because a single locus could retain higher than expected levels of diversity. In particular, certain positions in the mammalian D loop appear hypermutable. In our humpback analysis, for example, some positions changed four to eight times. Because these positions contribute disproportionately to intraspecific diversity, they may inflate θ. Removing the 14 sites with more than three intraspecific changes reduces diversity by about 25 to 33%, suggesting that this may be a source of error. Given the idiosyncratic features of this single locus, data from more loci are required before a fully accurate estimate of historical populations is possible. Unfortunately, no comparable nuclear data sets are yet available (9). Yet even if we assume that diversity is reduced by 50%, and rely on the lowest 95% confidence limit, our estimate of humpback populations would be about 75,000. Populations would also be halved if generation time estimates were doubled, but errors of this magnitude are unlikely. To bring our results completely in line with historical humpback population sizes of approximately 20,000 requires generation times of more than 45 years plus a substitution rate nearly four times higher than estimated (Fig. 2).

The genetic diversity of humpback, minke, and fin whales is inconsistent with the low historical population sizes currently assumed (9). The discrepancy of these values represents a crucial challenge. To reconcile these results requires genetic analyses of additional loci; more information about South Atlantic populations; and reevaluation of the time period, severity, and demographic impacts of North Atlantic whaling.

Reconciling these numbers is crucial, because the possibility that vast cetacean populations existed across deep ecological time has fundamental implications not only for their management but also for our perception of the world’s oceans. In its Revised Management Procedure, the International Whaling Commission (IWC) states, “catches should not be allowed on stocks below 54% of the estimated carrying capacity” (27). Genetic data cannot be used alone to define carrying capacity, because effective population sizes are often orders of magnitude lower than population censuses (5, 7, 9, 24), but they can be useful in setting a lower limit to these values. In light of our findings, current populations of humpback or fin whales are far from harvestable. Minke whales are closer to genetically defined population limits, and hunting decisions regarding them must be based on other data.

References and Notes
9. See supporting material on Science Online.
18. For juvenile abundance, we conservatively assumed that animals in year classes 1, 2, 3, 4, and 5 were equally abundant.
19. Based on annual female survival of 96% (40) over a reproductive life of 60 years.
27. IWC documents are available at www.iwcoffice.org/Estimate.htm.
35. J. Roman, S. R. Palumbi, data not shown.

Supporting Online Material
www.sciencemag.org/cgi/content/full/301/5632/508/DC1
Materials and Methods
Fig. S1
References
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Cannibalism by Sporulating Bacteria
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Sporulation by the bacterium Bacillus subtilis is an elaborate developmental process that is triggered by nutrient limitation. Here we report that cells that have entered the pathway to sporulate produce and export a killing factor and a signaling protein that act cooperatively to block sister cells from sporulating and to cause them to lyse. The sporulating cells feed on the nutrients thereby released, which allows them to keep growing rather than to complete morphogenesis. We propose that sporulation is a stress-response pathway of last resort and that B. subtilis delays a commitment to sporation by cannibalizing its siblings.

Some microorganisms respond to nutritional limitation by entering a resting state in which they remain inactive for an extended time. Bacillus subtilis produces a robust resting cell, the endospore, that can remain dormant for many years. Endospore formation is an elaborate and energy intensive process that requires several hours to complete (1–4). If during this period nutrients were once again to become plentiful, the sporulating cells would be at a disadvantage relative to cells able to resumed growth rapidly. Thus, bacteria could be expected to delay sporation until forced to do so by prolonged depletion of nutrients. Here we present evidence that cells that have entered the pathway to sporulate delay development by killing their siblings and feeding on the nutrients thereby released. Cannibalism is mediated by an extracellular killing factor and a novel intercellular signaling protein that act cooperatively to cause cell death and impede sporulation.
Entry into sporulation is governed by the regulatory protein Spo0A (5). While building mutants of genes under the control of Spo0A (6), we discovered two operons (Fig. 1A) that are strongly induced at the start of sporulation (fig. S1) and in which mutations accelerated sporulation (fig. 1B and C, and fig. S2). We refer to these operons as skf for sporulation killing factor and sdp for sporulation delaying protein.

