SpxB Is a Suicide Gene of *Streptococcus pneumoniae* and Confers a Selective Advantage in an In Vivo Competitive Colonization Model

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The human bacterial pathogen *Streptococcus pneumoniae* dies spontaneously upon reaching stationary phase. The extent of *S. pneumoniae* death at stationary phase is unusual in bacteria and has been conventionally attributed to autolysis by the LytA amidase. In this study, we show that spontaneous pneumococcal death is due to hydrogen peroxide (H\(_2\)O\(_2\)), not LytA, and that the gene responsible for H\(_2\)O\(_2\) production (spxB) also confers a survival advantage in colonization. Survival of *S. pneumoniae* in stationary phase was significantly prolonged by eliminating H\(_2\)O\(_2\) in any of three ways: chemically by supplementing the media with catalase, metabolically by growing the bacteria under anaerobic conditions, or genetically by constructing ΔspxB mutants that do not produce H\(_2\)O\(_2\). Likewise, addition of H\(_2\)O\(_2\) to exponentially growing *S. pneumoniae* resulted in a death rate similar to that of cells in stationary phase. While ΔlytA mutants did not lyse at stationary phase, they died at a rate similar to that of the wild-type strain. Furthermore, we show that the death process induced by H\(_2\)O\(_2\) has features of apoptosis, as evidenced by increased annexin V staining, decreased DNA content, and appearance as assessed by transmission electron microscopy. Finally, in an in vivo rat model of competitive colonization, the presence of spxB conferred a selective advantage over the ΔspxB mutant, suggesting an explanation for the persistence of this gene. We conclude that a suicide gene of pneumococcus is spxB, which induces an apoptosis-like death in pneumococci and confers a selective advantage in nasopharyngeal colonization.

*S. pneumoniae* is a gram-positive human pathogen that colonizes the nasopharynx of most children during infancy. Despite the availability of effective antibiotics and vaccines, pneumococci remain a major cause of morbidity and mortality, causing pneumonia, bacteremia, and meningitis. It is estimated that over 850,000 children die annually of pneumococcal infections worldwide (3).

*S. pneumoniae* grown in broth dies spontaneously when reaching stationary phase. How this process occurs and why it would persist in pneumococci are controversial and unresolved issues. Almost a century ago, McLeod and Gordon suggested that this phenomenon is due to accumulation of self-produced H\(_2\)O\(_2\) (18); however, their hypothesis was not widely accepted. In 1970, Tomasz et al. (39) reported that several antibiotic groups induce their bactericidal effect through the induction of lytA and that with loss of LytA function, pneumococci fail to lyse and lose viability at a substantially lower rate. Despite a subsequent report demonstrating that a LytA-independent component was responsible for cell death (19), the phenomenon of spontaneous death, or “suicide,” has been conventionally attributed to autolysis by the major autolysin of *S. pneumoniae*, an N-acetylmuramoyl-L-alanine amidase (LytA) (11, 14). LytA appears to be constitutively expressed (9, 11), but its activity is likely under the control of a complex and only partially understood regulatory mechanism to prevent continuous spontaneous autolysis. At the same time, the growth of pneumococcus on agar plates requires the presence of catalase, a well-known observation which implicates hydrogen peroxide produced by *S. pneumoniae* in limiting viability of the organism.

H\(_2\)O\(_2\) is produced by *S. pneumoniae* as a by-product of aerobic metabolism by the enzyme pyruvate oxidase (SpxB) (33). In our own studies on interspecies competition between *S. pneumoniae* and other microorganisms (28), we noted that H\(_2\)O\(_2\) is responsible for killing *Staphylococcus aureus*. We wondered whether H\(_2\)O\(_2\) could also be responsible for stationary-phase death of pneumococci. Finally, we wished to investigate whether the gene responsible for this process can confer any selective advantage in colonization, which may explain the persistence of this phenotype in all known clinical isolates of pneumococci.

Here we show that death of *S. pneumoniae* at stationary phase is in fact independent of LytA but dependent on the spxB gene and on H\(_2\)O\(_2\). We demonstrate that the effect of H\(_2\)O\(_2\) is to kill bacteria in a fashion that is reminiscent of apoptosis in eukaryotic cells. We also show that cells carrying spxB have a selective advantage over mutant ΔspxB cells in an infant rat model of pneumococcal colonization.

