



Cannibalism by Sporulating Bacteria

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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.

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Materials and Methods

Fig. S1

References

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Cannibalism by Sporulating Bacteria

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Spore formation 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 spore formation 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 resume growth rapidly. Thus, bacteria could be expected to delay spore formation 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.

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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 spore formation (Fig. 1, B and C, and fig. S2). We refer to these operons as *skf* for sporulation killing factor and *sdp* for sporulation delaying protein.

Clues that the eight-gene *skf* operon directs the production of an 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, subtilisin (10). Finally, the product of *skfD* contains a domain characteristic of the CAAX 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 dropped sharply upon entry into sporulation when grown in the absence of IPTG but not when grown in its presence (Fig. 2C).

We next asked whether the operon causes death in a homogenous population of wild-type cells. Cultures of cells sporulating in liquid medium show a characteristic drop in optical density shortly after the start of spore formation. This drop was associated with a dramatic (~70%)

decrease 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-CDEFGH* and the *sdpABC* operons [previously annotated as *ybcOPST ybdABDE* and as *yvaWXY*, respectively (23)]. The hairpin symbols represent transcriptional terminators. (B) Colonies of *skf* [$\Delta(skfABCDEF)::tet$; strain EG168] and *sdp* [$\Delta(sdpABC)::spc$; strain EG407] mutants, as well as the double *skf sdp* mutant (EG523), were brighter (an indication of spore 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 spore 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.

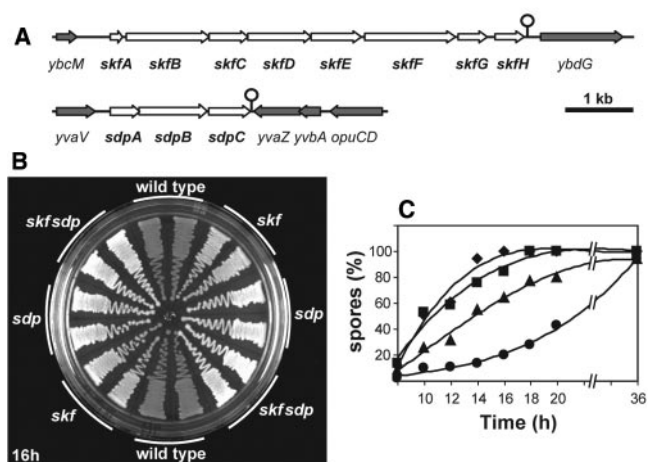
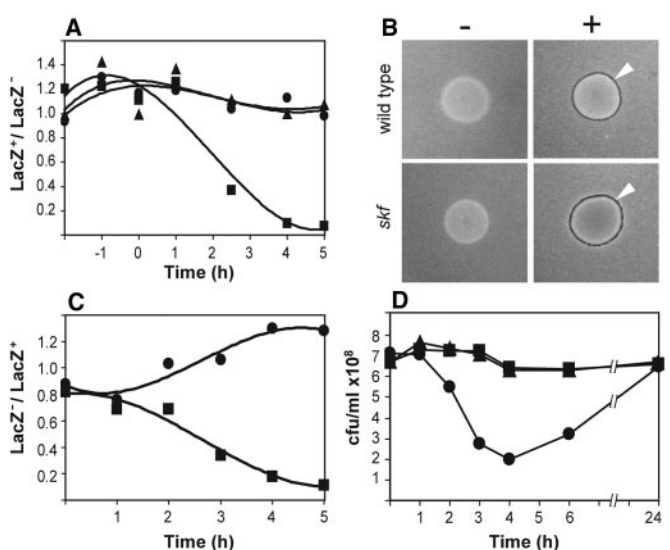


Fig. 2. The *skf* operon produces a sporulation killing factor. (A) *skf* mutant cells harboring a *lacZ* fusion [$\Delta(skfABCDEF)::tet amyE::cotD-lacZ$; EG169] and wild-type cells (PY79) were mixed in equal proportions and grown in liquid sporulation medium. The ratio of mutant to wild-type cells 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 EG208) were spotted on a lawn of wild-type or *skf* mutant cells growing on a rich (nonsporulation, Luria broth) medium. The engineered cells produced a halo of growth inhibition (arrow) 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 EG219) 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 EG219 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* (EG165) (■) and *skfABCDEF* (EG168) (▲) mutants in liquid sporulation medium at the indicated times after the start of sporulation.