Clauses that the eight-gene skf operon directs the production of the exported killing factor came from the similarity of its gene products to proteins involved in the production of peptide antibiotics (7–9). The first gene, skfA, encodes a small peptide, a characteristic of operons involved in the production of peptide antibiotics (7). The product of the second gene, skfB, is similar to a B. subtilis protein involved in the production of an antilisterial peptide, subtilosin (10). Finally, the product of skfD contains a domain characteristic of the CAAAX family of amino terminal proteases (11, 12). The operon also contains two genes, skfE and skfF, whose products resemble an ATP–binding cassette transport complex (ABC transporter) and could be responsible for exporting the peptide antibiotic and conferring resistance to it.

To investigate these possibilities, we asked whether wild-type cells would kill cells of a mutant of the skf operon that had been marked with a lacZ fusion. Mutant and wild-type cells were mixed in equal proportions and grown in liquid sporulation medium. The ratio of mutant to wild-type cells remained approximately constant during growth but dropped dramatically after the onset of sporulation (Fig. 2A). These results indicate that the skf operon is involved in the production of an extracellular killing factor during sporulation. The operon must also confer resistance to the factor, because the mutation rendered cells sensitive to it. In keeping with these ideas, cells engineered to express the skf operon during growth in response to IPTG (isopropyl β-D-1-thiogalactopyranoside) caused killing when spotted on a lawn of wild-type or skf mutant cells and did so in a manner that was dependent on the presence of the IPTG inducer (Fig. 2B). Evidence that skfE and skfF encode an export pump for the killing factor came from placing the genes under the control of an IPTG-inducible promoter and introducing the construct into a strain that lacked the skf operon. The mutant cells were mixed with wild-type cells (tagged with lacZ) and grown in liquid sporulation medium. The number of mutant cells decreased in the number of viable cells, and, of note, in a manner that was dependent on skf/ Fig. 2D and fig. S3). The simplest interpretation of these results is that the wild type produces a mixed population in which Spo0A is active (and directing transcription of skf) in some cells and not in others (fig. S4 and (13, 14)). Cells with active Spo0A would produce the killing factor and the pump that exports it. Cells with inactive Spo0A would produce neither the factor nor the pump, and they would be killed. Thus, the killing factor is responsible for killing genetically identical cells (siblings) in the population. This is contrary to the traditional paradigm of chemical warfare among microorganisms in which antibiotics are used to kill other, competing species.

Why do colonies of skf mutant cells exhibit accelerated sporulation (15)? We suggest that the killing factor causes cells in which Spo0A is inactive to lyse and release nutrients, which al-

Fig. 1. Mutants of the skf and sdp operons sporulate rapidly. (A) Gene organization of the skfab-cefgdfgh and the sdpABC operons (previously annotated as yycOPST yjbABDE and as ywaWXY, respectively (23)). The hairpin symbols represent transcriptional terminators. (B) Colonies of skfS[ΔskfABCDEF]:tet; strain EG168 and sdp[ΔsdpABC]:spc; strain EG407 mutants, as well as the double skf sdp mutant (EG523), were brighter (an indication of sporulation formation, see fig. S2) than those formed by the wild-type strain (PY79) after 14 hours of incubation on solid sporulation medium. Strains and plasmids used in our experiments are listed in table S2. (C) Time course of spor formation in solid medium by the wild-type (●), and the skf (▲), sdp (▲) and skf sdp (●) mutants. The percentage of heat-resistant, colony-forming units (spores) versus total viable cells was monitored at the indicated times after inoculation in solid sporulation medium.