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *S. pneumoniae* strains used were strain 603 (serotype 6B, a human clinical isolate) (16), Pn-20 (serotype 35B, a human nasopharyngeal isolate), TIGR4 (36), and Rx1 (32). Pyruvate oxidase (SpxB)-negative mutants of strain Pn-20 (Pn-20ΔspxB) and 603 (603ΔspxB) and LytA-negative mutants of strains Rx1 and Pn-20 (Rx1ΔlytA and Pn-20ΔlytA) were described previously (28). A LytA-negative mutant of strain 603 (603ΔlytA) was constructed by transforming a spontaneous streptomycin-resistant variant of...
603 with the Janus cassette (kan-<sup>plasmid</sup>) containing the appropriate flanking regions (28, 35).

Bacterial strains were routinely grown to mid-log phase at 37°C in brain heart infusion broth (BHI; Becton Dickinson, Sparks, MD) in 5% CO<sub>2</sub> microaerophilic conditions and stored at −70°C in broth supplemented with 10% glycerol. Bovine liver catalase (MP Biomedicals, Inc., Solon, OH) (1,000 U/ml) was added to growth media when specified. In experiments indicated, 1 mM of H<sub>2</sub>O<sub>2</sub> was added every 2 h, starting when cultures reached an optical density (OD) of 0.1 to 0.2. Anaerobic growth conditions were created using BBL GasPak anaerobic system envelopes (Becton Dickinson) in air-tight jars. Aerobic conditions were created by incubating a 5-ml culture in a 50-ml tube at 37°C with rocking. Heat killing was achieved by a 1-h incubation at 60°C.

**Growth curves.** Growth curves were determined by serial measurements of OD at 610 nm and/or counts of viable CFU.

*Inhibitory effect of H<sub>2</sub>O<sub>2</sub> on neighboring cells in vitro.* The inhibitory effect of H<sub>2</sub>O<sub>2</sub> on neighboring cells was determined by measuring the inhibitory effect of wild-type Pn-20, which produces 1.2 ± 0.4 mM of H<sub>2</sub>O<sub>2</sub>/10<sup>7</sup> CFU/ml, on its ΔlytA mutant, which does not produce detectable amounts of H<sub>2</sub>O<sub>2</sub> (28). We determined bactericidal activity on adjacent cells by measuring the killing effect of wild-type Pn-20 on Pn-20ΔlytA in coculture. Pn-20ΔlytA (10<sup>6</sup> CFU/ml) was cocultured with 0 and 10<sup>6</sup> CFU/ml of Pn-20 in a total volume of 100 µl. Cultures were incubated at 37°C in 5% CO<sub>2</sub> in BHI. Survival of Pn-20ΔlytA was measured by plating cultures of 6 h on tryptic soy agar supplemented with 5% sheep blood and 25 µg/ml kanamycin (selective for Pn-20ΔlytA) and counting viable CFU.

*Measures of apoptosis-like features.* (i) **Annexin V-FITC labeling.** Bacterial cells were grown for 6 h to 18 h in 5 ml BHI (initial inoculum = 10<sup>7</sup> CFU/ml) under microaerophilic conditions with or without bovine liver catalase (1,000 U/ml). To identify cells with features of apoptosis, bacteria were stained by using an annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (MBL Internationals Corporation, Woburn, MA) by adapting the manufacturer’s instructions for eukaryotic cells. Briefly, at various time points, bacterial cells were harvested, washed in 1 ml of cold phosphate-buffered saline (PBS), resuspended in binding buffer (supplied by the manufacturer), and then incubated for 10 min in the dark, at room temperature, with 5 µl annexin V. After extensive washing, the bacteria were analyzed by flow cytometry for fluorescence in the FL-1 channel. Bacteria with features of apoptosis were arbitrarily defined as those which stained above the 95th percentile of fluorescence of bacteria harvested after a 6-h culture (in which minimal cell death occurs). For each condition, 5,000 to 10,000 events were analyzed.

