the assay substrate before incubation with SpoIIIE, *E. coli* DNA topoisomerase I, and ATP. (Right) The distribution of DNA topoisomers in the assay product. Highly positively supercoiled topoisomers, in addition to the relaxed topoisomers in the starting DNA substrate, were formed upon incubation of the assay substrate with SpoIIIE, *E. coli* DNA topoisomerase I, and ATP. See the supplementary material (*6*) for experimental details.

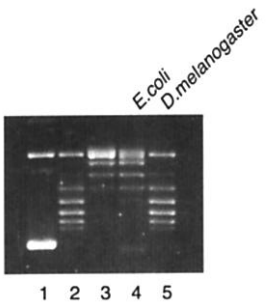

Fig. 3. A test of two alternative mechanisms of SpoIIIE-mediated supercoiling of DNA. Relaxed plasmid DNA (lane 3) was incubated with SpoIIIE, ATP, and either *E. coli* DNA topoisomerase I (lane 4) or *D. melanogaster* DNA topoisomerase I (lane 5). A fast-migrating positively supercoiled product was formed in the presence of the *E. coli* enzyme (compare lanes 3 and 4) but not in the presence of the *D. melanogaster* enzyme (lane 5). Lane 1 contained a sample of negatively supercoiled DNA, and lane 2 the same DNA after treatment with *D. melanogaster* DNA topoisomerase I. The difference in the distribution of topoisomers in the relaxed DNA samples run in lanes 2 and 5 was owing to the use of different buffers in the relaxation of the DNA by vaccinia virus topoisomerase and by *Drosophila* DNA topoisomerase I.

that it could mediate chromosome segregation by actively transporting the bulk of the chromosome destined for the prespore through the septum, acting as a DNA pump, or, alternatively, the protein could serve the role of a pore, through which DNA is driven by another effector (such as a DNA-condensing protein). To gain further insight into whether SpoIIIE can directly drive DNA movement across the septum, the COOH-terminal cytoplasmic portion of the protein was overexpressed and purified for biochemical studies.

A recombinant SpoIIIE fragment containing residues 177 to 787 of the intact protein plus a hexahistidine tag at the COOH-terminus was overexpressed and purified to near homogeneity [see supplementary material (*6*)]. Because the sequence of the protein suggested the presence of a nucleotide-binding motif (*5*), we first tested the purified protein for ATPase activity. The recombinant protein was indeed found to be a DNA-dependent ATPase (Fig. 1). In the absence of DNA, about two ATPs were hydrolyzed per SpoIIIE monomer per second. The presence of increasing amounts of DNA increased the rate of ATP hydrolysis to a plateau of about 10 ATPs per SpoIIIE monomer per second (Fig. 1). This experiment was repeated with purified SpoIIIE fragment carrying a mutation at codon 473, a lysine to alanine substitution (*7*). Replacing the conserved Lys^{473} in the nucleotide-binding motif of the protein with alanine is known to abolish SpoIIIE function in vivo (*7*). The mutant protein showed no detectable ATPase activity. Thus, SpoIIIE has functionally important ATPase activity.

The possibility that SpoIIIE might use ATP hydrolysis to effect relative movement between the protein and a DNA bound to it, along the longitudinal axis of the DNA, was then addressed. As a protein tracks along DNA, positive supercoils may accumulate in the region of the DNA ahead of the protein, and negative supercoils may be left in its wake (*8*). In the presence of bacterial DNA topoisomerase I, which removes negative, but not positive, supercoils (*9*), tracking of a protein along a DNA may thus yield a positively supercoiled DNA (*10–13*). Incubation of relaxed DNA with various combinations of SpoIIIE, *Escherichia coli* DNA topoisomerase I, and ATP was carried out, and the reaction products were analyzed by agarose gel electrophoresis (Fig. 2A). In the presence of SpoIIIE, *E. coli* DNA topoisomerase I, and ATP, a DNA product was generated that migrated slightly faster than the negatively supercoiled marker (Fig. 2A). The mobility of this product was consistent with its being positively supercoiled, and this identity was confirmed by two-dimensional gel electrophoresis (Fig. 2B). In the presence of SpoIIIE, *E. coli* DNA topoisomerase I, and ATP, the assay product contained highly positively supercoiled DNA topoisomers, in addition to the input relaxed topoisomers (Fig. 2B). In this and similar experiments, the positively supercoiled product accounted for 10 to 20% of the input DNA. No positively supercoiled DNA was produced in reactions in which either SpoIIIE or *E. coli* DNA topoisomerase I was omitted (Fig. 2A). Replacing ATP by its nonhydrolyzable β,γ-imido analog AMPPNP again abolished the formation of the positively supercoiled product (Fig. 2A), suggesting that ATP hydrolysis is required in this reaction.