Fig. 2. The skf operon produces a sporulation killing factor. (A) skf mutant cells harboring a lacZ fusion [ΔskfABCDEF]:tet amyE:cotD-lacZ; mE169 and wild-type cells (PY79) were mixed in equal proportions and grown in liquid sporulation medium. The ratio of mutant to wild-type cells was determined at the indicated times before and after the start of sporulation (hour 0, ●). As a control, ratios were determined for wild-type cells that had been mixed with wild-type cells that carried a lacZ fusion (strain PE29, ●) and for skf mutant cells (EG168) that had been mixed with skf mutant cells that carried a lacZ fusion (EG169, ▲). In these mixed cultures, the total number of viable cells during the time course was similar to that for individual cultures of the wild type or the skf mutant shown in (D). (B) Cells harboring the skf operon under the control of an IPTG-inducible promoter (strain EC208) were spotted on a lawn of wild-type or skf mutant cells growing on a rich (nonsporulation, Luria broth) medium. The enhanced growth seen in the presence (+) but not in the absence (−) of the inducer (1 mM IPTG). (C) Cells lacking the skf operon but containing a copy of skfE and skfF under the control of an IPTG-inducible promoter (strain EC219) were mixed in equal proportion with wild-type cells that carried a lacZ fusion (PE29), and the cell mixture was grown in liquid sporulation medium in the absence (●) or in the presence (▲) of the inducer (IPTG). The ratio of cells of strain EC219 to the cells of strain PE29 was determined at the indicated times after the start of sporulation. (D) Number of viable cells was measured in cultures of wild-type cells (●), and cells of skfA (EG163) (▲) and skfABCDEF (EG168) (▲) mutants in liquid sporulation medium at the indicated times after the start of sporulation.
low cells in which Sp00A is active but which have not yet committed to morphogenesis to keep growing. An sfp mutant, in contrast, does not cause killing and hence sporulation takes place without delay.

**Fig. 3.** The sdp operon encodes an extracellular signaling protein. (A) Wild-type cells and sdp mutant cells containing a *PyvbA-yvaZ-lacZ* fusion (EG381 and EG524, respectively) were grown on solid sporulation medium containing X-gal (5-bromo-4-chloro-3-indolyl B-D-galactopyranoside). (B) Cells of the sdp mutant harboring *PyvbA-yvaZ-lacZ* (EG524, white arrows) were streaked on solid sporulation medium in the vicinity of a streak of wild-type (strain PY79, left) or sdp mutant cells (EG407, right). (C) Eluates from reversed-phase chromatography of supernatant fluids from cultures of wild-type and sdp mutant (EG407) cells (at hour 1.5 of sporulation in liquid medium) were subjected to SDS-polyacrylamide electrophoresis in a 4 to 20% gradient gel. (D) Supernatant fluids were collected from cells of a strain (EG351) in which the sdp operon was under the control of an IPTG-inducible promoter and grown in the absence (●) or in the presence (●) of the inducer (IPTG). Eluates from reversed-phase chromatography of the supernatant fluids from the cultures were added to cells of an sdp mutant harboring *PyvbA-yvaZ-lacZ* (EG524) growing in minimal medium. Culture samples were collected at the indicated times and assayed for β-galactosidase activity.

Mutations in a second operon, *sdp* (Fig. 1A), also caused an accelerated sporulation phenotype and did so more rapidly than mutations in *sfp* (Fig. 1C and fig. S2). What is the mechanism by which this three-gene operon delays spore formation? To answer this question, we carried out microarray analysis to identify genes whose transcription was dependent on the operon (fig. S5 and table S1). Two genes whose transcription was strongly dependent on *sdp* were *yvbA* (whose inferred product is similar to the ArsR family of transcriptional regulators) and *yvaZ* (whose product is of unknown function but is inferred to contain multiple transmembrane segments). The *yvbA* and *yvaZ* genes constitute an apparent operon that is located immediately downstream of, and in convergent orientation to, the *sdp* operon itself (Fig. 1A). The use of lacZ fused to the promoter for *yvbA* and *yvaZ* (*P*~*yvbA*-yvaZ-lacZ) confirmed that transcription of the operon was almost completely dependent on *sdp* (Fig. 3A).