(ii) **PI staining for detection of bacterial DNA.** Bacterial cells were harvested as described above for annexin V-FITC staining, but with a permeabilization step. After harvest and washing, bacteria were permeabilized by the addition of 300 µl of 70% ethanol in PBS with 1% fetal bovine serum for 10 min at 4°C. Cells were then thoroughly washed in PBS with 1% fetal bovine serum and resuspended in 300 µl of a solution containing 50 µM propidium iodide (PI) and 500 µg/ml RNase (both from Sigma-Aldrich, St. Louis, MO) for 20 min at 4°C. Cell DNA content was determined by flow cytometry. In this assay, we defined cells with features of apoptosis as those with diminished intensity of PI staining, due to decreased DNA content. In some experiments, we stained nonpermeabilized bacteria with PI to distinguish dead from viable cells using flow cytometry.

(iii) **Transmission electron microscopy (TEM).** Bacterial cells were harvested as described above. Bacteria were fixed in 2.5% paraformaldehyde, 5% glutaraldehyde, 0.06% picric acid in 0.1 M cacodylate buffer overnight, washed in 0.1 M cacodylate buffer, resuspended with 1% osmium and 1.5% ferricyanide for 8 h, and then stained with uranyl acetate in maleate buffer overnight. Pellets were then dehydrated with increasing alcohol concentrations (50% to 100%), washed with propylene oxide, and infiltrated with 50% propylene oxide and 50% TAAB Epon overnight. Pellets were embedded in TAAB Epon and polymerized at 60°C for 24 h before slicing to thin sections.

**Penicillin tolerance test.** To determine whether bacteria were tolerant to the effect of penicillin, bactericidal effect was measured as follows. Bacteria harvested at mid-log cultures and subsequently frozen at −70°C were thawed and cultured in prewarmed BHI. Penicillin G was added after 1 h of incubation (during exponential growth). Cultures (100 µl) were incubated in a 96-well microtiter plate with or without 0.25 µg/ml of penicillin (10 times the MIC), with or without catalase (1,000 U/ml). Viable CFU were assessed before addition of penicillin (time zero) and after 4 h of incubation.

**Bile solubility test.** Sodium deoxycholate (10 µl, 10%) was applied dropwise on isolated colonies of Pn-20, Pn-20ΔlytA, and Pn-20ΔlytA ΔspxB grown on tryptic soy agar and 5% sheep blood. After 10 min of incubation at room temperature, the appearance of colonies was examined to determine if lysis had occurred.

**RESULTS**

Death at stationary phase is independent of LytA but dependent on SpxB. To evaluate the role of LytA in pneumococcal death at stationary phase, strains 603 and Pn-20 and their ΔlytA mutants were grown in BHI at 37°C under either microaerophilic or anaerobic conditions. Growth was monitored by determining both the OD and viable-cell counts. As shown in Fig. 1A, the ODs of the parent strains (603 and Pn-20) rapidly declined after cultures reached stationary phase (at 8 to 10 h of culture), whereas the ODs of the ΔlytA mutants did not decline, consistent with decreased lysis. However, the ΔlytA mutants spontaneously died at stationary phase at the same rate as their parent strains, with a 4-log decline in viable-cell count over 15 h in stationary phase (e.g., to 5 × 10<sup>4</sup> and 5 × 10<sup>5</sup> CFU/ml for 603 and ΔlytA, respectively [Fig. 1B]). Although the growth of Pn-20ΔlytA was delayed, it reached a peak count of 2 × 10<sup>6</sup> CFU/ml and demonstrated a death rate at stationary phase similar to that of the parent strain (counts declined to 8 × 10<sup>3</sup> and 1 × 10<sup>4</sup> CFU/ml for Pn-20 and Pn-20ΔlytA, respectively [Fig. 1C]). Thus, death at stationary phase is independent of LytA.

