The yefM-yoeB Toxin-Antitoxin Systems of Escherichia coli and Streptococcus pneumoniae: Functional and Structural Correlation†*

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Toxin-antitoxin loci belonging to the yefM-yoeB family are located in the chromosome or in some plasmids of several bacteria. We cloned the yefM-yoeB locus of Streptococcus pneumoniae, and these genes encode bona fide antitoxin (YefM) and toxin (YoeB) products. We showed that overproduction of YoeBpneumococcus is toxic to Escherichia coli cells, leading to severe inhibition of cell growth and to a reduction in cell viability; this toxicity was more pronounced in an E. coli B strain than in two E. coli K-12 strains. The YoeBpneumococcus-mediated toxicity could be reversed by the cognate antitoxin, YefM, but not by overproduction of the E. coli YefM antitoxin. The pneumococcal proteins were purified and were shown to interact with each other both in vitro and in vivo. Far-UV circular dichroism analyses indicated that the pneumococcal antitoxin was partially, but not totally, unfolded and was different than its E. coli counterpart. Molecular modeling showed that the toxins belonging to the family were homologous, whereas the antitoxins appeared to be specifically designed for each bacterial locus; thus, the toxin-antitoxin interactions were adapted to the different bacterial environmental conditions. Both structural features, folding and the molecular modeled structure, could explain the lack of cross-complementation between the pneumococcal and E. coli antitoxins.

The gram-positive, spherical bacterium Streptococcus pneumoniae (pneumococcus) is the cause of many human diseases, such as pneumonia, bacterial blood poisoning (bacteremia), inflammation of the membranes surrounding the brain and spinal cord (meningitis), middle-ear infection (otitis media), osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, and sinusitis; pneumonia is the most severe disease (15, 28). Although the pneumococcus can normally be found in the noses and throats of healthy individuals, it can grow and cause infection when the immune system is weakened. The people who are most at risk of developing pneumococcal pneumonia have a weakened immune system. These people include the elderly, infants, cancer patients, AIDS patients, postoperative patients, alcoholics, and people with diabetes. The global rate of mortality is more than 1,000,000 people per year, and this figure represents about 15 to 20% of the people infected. Epidemiological studies of community-acquired pneumonia have shown that S. pneumoniae is still one of the most significant etiologic agents in all age groups in developing and industrialized countries (28). A recently published analysis estimated that 1.6 to 2.2 million children die from acute respiratory infection worldwide each year and that about 30% of the deaths are in Southeast Asia (52). Historically, the treatment for pneumococcal pneumonia has been penicillin. However, an increasing number of pneumococcal strains are partially or completely resistant not only to penicillin but also to macrolides, trimethoprim-sulfamethoxazole, and cephalosporins, making it increasingly difficult to treat this disease (3, 50). Vaccination is an available solution, but unfortunately, the people for whom vaccination is most recommended are the people who are least likely to respond favorably to it. Therefore, the overall effectiveness of a vaccine remains questionable, and the need for novel drugs is increasing (51).

Seeking new approaches and finding new targets for pneumococci comprise an important field. In this sense, chromosomally encoded proteic toxin-antitoxin (TA) systems can be considered suitable targets for antibiotics (17). Originally, these systems were discovered in bacterial plasmids, but they are also abundant in the chromosomes of bacteria and archaea (37). They consist of two proteins that form a harmless complex in which the unstable antitoxin neutralizes the toxicity of the cognate stable toxin. When cells encounter nutritional or environmental stresses, cellular proteases (Lon or Clp) activate the toxins by actively degrading their antitoxin counterparts (1, 10, 11). However, the toxin activity does not necessarily lead to cell death provided that within a certain window of time synthesis of the antitoxin is resumed (16, 36, 38). The chromosomal TA systems seem to operate by modulating the global level of translation, with the toxins functioning as specific endoribonucleases (12, 20) that cleave either free mRNA, like YoeB or MazF (26, 55), or mRNA associated with actively translating ribosomes, like RelE (39). Thus, the bacterial toxins are potentially interesting as new antibiotics (17, 20, 36). Ideally, an inhibitor that mimics the most relevant toxin residues and their interactions with the antitoxin, leading to a new complex (compound-antitoxin) that frees the toxin to...
cause bacterial damage, would be an excellent antitoxinaceous agent.

In the chromosome of S. pneumoniae R6 (24), at least three TA loci have been identified. Two of these loci, relBE1_Spn and relBE2_Spn, exhibit homology with the relBE genes of Escherichia coli (19), whereas the third locus, yefM-yoeB_Spn, is homologous to the yefM-yoeB (also designated relBE3) genes of E. coli (5). A similar locus has been reported to be present in plasmid pRUM of Enterococcus faecium (22). DNA fragments containing the putative toxin genes relE1_Spn and relE2_Spn were cloned in an E. coli expression vector. Overproduction of both toxins showed that RelE2_Spn was toxic to E. coli, whereas RelE1_Spn was innocuous, indicating that relE2_Spn is a toxin gene (36). The relB2_Spn and relE2_Spn genes were shown to be organized in a single operon. Overexpression of the relE2_Spn toxin gene both in S. pneumoniae and in E. coli led to cell growth arrest and a concomitant reduction in the number of viable cells, which could be reversed by expression of the cognate antitoxin (36).

The yefM-yoeB E. coli TA locus was first identified on the basis of its similarity to the axe-tex TA system of plasmid pRUM (22). Later, the E. coli yefM-yoeB locus was analyzed, and the chromosomal proteins YefM and YoeB were characterized in detail. The YefM antitoxin of E. coli was shown to be unfolded in its native state (5), although the YoeB toxin was folded and formed a complex with the unfolded YefM antitoxin (6). YoeB exhibited endogenous endoribonuclease activity, and its interaction with YefM induced a conformational change in the toxin around the putative active site, leading to the inhibition of the RNase activity of YoeB (26). Although the antitoxins associated more efficiently with their cognate toxins, effective cross-complementation between Axe and YoeB and, to lesser extent, between YefM and Tex has been shown to occur in vivo (22), demonstrating the broad specificity of the TA of the two bacterial species and suggesting that there is a common mechanism of toxin-antitoxin interaction.