Remarkably, this dependence was mediated by intercellular signaling. Expression of *P*~*yvbA*-yvaZ-lacZ in cells mutant for *sdp* was restored when the mutant cells were grown in close proximity to wild-type cells on solid medium (Fig. 3B). No restoration of lacZ expression was observed when the mutant was grown close to cells mutant for *sdp*. Evidently, *sdp* is responsible for the production of an extracellular factor that is capable of inducing the transcription of *yvbA* and *yvaZ* in recipient cells. We purified from conditioned medium from a culture of wild-type cells a fraction containing a 5-kD protein that stimulated β-galactosidase synthesis when added to cells of an *sdp* mutant that harbored *P*~*yvbA*-yvaZ-lacZ [Fig. 3C (and (16))]. Neither the stimulatory activity nor the protein was present in conditioned medium from *sdp* mutant cells. A protein of similar size was obtained with cells engineered to express the *sdp* operon during growth in response to IPTG. Again, the fraction containing this protein stimulated β-galactosidase production (Fig. 3D).

Finally, sequential Edman degradation (GLYAV-VAGLYVVVNGAALQTAAAY) (12) revealed that the ~5-kD protein originated from the product of the *sdpC* gene of the operon, its N-terminal residue corresponding to residue 141 of the 203-residue-long protein.

Next, we asked whether induction of the *yvbA yvaZ* operon, and *yvbB* in particular, was responsible for the delay in sporulation caused by the signaling protein by engineering cells to express *yvbA* or *yvaZ* or both in response to IPTG. The results show that artificial induction of *yvbA* and *yvaZ* or of *yvbB* alone (but not *yvaZ* alone) was sufficient to delay sporulation (Fig. 4A). Transcriptional profiling with cells mutant for the *sdp* operon revealed candidates for genes that could be under the control of the YvbA transcription factor (above; fig. S5 and table S1). Among these were the ATP synthase operon (*atpB-EFHAGDC*), which is responsible for ATP synthesis.
production, and the yusLJK operon, whose inferred products are similar to lipid catabolism enzymes (fig. S5 and table S1). Use of lacZ fused to yusLJK confirmed that high-level expression of the operon was dependent on the signaling protein and on YvbA (Fig. 4B). Also, artificial induction of YvbA synthesis restored the expression of yusLJK to cells doubly mutant for the sdp and yvbA ysvZ operons (Fig. 4B). We propose that the signaling protein turns on the synthesis of YvbA, which, in turn, causes an increase in lipid oxidation and ATP production. The proposed increase in energy production could be responsible for delaying sporulation, which is triggered by depletion of energy reserves.

Finally, and coming full circle, we found that artificial induction of YvbA synthesis caused a marked drop in cell viability in a manner that was dependent on the sdp operon (Fig. 4C). Evidently, synthesis of the YvbA transcription factor causes enhanced sensitivity to the sporulation killing factor. It could do so by stimulating the expression of genes involved in energy production, as metabolically active cells are more sensitive to antibiotics than are quiescent cells (17, 18). Also, yvbA was previously identified in a screen for genes that inhibit the expression of the gene for σW, a regulatory protein that turns on genes involved in detoxification and resistance to antibiotics (16, 19, 20). Thus, YvbA-mediated repression of the gene for σW could heighten sensitivity to the killing factor by suppressing the antibiotics stress response.