To determine the role of H<sub>2</sub>O<sub>2</sub> in death at stationary phase, growth was assessed in cultures in which H<sub>2</sub>O<sub>2</sub> was eliminated by several methods. First, we constructed a ΔspxB mutant (Pn-20ΔspxB), which does not produce detectable amounts of H<sub>2</sub>O<sub>2</sub>. Compared to Pn-20, strain Pn-20ΔspxB showed prolonged survival (Fig. 1C). Because it was shown previously that spxB mutants differ from wild-type strains with respect to factors other than H<sub>2</sub>O<sub>2</sub> production (such as ATP production [33]), we confirmed the role of H<sub>2</sub>O<sub>2</sub> in spontaneous death by growing Pn-20 in medium depleted of H<sub>2</sub>O<sub>2</sub> by addition of excess catalase. Both parent and ΔlytA mutant strains demonstrated prolonged survival, with only a 10-fold decline over 15 h in stationary phase (to >10<sup>5</sup> CFU/ml) compared to a 10<sup>4</sup>-fold decrease when catalase was not added (Fig. 1B and C). When these strains were grown under anaerobic conditions, in which H<sub>2</sub>O<sub>2</sub> is not produced by *S. pneumoniae*, death in stationary phase was very slow, comparable to that observed under aerobic conditions with supplemental catalase. Under anaerobic conditions, the addition of catalase was not additionally advantageous (Fig. 1D). Similar growth patterns were observed for other *S. pneumoniae* strains and their lytA mutants (TIGR4 and Rxi1) (data not shown). Finally, when exogenous H<sub>2</sub>O<sub>2</sub> was added to exponentially growing Pn-20ΔspxB cultures, the cells died at a rate similar to that of the wild type at stationary phase (Fig. 1C). Overall, these findings demonstrate that spxB and its
metabolic product, H$_2$O$_2$, are responsible for death at stationary phase.

**Wild-type cells are lethal to neighboring spxB-deficient cells.** To determine whether production of H$_2$O$_2$ by a wild-type pneumococcus can kill adjacent cells, we first measured the effect in vitro by coculturing strain Pn-20, which produces 1.2 ± 0.4 mM H$_2$O$_2$/10$^7$ CFU, with its Pn-20ΔspxB mutant, which does not produce H$_2$O$_2$. To distinguish the mutant from its parent and determine the viable CFU, cultures were plated on selective medium. When grown in coculture for 6 h, over 99.9% of Pn-20ΔspxB was killed by 10$^8$ CFU/ml Pn-20, as measured by a 3.4-log decrease in CFU/ml, similar to the killing effect of exogenous H$_2$O$_2$ as observed in Fig. 1. Monoculture of Pn-20ΔspxB under identical conditions yielded an increase in viable counts of 0.8 log CFU/ml. The lethal effect of Pn-20 was mitigated by catalase, thus confirming the role of H$_2$O$_2$ in this killing process.

**H$_2$O$_2$-mediated killing of pneumococcus has features consistent with apoptosis: externalization of anionic phospholipids, reduction in DNA content, and typical morphological features.** H$_2$O$_2$ produced by S. pneumoniae has been implicated in eukaryotic cell death by apoptosis (2, 4, 42). Previous reports of bacterial apoptosis have shown that several bacterial species can undergo a death process that shares many features of eukaryotic apoptosis (10, 21, 27, 29, 30). Based on these observations, we asked whether pneumococcal death induced by H$_2$O$_2$ would have features consistent with bacterial apoptosis. An early marker of apoptosis in eukaryotic cells is externalization of anionic phospholipids to the membrane surface (8). This membrane change can be detected by binding of annexin V to the eukaryotic-cell membrane (13). This marker has been used to demonstrate apoptosis in Xanthomonas spp. (27). Bacteria were grown to stationary phase, stained with annexin V conjugated to FITC, and then analyzed by flow cytometry. When cells were harvested during stationary phase, 27% of bacterial cells were stained with annexin V. Addition of catalase to the medium significantly reduced this process, so that only 10% ($P < 0.01$) were stained with annexin V. Pn-20ΔspxB also demonstrated significantly lower staining (9% versus 27% in the wild type, $P < 0.01$), while Pn-20ΔspxB cells grown in cultures to which exogenous H$_2$O$_2$ was added were highly stained (21% positive), confirming that this phenotype is dependent on H$_2$O$_2$. As a control, heat-killed pneumococci were similarly incubated with annexin V-FITC, and only 4% of the cells were stained with annexin V ($P < 0.001$ compared to the wild type) (Fig. 2A).