Two types of mechanisms can account for the accumulation of positive supercoils in the DNA in these reactions. In one, SpoIIIE tracks along DNA to generate supercoils; in the other, the protein stoichiometrically binds DNA in a way that alters its writhe and/or twist. Whereas positive and negative supercoils generated by tracking are accessible to a topoisomerase, local changes in the writhe and/or twist of a DNA segment by its binding to a protein would be constrained by the protein and could not be altered by a topoisomerase. In the latter case, formation of the positively supercoiled product in the SpoIIIE reaction would persist even if *E. coli* DNA topo-

isomerase I is replaced by eukaryotic DNA topoisomerase I, which can remove unconstrained positive and negative supercoils (*14*). No positively supercoiled product was generated when a relaxed plasmid was incubated with SpoIIIE, ATP, and *Drosophila* DNA topoisomerase I (Fig. 3); nevertheless, a positively supercoiled product was again observed in the reaction with SpoIIIE, ATP, and *E. coli* DNA topoisomerase I. The failure of *Drosophila* DNA topoisomerase I to substitute for the *E. coli* enzyme demonstrated that the positive supercoils generated by SpoIIIE were unconstrained, which is consistent with a mechanism in which SpoIIIE would generate positive supercoils by ATP-dependent tracking along the DNA.

The ability of SpoIIIE to transport DNA from the mother cell to the prespore presumably depends not only on the ability of its COOH-terminal part to track along DNA but also on the specific localization of the protein to the cell division septum. In sporulating cells expressing residues 2 to 183 of SpoIIIE fused to the green fluorescent protein, the fusion protein was seen to localize to the division septa in both vegetative and sporulating cells, indicating that the segment comprising residues 2 to 183 of SpoIIIE is sufficient for the specific localization of the protein [see Web fig. 1 (*6*)].

Although SpoIIIE is not essential for vegetative growth of *B. subtilis*, it is required under conditions where chromosome segregation is not complete at the time of septation (*15*). The DNA tracking activity described here could facilitate the clearance of DNA from the septum, allowing cell separation without chromosome breakage. The COOH-terminal and, to a lesser extent, the NH_2-terminal domains of SpoIIIE are conserved across a broad range of bacteria, suggesting that these organisms may use a similar mechanism for moving chromosomal DNA away from division septa. SpoIIIE is also weakly homologous to the Tra proteins encoded by mobile plasmids of various Gram-positive bacteria (*7*). It is plausible that the Tra proteins catalyze DNA transfer between donor and recipient cells.

References and Notes

1. P. J. Lewis, J. Errington, *Mol. Microbiol.* **25**, 945 (1997).
2. C. D. Webb *et al.*, *Cell* **88**, 667 (1997).
3. G. S. Gordon *et al.*, *Cell* **90**, 1113 (1997).
4. L. J. Wu, J. Errington, *Science* **264**, 572 (1994).
5. ———, *EMBO J.* **16**, 2161 (1997).
6. Supplementary material is available to *Science* Online subscribers: www.sciencemag.org/feature/data/1054624.shl.
7. L. J. Wu, P. J. Lewis, R. Allmansberger, P. M. Hauser, J. Errington, *Genes Dev.* **9**, 1316 (1995).
8. L. F. Liu, J. C. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7024 (1987).
9. J. C. Wang, *J. Mol. Biol.* **55**, 523 (1971).
10. Y. P. Tsao, H. Y. Wu, L. F. Liu, *Cell* **56**, 111 (1989).
11. L. Yang, C. B. Jessee, K. Lau, H. Zhang, L. F. Liu, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6121 (1988).
12. E. A. Ostrander, P. Benedetti, J. C. Wang, *Science* **249**, 1261 (1990).
13. H. S. Koo, L. Claassen, L. Grossman, L. F. Liu, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1212 (1991).
14. J. J. Champoux, R. Dulbecco, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 143 (1972)
15. M. E. Sharpe, J. Errington, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8630 (1995).

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