We conclude that sporulating cells of B. subtilis are cannibalistic, feeding on their siblings in order to delay committing to spore formation. Because sporulation becomes irreversible after its earliest stage, delaying spore formation as long as possible might be beneficial, as a cell that is committed to spore formation could be at a disadvantage relative to other cells should nutrient deprivation prove to be fleeting. Wild (but not laboratory) strains have been found to assemble into multicellular structures in which spore formation preferentially takes place at the apical tips (21). Perhaps the killing factor and signaling protein influence the timing and localization of spore formation in these fruiting-body-like structures. Fruiting body formation by the unrelated spore-forming bacterium Myxococcus xanthus is reported to involve lysis of nonsporulating cells (22). Conceivably, this killing is mediated by cells in the developing fruiting body that have entered the pathway to sporulate. It will be interesting to see whether the killing of genetically identical siblings is a widespread feature of the dynamics of bacterial populations.

References and Notes


12. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; 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; W, Trp; X, any amino acid; and Y, Tyr.


15. Of particular interest, accelerated sporulation was observed to be restricted to solid but not in liquid medium; see supporting text on Science Online.

16. J. E. González-Pastor, E. Hobbs, R. Losick, data not shown.


24. We are grateful to S. Ben-Yehuda for the sdpC mutant and contributing to its characterization; P. Eichenberger and J. M. Ranz for help with the DNA microarray experiments; W. Lane for Edman sequencing; and A. L. Sonenshein, P. Piggot, D. Harte, D. Haig, A. Murray, M. Fujita and members of the Losick laboratory for helpful advice. This work was supported by NIH grant GM18568 to R.L.J.E.G.-P. was supported by a Ministerio de Educación y Ciencia Postdoctoral Fellowship [Spain]. E.C.H. was supported by an NSF Graduate Research Fellowship.

Supporting Online Material

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Material and Methods

SOM Text

Sigs. 51 to 55

Tables S1 and S2

References

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VDAC2 Inhibits BAK Activation and Mitochondrial Apoptosis

Emily H.-Y. Cheng, Tatiana V. Sheiko, Jill K. Fisher, William J. Craigen, Stanley J. Korsmeyer*

The multidomain proapoptotic molecules BAK or BAX are required to initiate the mitochondrial pathway of apoptosis. How cells maintain the potentially lethal proapoptotic effector BAK in a monomeric inactive conformation at mitochondria is unknown. In viable cells, we found BAK complexed with mitochondrial outer-membrane protein VDAC2, a VDAC isoform present in mammalian cell death pathway. The upstream

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The multidomain proapoptotic molecules BAK or BAX are required to initiate the mitochondrial pathway of apoptosis. How cells maintain the potentially lethal proapoptotic effector BAK in a monomeric inactive conformation at mitochondria is unknown. In viable cells, we found BAK complexed with mitochondrial outer-membrane protein VDAC2, a VDAC isoform present in low abundance that interacts specifically with the inactive conformer of BAK. Cells deficient in VDAC2, but not cells lacking the more abundant VDAC1, exhibited enhanced BAK oligomerization and were more susceptible to apoptotic death. Conversely, overexpression of VDAC2 selectively prevented BAK activation and inhibited the mitochondrial apoptotic pathway. Death signals activate “BH3-only” molecules such as BID, BIM, or BAD, which displace VDAC2 from BAK, enabling homo-oligomerization of BAK and apoptosis. Thus, VDAC2, an isoform restricted to mammals, regulates the activity of BAK and provides a connection between mitochondrial physiology and the core apoptotic pathway.

The BCL-2 family of pro- and antiapoptotic proteins constitutes a critical control point for apoptosis (1–2). A combination of genetic and biochemical approaches has helped to order the components of the mammalian cell death pathway. The upstream "BH3-only" family members respond to select death signals and subsequently trigger the activation of the multidomain death effectors BAX and BAK (3–5). BAX and BAK constitute an essential gateway to the intrinsic death pathway operating at the level of both mitochondria and endoplasmic reticulum (ER) Ca2+-dynamics (6, 7). Activated homo-oligomerized BAX or BAK results in the permeabilization of the mitochondrial outer membrane (MOM) and the