A later marker of eukaryotic apoptosis is loss of DNA due to degradation (31). PI staining of permeabilized cells was used to stain DNA (6). The amount of DNA per cell diminished with time. At 12 h into stationary phase, 30% of Pn-20 cells had low DNA content. In contrast, significantly fewer cells grown in media supplemented with catalase demonstrated this feature

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**FIG. 1.** Representative growth curves of strains 603 and Pn-20 and their mutants under different conditions. (A and B) Optical density (A) and cell viability (B) over time of strains 603 (squares) and 603ΔhtA (triangles), under microaerophilic conditions, in BHI (solid lines) or in BHI supplemented with catalase (dotted lines). (C) Cell viability over time of Pn-20 (diamonds), Pn-20ΔhtA (triangles), and Pn-20ΔspxB (circles), under microaerophilic conditions, in BHI (solid lines) and in BHI supplemented with catalase (dotted lines) and of Pn-20ΔspxB in BHI supplemented with exogenous H$_2$O$_2$ (×). (D) Cell viability over time of these strains under anaerobic conditions. Each experiment was repeated independently at least three times.
(8.5% versus 30%, \( P < 0.001 \)). The Pn-20ΔspxB mutant, harvested at 12 h into stationary phase, also had fewer cells with low DNA content (8% versus 30%, \( P < 0.001 \)), while Pn-20ΔspxB grown in cultures to which exogenous H2O2 was added showed significantly greater proportions of cells with low DNA content (25% versus 8%, \( P < 0.001 \)). Heat-killed cells did not show diminished DNA content and appeared similar to exponential-phase Pn-20 (percentage of cells with low DNA content, 4.6% versus 30% in wild type, \( P < 0.001 \)) (Fig. 2B).

To further evaluate whether H2O2-mediated pneumococcal death has morphological features of apoptosis, bacteria were grown under various conditions, harvested, and examined by TEM. Wild-type bacteria harvested 12 h into stationary phase displayed very different features compared to bacteria harvested in logarithmic phase. The most prominent characteristic of wild-type bacteria from stationary phase was condensed DNA that concentrated at the periphery of the cells, proximal to the cell membrane (Fig. 3A to C). At a later time in stationary phase (12 h or longer), in addition to cells with this apoptosis-like morphology, cells with clearly disrupted structures, with loss of coccoid shape and clearing of the cytoplasm (reminiscent of “ghost cells”), were also evident (Fig. 3C). Heat-killed cells did not demonstrate these features: the DNA was not condensed, and the contours of the cell were well preserved (Fig. 3D).

The apoptotic features were observed with increased frequency at later stages of growth: at exponential growth phase (6 h culture), fewer than 5% of bacterial cells demonstrated this phenotype; 6 h and 12 h into stationary phase, >90% and >99% of cells demonstrated this phenotype (Fig. 4A to C).

Heat-treated Pn-20 (60°C for 1 h) were apparent; however, no ghost cells could be seen at this time point (Fig. 4F), and only few cells resembling ghost cells were observed after 12 h in stationary phase (data not shown). These results suggest that the ghost cells seen in lytA-sufficient cultures are most likely bacteria in the process of lysing, which occurs as a consequence of the effect of the autolytic enzyme on dying pneumococci.

**LytA activity does not directly depend on H2O2.** An unexpected feature of pneumococci grown in the absence of H2O2 (by growth with catalase or culture of the ΔspxB mutant) was the formation of chains consisting of four to eight cells (Fig. 4D and E), which was not observed at any stage of growth of wild-type cultures (Fig. 4A to C). Formation of short (four- to eight-cell) chains is usually seen with pneumococci deficient in LytA (14). This suggested the possibility that certain cellular activities that depend on LytA might also require H2O2. To test this hypothesis, we examined whether two processes that depend on LytA, namely, penicillin- and deoxycholate-induced lysis, are also dependent on H2O2. A total of 10^7 CFU of Pn-20 were rapidly killed by 0.25 \( \mu \)g/ml penicillin G (10 times the MIC of the isolate), with a 2-log reduction in viable cells at 4 h of incubation. In contrast, Pn-20ΔlytA was tolerant to the bactericidal effect of penicillin G, consistent with previous reports.