In this paper, we describe cloning, characterization, and analysis of the yefM-yoeB_Spn pneumococcal TA system. We analyzed the cross-complementation between the two TA systems from E. coli and S. pneumoniae with the corresponding heterologous YoeB toxins. Unlike the interaction of Axe and YefM, the YefM_Spn antitoxin was able to interact only with its cognate toxin, indicating that there are some structural differences between the antitoxins which could hinder heterologous TA interactions. Far-UV circular dichroism (CD) and molecular modeling analyses indicated that even though the toxins of E. coli and S. pneumoniae exhibited a high level of similarity, the YefM antitoxin counterparts seemed to be structurally different, thus explaining the lack of cross-complementation. Therefore, similar TA systems harbored by different bacteria may have specifically evolved to respond to different environmental conditions, thereby optimizing the TA interactions in their hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The E. coli strains used were TOP-10 [F− mcrA Δ(mar-hsdRMS-mcrBC) ΔlacIΔZ15 ΔlacX74 recA1 deoR araD139 (ara-lac)7697 galU galK rpsL (Str−) endA1 mupG] (Invitrogen), BL21 [F−ompT gal (dcm) (lon)] hsdSR (rB mB)] (an E. coli B strain) (47), BL21(DE3)(pLysS) (Novagen), MG1655 (wild-type E. coli) (53), and two isogenic derivatives of MG1655, SC56 (MG1655Carf-yefB) (7) (provided by K. Gerdes) and MG1655lon (a gift from L. van Melder). For bacterial two-hybrid assays, E. coli XL-Blue MRF’ Km (Stratagene) was used as the host during construction of the recombinant bait and target pairs. The recombinant bait and target pairs were then cotransformed into competent cells of the Bacteriophagm II E. coli validation reporter (Stratagene), a derivative of E. coli XL-Blue MRF’ [Δ(mcrAΔmcrBC) Δ(araBAD)](mcrCB-hsdSMB-mcrBC)175 endA1 hisB supE44 thi-1 recA1 gyrA106 relA1 lac (F− lacIΔ7 his-3 adaA1 KAN), which allowed detection of protein-protein interactions through transcriptional activation of the HIS reporter gene. All cultures were grown in TY medium (33), which was supplemented with 100 μg/ml ampicillin or with 50 μg/ml kanamycin. All cultures were grown at 37°C. The source of S. pneumoniae chromosomal DNA was strain R6, which was grown as reported previously (29) and which is a derivative of the R6 sequenced strain (24). The growth conditions and details concerning induction of synthesis of the pneumococcal toxin and its cognate antitoxin in E. coli have been described elsewhere (36); briefly, the procedures included measuring the turbidity of the cultures by monitoring the optical density at 600 nm (OD600) and determining the number of CFU by plating suitable dilutions of the cultures.

Plasmids used. Plasmids used in this work were constructed as follows.

(i) pBS2. The yoeBE_Spn gene with its own Shine-Dalgarno sequence was amplified by PCR from chromosomal DNA (prepared from S. pneumoniae R6) using primers yoeB_Spn_F (5’-CTGGAAATTCGCCAGGGTCATGTTAGGAGGA-3’), yoeB_Spn_R (5’-CCGGAGATCCGGTAGAATTGAGAACAGA-3’). The resulting 338-bp PCR fragment was doubly digested with EcoRI and BamHII and ligated into plasmid pFUS2 (31) digested with EcoRI and BglII (compatible ends with BamHII sites). This construction placed the yoeBE_Spn gene under control of the arabinose-induced PBAD promoter. Thus, when cells were grown in an arabinose-containing medium, AraC was inactive and promoter transcription from the PBAD promoter, whereas when cells were grown in the presence of glucose, AraC was inactive and transcription from PBAD was switched off (54).

(ii) pYS10. A DNA fragment encompassing the yefMSpn gene with its putative Shine-Dalgarno sequence was amplified by PCR from pneumococcal chromosomal DNA by PCR using primers yefM_N (5’-CGCGGATCCGTTAATTAACGCTCATCATCGTTG-3’), yefM_N_R (5’-CCGGGAATCTCCGTTTACGGAACAAGGAACGCGAAA-3’), which allowed detection of protein-protein interactions through transcriptional activation of the IPTG-inducible Pbad promoter (21).

(iii) pYBET. The yoeBC_Spn gene was amplified from E. coli genomic DNA by PCR using primers yoeB_N (5’-CTGGAAATTCGCCAGGGTCATGTTAGGAGGA-3’), yoeB_N_R (5’-GGATCCCTGAAAAATGATGATGATG-3’). The resulting 350-bp PCR product was digested with EcoRI and BamHII before ligation into the equivalent sites of pBAD-LacZ, which is a derivative of pBAD promoter, whereas when cells were grown in the presence of glucose, AraC was inactive and transcription from PBAD was switched off (34).

(iv) pPYME. The yefMSpn gene was amplified from E. coli genomic DNA by PCR using primers yefM_N (5’-CGCGGATCCGTTAATTAACGCTCATCATCGTTG-3’), yefM_N_R (5’-CCGGGAATCTCCGTTTACGGAACAAGGAACGCGAAA-3’), which allowed detection of protein-protein interactions through transcriptional activation of the IPTG-inducible Pbad promoter (21).