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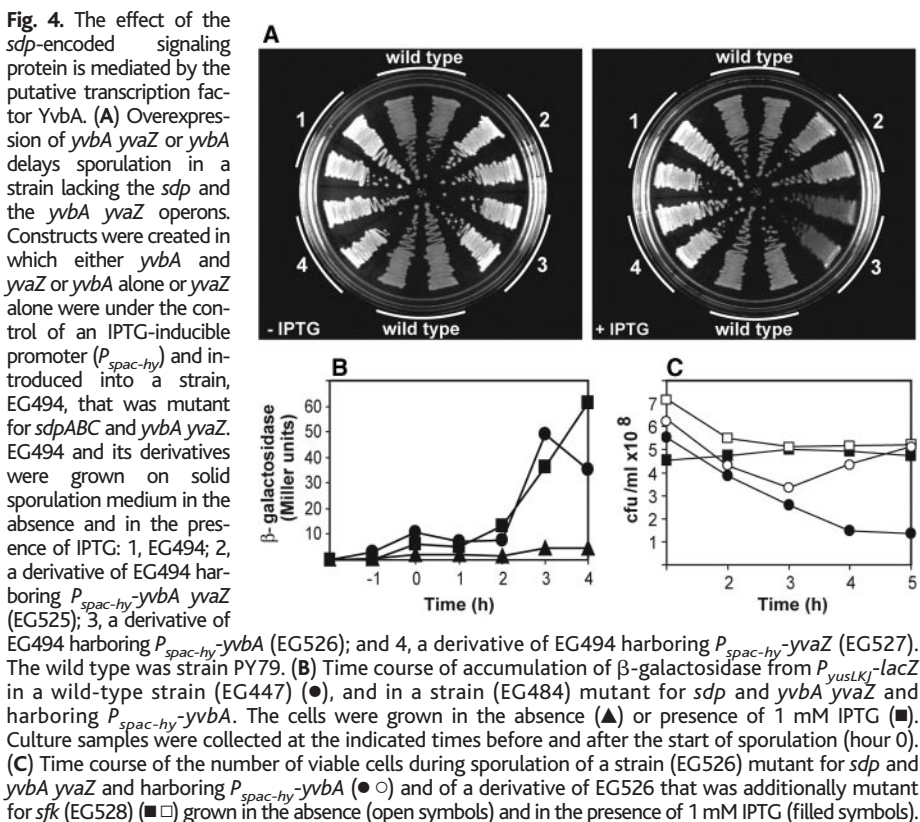
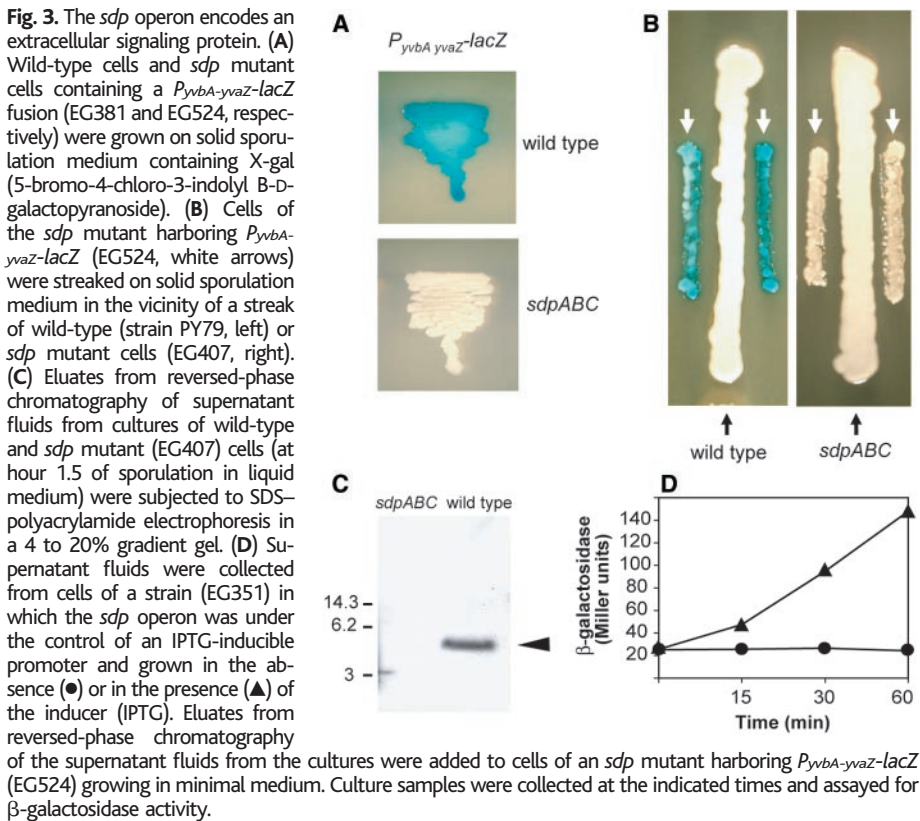
low cells in which Spo0A is active but which have not yet committed to morphogenesis to keep growing. An *skf* mutant, in contrast, does not cause killing and hence sporulation takes place without delay.

Mutations in a second operon, *sdp* (Fig. 1A), also caused an accelerated sporulation phenotype and did so more rapidly than mutations in *skf* (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-VAAGYLYVVGVNAAALQTAATAAV) (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 *yvbA* 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 *yvbA* 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 (*atp1BE-FHAGDC*), which is responsible for ATP



production, and the *yusLJKJ* operon, whose inferred products are similar to lipid catabolism enzymes (fig. S5 and table S1). Use of *lacZ* fused to *yusLJKJ* 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 *yusLJKJ* to cells doubly mutant for the *sdp* and *yvbA yvaZ* 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 *skf* 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 σ^W , 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 σ^W could heighten sensitivity to the killing factor by suppressing the antibiosis 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.

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VDAC2 Inhibits BAK Activation and Mitochondrial Apoptosis

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William J. Craigen,² Stanley J. Korsmeyer^{1*}

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 tBID, 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 mam-

malian 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 (3, 6). Activated homo-oligomerized BAX or BAK results in the permeabilization of the mitochondrial outer membrane (MOM) and the

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