FIG. 2. Quantification of cells at stationary phase with features of apoptosis. The percentage of cells with features of apoptosis was determined by annexin V-FITC staining 6 h into stationary phase (A) or by PI staining of permeabilized cells 12 h into stationary phase (B). Data for Pn-20 in BHI and in BHI supplemented with catalase, Pn-20ΔspxB in BHI, Pn-20ΔspxB in BHI supplemented with 1 mM exogenous H2O2, and heat-treated Pn-20 (60°C for 1 h) are shown. **, \( P < 0.01 \); $$$, \( P < 0.001 \).
The bactericidal effect of penicillin G was sustained when H$_2$O$_2$ was eliminated (either by using a ΔspxB mutant or by supplementing the media with catalase), with similar reductions in viable cells. Similarly, resistance to lysis by deoxycholate was observed only with the ΔlytA mutant, while lysis was immediate in both the wild type (Pn-20) and the ΔspxB mutant. These results indicate that the lytic activity of LytA is not directly dependent on H$_2$O$_2$.

The spxB gene confers a competitive advantage over non-H$_2$O$_2$-producing bacteria in vivo. Our findings that spxB is responsible for pneumococcal death at stationary phase raise the question of why this gene would persist in the pneumococcal population. A possible clue to this puzzle has been offered by previous investigators (25), who showed that spxB is required for resistance to the toxic by-product of its own activity in vitro. This raised the possibility that in a population of wild-type pneumococci, newly arising spxB-deficient strains would be at a disadvantage due to heightened susceptibility to killing by their wild-type neighbors, as was the case in our mixed-culture studies in vitro. To investigate this further, we developed an infant rat model of nasopharyngeal colonization to test competition between a wild-type S. pneumoniae strain and its ΔspxB mutant. Inoculation with $1 \times 10^7$ CFU of 603 or 603ΔspxB alone yielded similar median colonization densities (3.9 $\times$ 10$^4$ CFU/nasal wash and 3.3 $\times$ 10$^4$ CFU/nasal wash, respectively) (Fig. 5), indicating that the deficiency of spxB is not associated with a reduced capacity to colonize infant rats. However, when the two strains were simultaneously inoculated at $5 \times 10^6$ CFU each, the wild type colonized significantly better than the ΔspxB strain (2.4 $\times$ 10$^4$ versus 1.6 $\times$ 10$^3$ CFU/nasal wash, respectively; $P = 0.001$) (Fig. 5). Thus, these experiments show that the presence of the spxB gene is associated with a colonization advantage in competition with spxB-deleted strains, in a pattern consistent with our in vitro results.

**DISCUSSION**

*S. pneumoniae* has long been known to possess a pathway leading to the lytic death of a large proportion of cells. LytA has been shown to be the lytic enzyme, yet the regulation of this enzyme has been only partially defined (12, 22, 23). It has been suggested that death at stationary phase is also dependent on LytA (11, 14). In this study we show that *S. pneumoniae* cells die by a mechanism independent of LytA but dependent on spxB and its by-product H$_2$O$_2$, as was proposed initially by McLeod and Gordon in 1922 (18). We used three approaches to demonstrate the role of this gene and H$_2$O$_2$ in the death process of *S. pneumoniae*: genetically by creation of a mutant that does not produce H$_2$O$_2$ (ΔspxB), chemically by elimination of H$_2$O$_2$ with catalase, and environmentally by prevention of generation of H$_2$O$_2$ by growth under anaerobic conditions. In addition, we demonstrated that the exogenous addition of H$_2$O$_2$ at concentrations similar to those produced by the cells in vitro induces death of exponential-phase cells at a rate similar to that of wild-type bacteria at stationary phase.

We also demonstrate that H$_2$O$_2$-mediated death of pneumococci has several features that are consistent with apoptosis. The term “apoptosis” has conventionally been used to describe...
the eukaryotic phenomenon of programmed cell death, a genetically regulated process of active cell suicide that is required for the homeostasis and development of tissues. Previous reports of bacterial apoptosis have shown that under specific conditions bacterial cells may also undergo a death process that shares many of the features of apoptosis (21, 27, 30). Furthermore, regulatory systems that control cell viability at stationary phase and lysis have been described, adding genetic evidence in support of the existence of bacterial apoptosis. The first well-described system was the *mazEF* operon in *E. coli* (1).

Another recently described system is the *cid* operon, which controls cell viability in stationary phase, lysis, and antibiotic tolerance in *S. aureus* (24, 41).