(v) pRES2. A DNA fragment obtained from the chromosome of S. pneumoniae containing the relE2_Spn gene with its own Shine-Dalgarno sequence was generated using primers relE2N (5’-GGATCCCTGAAAAATGATGATGATG-3’), relE2R (5’-GGATCCCTGAAAAATGATGATGATG-3’). The resulting 341-bp product was digested with EcoRI and BamHII before ligation into the equivalent sites of pBAD-LacZ.

(vi) pPMYE. The yefMSpn gene from the S. pneumoniae chromosome was amplified by PCR using primers yefM_N (5’-GGATCCCTGAAAAATGATGATGATG-3’), yefM_N_R (5’-GGATCCCTGAAAAATGATGATGATG-3’). The resulting 353-bp PCR fragment was doubly digested with XbaI and EcoRI and inserted into plasmid pET28a digested with Nhel (compatible with XbaI) and EcoRI. This construction yielded a His-tagged YefMSpn protein.

(vii) pEHB11. A DNA fragment from the pneumococcal chromosome spanning both the yefMSpn and yoeBSpn genes was PCR amplified using primers yefM_N (5’-GGATCCCTGAAAAATGATGATGATG-3’) and yoeB_N (5’-GGATCCCTGAAAAATGATGATGATG-3’). The resulting 353-bp fragment was doubly digested with XbaI and Aval and ligated into plasmid pET24b digested with Nhel and XbaI. This construction yielded His-tagged pneumococcal toxin-antitoxin proteins.

(viii) pYBETF. A DNA fragment containing the coding sequence of the yefMSpn gene was obtained by PCR using the chromosomal DNA of S. pneumoniae as the template and primers yefMSpn_F (5’-GGATCCCTGAAAAATGATGATGATG-3’) and yefMSpn_R (5’-GGATCCCTGAAAAATGATGATGATG-3’). The resulting 353-bp fragment was analyzed for PCR product size and sequence before ligation into the equivalent sites of pBAD-LacZ.

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TAGGTGGAAAAGCTTAAAAC-3’) and yefM-spR (5’-CTCGAGTCTAC
TCAATCATGTT-3’). The resulting 262-bp PCR fragment was digested with EcoRI and Xhol and ligated into the equivalent sites of pBT, generating pBT-YefMsp, which expressed YefM sp as a fusion protein with xcl at the N terminus.

(ii) pBT-YoeBsp. The yoeBsp gene was amplified from chromosomal DNA of S. pneumoniae using primers yoeBSpl-F (5’-GAATCCCATGCTAC
TCAATGTTAC-3’) and yoeBspR (5’-CTCGAGTATAATAGGGAAAGAGGAC-3’) and yeoBspR (5’-CTCGAGTCTACCCTAAA
CTACATGTT-3’). The resulting 257-bp PCR fragment was digested with EcoRI and Xhol and ligated into similarly digested pBT, yielding pBT-YoeBsp, which produced Yoebsp as a fusion protein with xcl at the N terminus.

(iii) pTRG-YefMsp. The yefMsp gene from the S. pneumoniae chromosome was amplified by PCR from primers yeoMSpl-F (5’-GAATCCCATGGTAC
TCAATGTTAC-3’) and yeoMspR (5’-CTCGAGTATAATAGGGAAAGAGGAC-3’) and yeoMspR (5’-CTCGAGTCTACCCTAAA
CTACATGTT-3’). The resulting 257-bp fragment was digested with EcoRI and Xhol prior to ligation into EcoRI- and Xhol-digested pTRG to generate the pTRG-YefMsp recombinant, which expressed YefMsp as an N-terminal fusion protein with the RNA polymerase α subunit (RNAPs).

(iv) pTRG-YoeBsp. A DNA fragment encompassing the yoeBsp gene was PCR amplified from DNA of the S. pneumoniae chromosome using primers yoeBSpl-F (5’-GAATCCCATGGTAC
TCAATGTTAC-3’) and yoeBspR (5’-CTCGAGTATAATAGGGAAAGAGGAC-3’). The resulting 258-bp fragment was digested with EcoRI and Xhol and then ligated with similarly digested pTRG to obtain the pTRG-YoeBsp recombinant, which expressed YoeBsp as a fusion protein with RNAPs at its N terminus.

Purification of S. pneumoniae Hiss-YeFm antitoxin and the YeFm-YoeB anti-toxin-tetrin pair. The pEMH10 vector, containing the coding sequence of yoeBsp, was transformed into E. coli BL21 (DE3) using the lysothetin method (23). Reduced and denatured proteins were eluted from the column in a single peak using buffer B containing 500 mM imidazole. Fractions containing the His-YefM protein or the coeluted YoeB protein were then stored at 20°C. For the proteins were identified by Western hybridization using anti-His antibodies. Protein concentration was determined using a bicinchoninic acid protein assay kit.

RESULTS

Chromosomal location of the yefM-yoeBsp operon. Genome analyses showed that the two sequenced pneumococcal strains, TIGR4 and R6, harbor homologs of the E. coli yefM-yoeB and E. faecium plasmid pRUM-encoded ace-tex TA systems (5, 22, 37). By PCR amplification of chromosomal DNA of the R6 pneumococcal strain, we were able to clone the region spanning the yefM-yoeB loci in a pneumococcal plasmid vector and show that (i) the genes constitute an operon and (ii) the toxin was able to stop the growth of pneumococcal cells (unpublished results).

The antitoxins exhibited more sequence divergence than the toxins; YeFmRec exhibited 24% identity with YeFmsp and 27% identity with Axe. For the toxin inter-

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action domains, the levels of similarity were higher, 67% for YefM _Spn_ and YefM _Eco_, and 56% for Axe and YefM _Eco_.

**Overproduction of YoeB _Spn_ inhibits cell growth and colony formation in different strains of _E. coli_.** The yoeB _Spn_ gene was amplified by PCR from the pneumococcal chromosome and cloned in a plasmid vector, pFUS2 (31), which placed the toxin gene under control of the arabinose-inducible P araBAD promoter and kanamycin to an OD600 of 0.03 to 0.04. Cultures were divided in two. One half of each culture was grown in the presence of glucose (open symbols; for clarity, only the data for strain MG1655 are shown), and the other half was induced by addition of 0.2% arabinose (solid symbols). Growth was monitored by determining the OD600 of the cultures. (B) At the times indicated, the numbers of CFU in the cultures were determined by plating appropriate dilutions on TY agar supplemented with 0.4% glucose and kanamycin and incubating the preparations overnight at 37°C. Neither cell growth arrest nor a reduction in the number of CFU was observed for _E. coli_ TOP-10 cultures containing the pYFS2 plasmid (YefM _Spn_ (■)) after induction of antitoxin synthesis by addition of 2 mM IPTG. This culture was grown and plated as described above but using ampicillin instead of kanamycin for selection. All experiments were performed at least in duplicate.

**FIG. 1.** Effect of yoeB _Spn_ expression in _E. coli_. (A) Cell growth arrest after induction of yoeB _Spn_ expression in strains MG1655 (○ and ●), TOP-10 (■), and BL21 (▲) harboring pYBS2 (yoeB _Spn_). Cells were grown exponentially in medium containing 0.2% glucose (repression conditions) and kanamycin to an OD600 of 0.03 to 0.04. Cultures were divided in two. One half of each culture was grown in the presence of glucose (open symbols; for clarity, only the data for strain MG1655 are shown), and the other half was induced by addition of 0.2% arabinose (solid symbols). Growth was monitored by determining the OD600 of the cultures. (B) At the times indicated, the numbers of CFU in the cultures were determined by plating appropriate dilutions on TY agar supplemented with 0.4% glucose and kanamycin and incubating the preparations overnight at 37°C. Neither cell growth arrest nor a reduction in the number of CFU was observed for _E. coli_ TOP-10 cultures containing the pYFS2 plasmid (YefM _Spn_ (●)) after induction of antitoxin synthesis by addition of 2 mM IPTG. This culture was grown and plated as described above but using ampicillin instead of kanamycin for selection. All experiments were performed at least in duplicate.
the pneumococcal antitoxin, a similar approach was used with *E. coli* cells that harbored different pairs of plasmids, either pFYBE (yoeBEco) and pNMYE (yefMEco) or pFYBE (yoeBEco) and pYFS10 (yefMSpn). *E. coli* cells harboring only plasmid pFYBE (yoeBEco) were used as a toxicity control. The results (Fig. 2C and D) confirmed that as in the previous experiment, there was no interaction between the noncognate toxin and the antitoxin proteins.

**Recovery of cell viability after induction of YefM<sub>Spn</sub> antitoxin synthesis in cultures treated with the YoeB<sub>Spn</sub> toxin.** To determine whether the toxicity of the YoeB<sub>Spn</sub> pneumococcal toxin in *E. coli* could be eliminated by the cognate antitoxin YefM<sub>Spn</sub>, *E. coli* cells harboring plasmids pYBS2 (yoeB<sub>Spn</sub>) and pYFS10 (yefM<sub>Spn</sub>) were grown in the presence of 0.2% arabinose to induce toxin synthesis. Bacterial growth was measured by monitoring the optical densities of the cultures, and the number of CFU was determined by plating samples on glucose-containing medium (repressed conditions for the toxin) supplemented or not supplemented with IPTG (to induce antitoxin synthesis). The number of CFU was plotted as a function of the time of toxin induction, and the results indicated the fraction of surviving cells (Fig. 3). It was apparent that the number of viable cells decreased after induction of toxin expression, although the reduction was more dramatic when there was no induction of the antitoxin (plates without IPTG). After 6 h of toxin overproduction, plates with IPTG contained 3.5% of the original number of *E. coli* CFU, compared with the 0.3% survivors when the cells were grown in IPTG-free plates.

**Effect of YoeB<sub>Spn</sub> overproduction in lon<sup>+</sup> and lon E. coli strains.** Overproduction of the Lon protease in *E. coli* triggers bacterial growth inhibition mediated by YoeB<sub>Ec</sub> (7). Furthermore, indirect results obtained with a strain in which *lon* was deleted suggested that this strain was less sensitive to YoeB<sub>Ec</sub>-mediated lethality (7). For pneumococcal YoeB<sub>Spn</sub> overproduction in *E. coli* resulted in a reduction in bacterial growth, and this effect could not be alleviated by expression of the host yefM<sub>Ec</sub> gene (Fig. 2). In addition, when *E. coli* strain SC36, which lacked the yefM-yoeB locus (7), was tested to determine the toxicity of pneumococcal YoeB<sub>Spn</sub>, we found no differences between this strain and strain MG1655, which is wild type for the yefM-yoeB locus (not shown). Both sets of results suggested that degradation of the YefM<sub>Ec</sub> host antitoxin by the Lon protease was not necessary for YoeB<sub>Spn</sub> activity. To test directly whether the Lon protease has a role in YoeB<sub>Spn</sub> toxicity in *E. coli*, two isogenic strains that were defective and not defective for the *lon* gene (MG1655 and MG1655<sup>lon</sup>, respectively) were transformed with the yoeB<sub>Spn</sub>-containing plasmid pYBS2 (yoeB<sub>Spn</sub>). Cells were treated identically by growing them under toxin induction conditions (with arabinose), and the effect of pneumococcal toxin overproduction was determined by measuring cell growth and CFU counting. The results showed that synthesis of the

![Figure 2](https://example.com/figure2.png)