Reactive oxygen species, and among them mainly H$_2$O$_2$, have been shown to participate in both early and late steps of apoptosis pathways in eukaryotic cells, yeasts, and amitochondrial parasites (15, 17, 26). Reactive oxygen species have been suggested to serve as intracellular messengers that stimulate unknown proapoptotic regulatory machinery. The fact that eliminating H$_2$O$_2$ significantly inhibited the death process indicates that H$_2$O$_2$ plays a causal role, not just a secondary one. In addition, the death process induced by H$_2$O$_2$ is a lengthy process, suggesting the necessity for protein synthesis or activation rather than just direct, rapid cellular damage. Previous studies on the effect of exogenous H$_2$O$_2$ on bacterial cells have shown that high concentrations of H$_2$O$_2$ (~100 mM) induce DNA damage that may lead to bacterial cell death (30). Our study is novel in that we demonstrate that H$_2$O$_2$ produced by the bacteria itself, at relatively low concentrations (~1 mM), has a causal role in suicide via a process that has morphological features of apoptosis. Whether these apoptosis-like features are also associated with a specific genetic program remains to be determined.

![Figure 4](image4.png)

**FIG. 4.** Transmission electron micrographs showing the morphological appearance of Pn-20 cell cultures (magnification, ×4,800). (A) Exponential phase (<5% appear apoptotic); (B) 6 h into stationary phase (>90% appear apoptotic); (C) 12 h into stationary phase (>99% appear apoptotic or have a ghost cell appearance). (D to F) TEM of bacteria 6 h into stationary phase; (D) Pn-20 in culture supplemented with catalase; (E) Pn-20ΔgrpB; (F) Pn-20ΔlytA.

![Figure 5](image5.png)

**FIG. 5.** Density of *S. pneumoniae* 603 and 603ΔgrpB colonization determined 7 days after intranasal inoculation of infant Sprague-Dawley rats. Group 1 was inoculated with 1 × 10$^7$ CFU of strain 603. Group 2 was inoculated with a mixture of 5 × 10$^5$ CFU of 603 and 603ΔgrpB; 2a shows colonization by strain 603 as determined by plating on streptomycin blood agar plates, and 2b shows colonization by 603ΔgrpB as determined by plating on kanamycin blood agar plates. Group 3 was inoculated with 1 × 10$^7$ CFU of strain 603ΔgrpB. Solid lines indicate group medians (CFU/nasal wash). The dashed line indicates the limit of detection. P values were determined by Mann-Whitney tests for differences in the distributions of CFU/nasal wash between groups.
Our findings offer clues to an intriguing evolutionary enigma: why would a bacterial species carry a gene that results in its death? One can speculate regarding some potential benefits of \( \text{H}_2\text{O}_2 \) production, such as the elimination of damaged cells, the release of toxins that may reduce immune pressure, or the transfer of genetic material with surviving cells (7, 20, 29). Other possible evolutionary advantages for \( S. \text{ pneumoniae} \) of carrying the \( \text{spxB} \) gene may be the role of \( \text{H}_2\text{O}_2 \) in competition with other species (as shown for \( S. \text{ aureus} \) [28]) and in its role in stimulating a host response that may eventually help spread the bacteria among potential hosts by inducing epithelial cell apoptosis and shedding (34). Our studies provide empirical support for an explanation for the persistence of this gene despite its role in pneumococcal suicide. Here we demonstrate that the mutant lacking this gene has a disadvantage when in competition with the wild-type parent in an in vivo model of nasopharyngeal colonization. Although \( \text{spxB} \) mutants are known to be defective in more than just \( \text{H}_2\text{O}_2 \) production (such as reduced ATP under aerobic conditions [33]), the mutant we studied was fully able to colonize infant rats (and as such efficiently as the wild-type strain, when these bacteria were administered alone), arguing against a global defect in colonization. A reasonable explanation, therefore, for the persistence of the \( \text{spxB} \) gene is that the \( \text{spxB} \)-dependent system of aerobic metabolism was established in pneumococci, \( \text{spxB} \)-deficient strains may have been unable to increase in frequency in environments where they occurred with wild-type bacteria.

We conclude that \( \text{spxB} \) is a suicide gene of \( S. \text{ pneumoniae} \) which induces spontaneous death at stationary phase via its main by-product, \( \text{H}_2\text{O}_2 \). This spontaneous death has features that are consistent with bacterial apoptosis. The presence of the \( \text{spxB} \) gene confers a competitive advantage in an in vivo model of nasopharyngeal colonization, which may explain the persistence of this gene in the general pneumococcal population.

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