**FIG. 2.** Toxicity of YoeB<sub>Spn</sub> is neutralized only by combined expression of the cognate antitoxin in *E. coli*. (A and B) *E. coli* TOP-10 strains harboring pYBS2 (yoeB<sub>Spn</sub>) (○), pYBS2 (yoeB<sub>Spn</sub>) and pNMYE (yefM<sub>Ec</sub>) (●), or pYBS2 (yoeB<sub>Spn</sub>) and pNMYE (yefM<sub>Ec</sub>) (▲) were grown exponentially in TY medium containing 0.2% glucose, kanamycin, and ampicillin to an OD<sub>600</sub> of 0.03 to 0.04. Then 0.2% arabinose (toxin synthesis induction) and 1 mM IPTG (antitoxin synthesis induction) were added. (A) Absorbance at 600 nm was used to monitor the growth of the cultures. (B) At the times indicated, samples were diluted and plated on TY agar containing 0.4% glucose, kanamycin, and ampicillin and incubated overnight at 37°C. (C and D) *E. coli* TOP-10 strains harboring pFYBE (yoeB<sub>Ec</sub>) (○), pFYBE (yoeB<sub>Ec</sub>) and pNMYE (yefM<sub>Ec</sub>) (●), or pFYBE (yoeB<sub>Ec</sub>) and pYFS10 (yefM<sub>Spn</sub>) (▲) were grown and treated as described above. (C) Absorbance at 600 nm was used to monitor the growth of the cultures. (D) At the times indicated, samples were diluted and plated on TY agar containing 0.4% glucose, kanamycin, and ampicillin and incubated overnight at 37°C. All the experiments were performed at least in duplicate.
pneumococcal toxins led to growth arrest in both strains (Fig. 4A), indicating that inhibition of growth by YoeBSpn does not seem to require lon expression. However, plotting the percentages of surviving cells of the lon/H11001 and lon/strains showed that there were differences between the Lon-proficient strain and the mutant. After 1 h of toxin synthesis, about 85% of the lon mutant bacteria were able to form colonies, but only 25% of the wild-type bacteria were able to form colonies (Fig. 4B). This result could be explained by a direct effect of the lon mutation, although at present we cannot rule out the possibilities that the two strains had different growth rates and that there were different levels of expression in the two strains. However, these possibilities seem unlikely since (i) the two strains had similar doubling times and at time zero the numbers of CFU were almost identical for the two strains (around 10^7 CFU/ml) and (ii) expression of the relE2Spn

FIG. 3. Recovery of cell viability due to antitoxin synthesis after overproduction of YoeBSpn. E. coli TOP-10 cells harboring plasmids pYBS2 (yoeBSpn) and pYFS10 (yefMSpn) were grown in TY medium without glucose containing kanamycin and ampicillin. When the culture reached an OD_{600} of 0.13, 0.2% arabinose was added. Absorbance data were obtained for 7 h after induction of toxin synthesis (inset). At different times, samples were plated on TY agar containing 0.4% glucose, kanamycin, and ampicillin supplemented (■) or not supplemented (○) with IPTG.

FIG. 4. Differences in YoeBSpn and RelE2Spn toxicity in E. coli lon/H11001 and lon strains. (A) Inhibition of cell growth by overproduction of the pneumococcal toxins YoeBSpn (○ and ■) and RelE2Spn (▲ and △) in E. coli strain MG1655 (lon/H11001) (solid symbols) or in the lon isogenic mutant MG1655lon (open symbols). E. coli MG1655 (○ and ■) or MG1655lon (△) was grown in TY medium supplemented with kanamycin and 0.2% glucose (■) or arabinose (○, △, ▲, and ▼). Absorbance data were obtained at different times after the addition of arabinose. (B) To determine the number of CFU, samples were removed at various times and spread on TY agar plates containing 0.4% glucose and kanamycin. The percentages of viable cells were calculated by comparison of the numbers of CFU in the induced cultures to the numbers of CFU in the noninduced cultures at time zero.
gene (encoding the pneumococcal toxin RelE<sub>Spn</sub>), using the same expression vector, resulted in up to a 5% reduction in the number of viable cells of a lon strain after 1 h of toxin overproduction (36).

**Purification of YefM<sub>Spn</sub> and of the (YefM-YoeB)<sub>Spn</sub> complex.** A DNA fragment spanning the pneumococcal region that included either the (yefM-yoeB)<sub>Spn</sub> genes or only the yefM<sub>Spn</sub> gene was cloned in two different pET vectors (see Materials and Methods). The pneumococcal genes were placed under control of promoter φ10 of phage T7 (47), so IPTG induction led to synthesis of a His-tagged toxin-antitoxin complex or only a His-tagged antitoxin. The YefM<sub>Spn</sub> antitoxin (9,953 Da) co-precipitated with the His-tagged YoeB<sub>Spn</sub> toxin (11,467 Da) and eluted in a single broad peak (Fig. 5A). The proteinaceous yield of the purified complex was relatively high, as a considerable amount of complex (>15 mg) could be obtained from 2 liters of a bacterial culture. Compared to the yield of the complex, the proteinaceous yield of the His-tagged YefM<sub>Spn</sub> antitoxin (12,457 Da) was lower (Fig. 5B). This was unexpected since the YefM<sub>Eco</sub> antitoxin exhibited high solubility on the one hand and should not have provoked transcriptional or translational inhibition on the other hand, which may have led to such poor yields. However, we speculated that susceptibility of the antitoxin to proteases (due to partial unfolding [see below]) without an available cognate partner to stabilize it could have been the reason for the results observed.

**Analysis of YefM<sub>Spn</sub> and YoeB<sub>Spn</sub> interactions.** Crude lysates of E. coli cells overproducing the pneumococcal toxin and
antitoxin were loaded onto a nickel affinity column under native conditions. Two distinct protein bands were observed during an SDS-PAGE analysis following elution with 500 mM imidazole, and the positions of these bands corresponded to the expected sizes of YefM<sub>Spn</sub> and YoeB-His<sub>Spn</sub>, indicating that under native conditions the two proteins copurified (Fig. 5A). This, in turn, suggests that the YefM<sub>Spn</sub> protein may have formed a tight complex with its toxin counterpart. This inference was validated when the purified proteins were subjected to size exclusion chromatography. A single peak at 49.5 kDa was detected, suggesting that YefM<sub>Spn</sub> and YoeB<sub>Spn</sub> generated a protein complex with an apparent stoichiometry of (YefM<sub>Spn</sub>)<sub>2</sub> (YoeB<sub>Spn</sub>)<sub>2</sub> (Fig. 5C). This finding agrees with the 2:2 stoichiometry observed for the <i>E. coli</i> YefM-YoeB complex based on size exclusion chromatography of approximately 0.2 mg/ml of the YefM–YoeB-His complex, although 2:1 and 2:4 stoichiometries were also observed, but with fewer occurrences (I. Cherny and E. Gazit, unpublished). In the case of the Kis-Kid proteins, 1:2, 2:2, and 2:1 stoichiometries were also found, although the results depended on the relative protein concentrations (27). Subsequent Western hybridization using an anti-His<sub>6</sub> antibody confirmed the identity of the protein.

To detect whether the two proteins interacted in vivo, a two-hybrid system was employed. To this end, the <i>yefm</i><sub>Spn</sub> and <i>yoeB<sub>Spn</sub></i> reading frames were cloned into both the pBT bait and pTRG target vectors for the two-hybrid assay as described in Materials and Methods. Positive protein-protein interactions, which were indicated by growth of the indicator strain harboring both the bait and target recombinants on the selection medium containing 5 mM 3-AT, were detected for both the positive control pBT-LGF2 and pTRG-Gal11<sup>P</sup> (Fig. 5D, streak 1) and for the pBT-yoeB<sub>Spn</sub>–pTRG-yefM<sub>Spn</sub> pair (Fig. 5D, streak 6). However, when the interaction pair was changed (i.e., pBT-yefM<sub>Spn</sub> and pTRG-yoeB<sub>Spn</sub> instead of pBT-yoeB<sub>Spn</sub> and pTRG-yefM<sub>Spn</sub>), there was sparser growth of the indicator strain harboring both plasmids on the 3-AT-containing selective medium (Fig. 5D, streak 5).

Far-UV CD analyses of YefM<sub>Spn</sub> and the (YefM–YoeB-His)<sub>Spn</sub> complex. The CD spectra of His-YefM<sub>Spn</sub> (A) and (YefM–YoeB-His)<sub>Spn</sub> complex (B) at 4°C (solid line), 37°C (dashed line), and 60°C (dotted line). (C) CD ellipticity of His-YefM<sub>Spn</sub> (<i>Œ</i>) and (YefM–YoeB-His)<sub>Spn</sub> (<i>E</i>) was measured at 220 nm at various temperatures to estimate the thermal stability. The values were normalized between 0 and 1 and are expressed as fractional changes. (D) SDS-PAGE analysis of (YefM–YoeB-His)<sub>Spn</sub> sample that was examined by CD analysis before and after exposure to 85°C. Lane M, marker; lane 1, before treatment (sample at room temperature); lane 2, after treatment (soluble fraction after exposure to 85°C).

FIG. 6. Far-UV CD spectra of His-YefM<sub>Spn</sub> (A) and (YefM–YoeB-His)<sub>Spn</sub> complex (B) at 4°C (solid line), 37°C (dashed line), and 60°C (dotted line). (C) CD ellipticity of His-YefM<sub>Spn</sub> (<i>Œ</i>) and (YefM–YoeB-His)<sub>Spn</sub> (<i>E</i>) was measured at 220 nm at various temperatures to estimate the thermal stability. The values were normalized between 0 and 1 and are expressed as fractional changes. (D) SDS-PAGE analysis of (YefM–YoeB-His)<sub>Spn</sub> sample that was examined by CD analysis before and after exposure to 85°C. Lane M, marker; lane 1, before treatment (sample at room temperature); lane 2, after treatment (soluble fraction after exposure to 85°C).
structures and, to some extent, in favor of an unordered conformation, as the CD minimum signals were weakened at both 221 and 208 nm and shifted to 218 and 207 nm.

The thermal stabilities of the antitoxin and the complex were estimated by monitoring the ellipticity at 220 nm (Fig. 6C) at various temperatures between 4 and 90°C. Both the antitoxin and the complex seemed to undergo unfolding when the temperature increased. However, two noteworthy differences were observed: (i) the unfolding rate of the antitoxin was higher than that of the complex (the difference in the melting temperatures was greater than 10°C, as estimated from the inflection points of the two melting curves), and (ii) following thermal melting the sample containing the complex became turbid, indicating that YoeB-His
Spn aggregation occurred during the melting. SDS-PAGE analysis of the soluble fraction which remained after heating (Fig. 6D) showed that the aggregation was predominantly related to the toxin, while the antitoxin remained soluble.

**Molecular modeling of YoeB-YefM-like protein structures.**

To shed some light on the structural basis of the interactions between members of the YefM and YoeB families of proteins, we constructed structural models for YefM
\textit{E. coli} and Txe (Fig. 7), based on the previously determined crystal structures and, to some extent, in favor of an unordered conformation, as the CD minimum signals were weakened at both 221 and 208 nm and shifted to 218 and 207 nm. One site is composed of the H3 and H4 helices, and the other site is composed of an extended \( \beta \)-strand (26). For the toxin models, we used the YefM
Streptococcus pneumoniae model (Fig. 7A, B, and C), and the template (24% and 27%, respectively) are in the so-called “twilight zone” for reliable homology modeling, although the levels of sequence similarity for the residues encompassing the two sites for recognition of YoeB by YefM are 67% and 56%, respectively. Additionally, the intrinsic qualities of the YefM
Streptococcus pneumoniae and Axe models were probed and confirmed by using several specific programs (WHAT_CHECK, PROCHECK, and Verify 3D).

With all the considerations described above, we focused on the interaction domain of YefM-YoeB, and we performed an analysis of the surface electrostatic potentials of the structural models. The positively charged hindrance defined by the YefM
Streptococcus pneumoniae R72 residue in the neutral H4 helix was shown to be the main part of the electrostatic interaction with the negatively charged pocket on the interface of YoeB
Streptococcus pneumoniae (26); the equivalent residue is K64 in the pneumococcal antitoxin. This positively charged moiety seems to be also exposed in the Axe model but not in the YefM
Streptococcus pneumoniae model (Fig. 7A, B, and C), and there are no other apparent differences between the surface electrostatic potentials of the antitoxins. The change in the distribution of charges in the residues that configure this region in the pneumococcal antitoxin could indicate that single amino acid substitutions may not necessarily lead to a total loss of antitoxin activity. The lack of this positively charged patch in the YefM
Streptococcus pneumoniae antitoxin could explain its failure to neutralize the toxic effect; in addition, it suggests that the interaction domain of YoeB
Streptococcus pneumoniae TA could involve other residues. A detailed mutational analysis of this region should clarify this possibility. When the models of the toxins were examined, an analysis of the surface electrostatic potentials showed that there were no significant differences (Fig. 7D, E, and F). However, a quantitative analysis of the solvation energies of the surface areas calculated with the program GETAREA (http://www.scsb.utmb.edu/getarea/) proved that the YoeB
Streptococcus pneumoniae structural model has a larger exposed surface (7% larger polar area) than the other two toxins as a result of 9 surface atoms more than the YoeB
Streptococcus pneumoniae-ordered monomer and 18 buried at-

**TABLE 1. Secondary structure contents determined from CD spectra**

| Protein(s) | Method\(^a\) | \(\alpha\)-Helices | \(\beta\)-Strands | Coils\(^b\) |
|------------|-------------|------------------|------------------|------------|
| YefM       | K2d         | 26 27 25         | 17 21 20         | 57 52 55   |
|            | Selcon3     | 26 24 19         | 19 20 23         | 55 56 58   |
|            | ContinLL    | 27 25 20         | 18 20 22         | 55 55 58   |
|            | CDssr       | 25 23 17         | 19 19 24         | 56 58 59   |
| Avg        | 26 24.75 20.25 | 18 25 20 22.25   | 55.75 55.25 57.5 |
| YefM-YoeB  | K2d         | 55 37 28         | 9 15 15          | 36 50 57   |
|            | Selcon3     | 50 32 30         | 8 13 14          | 42 55 56   |
|            | ContinLL    | 38 39 34         | 6 11 14          | 56 50 52   |
|            | CDssr       | 45 42 38         | 17 15 24         | 38 43 38   |
| Avg        | 47 37.5 32.5 | 10 13.5 16.75    | 43 49.5 50.75    |

\(^a\)K2d, K2d program developed by Andrade et al. (2); Selcon3, method developed by Sreerama and Woody (46); ContinLL, method developed by Provencher and Glockner (41); CDssr, method developed by Johnson (25).

\(^b\)Coils consist of random conformations and turns.
antitoxin for counteracting the toxic effect of the pneumococcal toxin. Definite insights into the structural interface of the pneumococcal YefM-YoeB complex, however, await X-ray crystallographic resolution data for this TA system.

**DISCUSSION**

The yefM-yoeB<sub>Spn</sub> locus has been identified as a new chromosomal TA system of *S. pneumoniae*. Overproduction of the pneumococcal YoeB<sub>Spn</sub> toxin in *E. coli* cells resulted in cytotoxic effects commonly linked to toxin activity. However, the size of the negative effect mediated by YoeB<sub>Spn</sub> depended on the strain used; whereas in the K-12 strains used, TOP-10 and MG1655, the reductions in the number of CFU were more than 10<sup>4</sup>- to 10<sup>5</sup>-fold, respectively, in the *E. coli* B strain (BL21) the reductions in the number of CFU were more than 10<sup>8</sup>-fold (Fig. 1). This influence of the type of strain used on the YoeB toxicity could explain the previous inability to obtain transformants from plasmid DNA harboring the yoeB<sub>Eco</sub> gene in an *E. coli* B strain (26). The differences in the reduction in the number of viable cells for the *E. coli* strains used could have been due to different levels of expression of yoeB<sub>Spn</sub> or to different sensitivities of the genetic backgrounds. The yefM-yoeB<sub>Spn</sub> cassette functions as an antitoxin-toxin module both in *S. pneumoniae* (unpublished results) and in *E. coli* (Fig. 1 to 4), and it seems likely that degradation of the pneumococcal antitoxin by a Lon-type protease could trigger the YoeB<sub>Spn</sub> toxic activity in *S. pneumoniae*. In the case of *E. coli*, it has been shown that Lon overproduction stimulates the toxic activity of YoeB<sub>Eco</sub> and that a Δlon strain is less sensitive to YoeB<sub>Eco</sub> overproduction than a lon<sup>−</sup> strain is, indicating that the Lon protease could be responsible for antitoxin degradation and the subsequent activation of YoeB<sub>Eco</sub> (7). Our results (Fig. 4) suggested that the activity of YoeB<sub>Spn</sub> did not require degradation of the *E. coli* antitoxin. We hypothesized that Lon could mediate the toxicity of YoeB-like proteins by antitoxin degradation and/or by an antitoxin degradation-independent mechanism.

Overproduction of YoeB<sub>Spn</sub> resulted in cessation of cell growth and a substantial decrease in the number of CFU, which then increased gradually as the time of toxin synthesis increased (Fig. 1 and 2). The reduction in cell viability was alleviated by antitoxin expression for almost 2 h, so that at this time the number of CFU was reduced only 60%. After 4 h of exposure to the toxin, neutralization by the antitoxin resulted in 4% viability, compared to the 0.4% viability in the absence of antitoxin synthesis (Fig. 3). The YoeB<sub>Spn</sub> toxin could inhibit protein synthesis by mRNA degradation like its *E. coli* homolog (7, 26). Overproduction of the pneumococcal toxin for extended periods of time could lead to very low levels of translation, thus favoring the senescence processes and finally leading to cell death. We believe that the function of the YoeB<sub>Spn</sub> toxin is to promote cell growth arrest under stress conditions; in a physiological situation, the amount of free toxin could be enough to reduce cell growth but permit a residual level of protein synthesis, so that the cells could be protected from irreversible damage and the metabolism could adapt to the unfavorable conditions.

The toxic effect of YoeB<sub>Spn</sub> could be neutralized by its cognate antitoxin, YefM<sub>Spn</sub>, but not by the *E. coli* counterpart.
relBE2Spn

of strain R6 of
Far-UV CD analysis of the YefM
gut for
survival in the different niches that the bacteria colonize (e.g., the
with its cognate antitoxin could be related to the mechanism of

apparent from the molecular modeling of the proteins (Fig. 7).
between the toxins and the heterologous antitoxins, which was
proteins and may also explain the lack of complementation
The fact that there is a considerable unstructured region in
YefM E. coli
may explain the structural difference between the two
proteins and may also explain the lack of complementation
between the toxins and the heterologous antitoxins, which was
apparent from the molecular modeling of the proteins (Fig. 7).
This in turn may indicate that the specific interactions of a toxin
with its cognate antitoxin could be related to the mechanism of
survival in the different niches that the bacteria colonize (e.g., the
gut for E. coli and the nasopharynx for pneumococci). In the case of
strain R6 of S. pneumoniae (24), two different functional TA
loci, namely relBE2Spn and yefM-yoeBSpn, the residual nonfunc-
tional relBE1Spn genes (36), and a putative homolog of omega-
epsilon-zeta (13; J. C. Alonso, personal communication) are
present in the chromosome. In the case of virulent strain TIGR4
(48), in addition to these three loci, there are two more putative
TA loci (37), one locus homologous to P1 phage phd/doc (30) and
the other homologous to Rts1 plasmid higBA (49). YoeB and
ReI toxins have similar toxic activities, but they have
different mRNA cleavage patterns and sets of features that
could enable the two TA systems to play different roles. First,
ReI cleaves at UAG, UAA, UGA, UCG, and CAG sequences with preference for the stop codon UAG (39), and a
similar pattern has been observed for pneumococcal RelE2Spn
(9); cleavage of mRNA by YoeB E. coli occurs predominantly at
the 3' end of adenine or guanine residues (26). Second, ReI-
dependent cleavage occurs only in the ribosome (39), whereas
YoeB E. coli has an intrinsic RNase activity and is able to cleave
RNA in the absence of ribosomes (26). Third, structure-based
comparisons (26) revealed that the residues involved in the
RNase activity of YoeB (D46, H83, and Y84) are not con-
served in ReI, with the exception of R65, suggesting that
ReIE could be an incomplete RNase which lacks the essential
residues for catalytic activity. Finally, the signal that triggers
mRNA cleavage could be different, since ReI activity in E.
coli is induced by amino acid starvation (10), whereas the Lon
protease triggers YoeBEco-dependent RNA cleavage (7).
Thus, either TA systems could act through independent mech-
anism or they could work together under stress conditions
(i.e., amino acid starvation), as has recently been suggested for
RelE and MazF (12). The combined action of the two toxins
with RNase activity (namely, YoeB E. coli with free mRNA and
ReI with mRNA bound to ribosomes) could lead to signifi-
cant depletion of mRNA and an effective, but not total, block
of translation, leaving low levels of antitoxin synthesis which
could result in toxin-mediated arrest of cell growth but not in
induction of a bacterial death response.

Even though TA loci appear to be nonessential, it is curious
that in many bacterial species more than one TA locus is
conserved in the chromosome and in plasmids. The role of the
TA systems in the extrachromosomal elements is related to
stable inheritance, or the TA systems may be triggered when a
plasmid cannot replicate (4, 14). Furthermore, all E. coli TA
loci can be deleted without causing significant growth differ-
ences in the wild-type strains (7, 8, 36, 38). However, antibi-
otics that inhibit transcription and/or translation in E. coli and
induce mazEF-dependent cell death do not reduce cell viability
in mazEF mutants (42). Mutations affecting both MazF and
ReI activities in Streptococcus mutans resulted in cells whose
cell acid tolerance and growth in glucose-based medium were
affected (32). In the case of Neisseria gonorrhoeae workers have
identified a mutant in a mutation in a putative TA system (desig-
nated FitAB for fast intracellular trafficking; function
unknown) which grows normally extracellularly but has a
higher rate of intracellular replication, and there is a com-
mentary increase in the rate at which this mutant traverses a
monolayer of polarized epithelial cells (34).

Taking into account the complexity of the “biological
niches” of S. pneumoniae and other pathogenic bacteria, it is
very simplistic to attempt to ascertain the function of the TA
system when cells are growing in a culture medium. Bacteria
grow in communities with other living cells and surely compete
with each other for the surrounding nutrients. In these bacte-
rial environments the levels of nutrients are not as high as
those used in laboratory conditions. For S. pneumoniae, the
mucosal epithelium of the nasopharynx is the primary site of
colonization, where it does not cause any symptoms of illness.
Occasionally, bacteria can switch from colonization to infec-
tion in the lungs, blood, and other tissues, and many times they
traverse the blood-brain barrier and infect the meninges. Con-
sequently, S. pneumoniae has to adapt to different situations,
and perhaps in these different situations the TA systems play
an important role in survival when the bacteria are exposed to
host defenses.

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