The relBE2Spn Toxin-Antitoxin System of *Streptococcus pneumoniae*: Role in Antibiotic Tolerance and Functional Conservation in Clinical Isolates

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Abstract

Type II (proteic) chromosomal toxin-antitoxin systems (TAS) are widespread in Bacteria and Archaea but their precise function is known only for a limited number of them. Out of the many TAS described, the relBE family is one of the most abundant, being present in the three first sequenced strains of *Streptococcus pneumoniae* (D39, TIGR4 and R6). To address the function of the pneumococcal relBE2Spn TAS in the bacterial physiology, we have compared the response of the R6-relBE2Spn wild type strain with that of an isogenic derivative, AREI2Spn under different stress conditions such as carbon and amino acid starvation and antibiotic exposure. Differences on viability between the wild type and mutant strains were found only when treatment directly impaired protein synthesis. As a criterion for the permanence of this locus in a variety of clinical strains, we checked whether the relBE2Spn locus was conserved in around 100 pneumococcal strains, including clinical isolates and strains with known genomes. All strains, although having various types of polymorphisms at the vicinity of the TA region, contained a functional relBE2Spn locus and the type of its structure correlated with the multilocus sequence type. Functionality of this TAS was maintained even in cases where severe rearrangements around the relBE2Spn region were found. We conclude that even though the relBE2Spn TAS is not essential for pneumococcus, it may provide additional advantages to the bacteria for colonization and/or infection.

Introduction

Chromosomally-encoded Type II toxin-antitoxin systems (TAS), composed of two proteins, are widely spread among Bacteria and Archaea. Typically, they are organized as operons in which the first gene encodes the antitoxin (A) and the second the toxin (T). Both proteins interact to generate a harmless TA complex that auto-regulate their own synthesis. The A protein by itself is metabolically unstable and is constitutively degraded by ATP-dependent proteases, releasing a free and stable T protein that would kill or stop the growth of the cells by disruption of key cellular processes [1]. A puzzling observation derived from bio-informatics approaches is that many bacteria and archaea harbour multiple copies of various TAS (e.g. around 60 TAS in *Mycobacterium tuberculosis* [2]), being even more abundant than previously envisaged [3,4]. Notwithstanding the knowledge on the mechanisms of action of TAS [5] and the three-dimensional structure of various TA protein complexes [6–12], little is known on the role of these systems in the bacterial cell lifestyle. In the case of plasmid-encoded TAS, they seem to be involved in the stable maintenance (“addiction”) of the replicons by increasing their chances of vertical transmission [13]. For the chromosomally-encoded TAS, several interpretations have been given to their ubiquity and abundance, though none has been demonstrated thus far [14]. First, it has been proposed that TAS could act as stress response elements that modulate growth by reducing macromolecular synthesis. Hence, induction of these systems results in cell stasis rather than in cell death, leading to viable but not cultivable cells [5,15]. Inhibition of bacterial growth induced by the toxin was reversed by expression of the cognate antitoxin or by the transfer-messenger mRNA (mRNA). Thus, toxins would induce a reversible stasis that improves bacterial cell survival under extreme conditions [15–17]. Second, some chromosomal TAS such as mazEF has been considered as mediators of bacterial programmed cell death [18,19]. Unfavourable cell growth conditions could trigger this pathway and, as a consequence, a subpopulation of bacterial cells would die. Death of these cells would i) preserve the food for the remaining population, ii) serve as a defence mechanism to restrict phage spreading, and iii) act as a mechanism to eliminate cells with deleterious mutations. It would seem that mazEF-mediated cell death is a population-dependent phenomenon requiring a quorum sensing molecule, termed extracellular death factor, which is a linear pentapeptide (NNWNN) important for mazEF-mediating killing activity [20]. *E. coli* strains defective in mazEF showed lower sensitivity to antibiotics than the wild type, indicating that antibiotic addition could induce mazEF-mediated cell death [21]. And third, comparison of the fitness of two isogenic *E. coli* strains, one wild type (wt) and the other having...
deletions in five TAS (mazEF, relBE, cheB, yefM-yoeB, dinJ-yafQ) subjected to short-term stress conditions (amino acid starvation, acidic stress, antibiotic treatment, and long-term stationary phase) showed no significant differences among them [22], pointing that TAS could be involved only in long-term evolution [1]. However, some findings have complicated further the interpretation of the TAS role: i) TAS-defective cells showed a reduced ability for biofilm formation [23,24]; ii) TA-cassettes have been localized in both integrative and conjugative genome elements that could have incorporated into the bacterial chromosome and, within this context, they could promote plasmid maintenance [2,25–28]; iii) TAS can work as anti-addiction modules [29]; iv) they may play an essential role in development of programmed cell death which leads to Myxococcus multicellular development [30], and v) they may be linked to bacterial persistence upon antibiotic exposure [31].

Genes for at least eight putative TAS are present in the chromosome of the Gram-positive bacterium Streptococcus pneumoniae (the pneumococcus): relBE1Spn, relBE2Spn, yefMYoeBSpn, higAB, phd/doc, pezAT, tasAB, and hicAB [3,17,32]. Among these, only three of them, namely relBE2Spn [33], yefMYoeBSpn [34], and pezAT [7] have been shown to be genuine TAS, whereas relBE1Spn was shown to be non-functional [33]. Exposure of E. coli cells to RelE2Spn toxin resulted in the arrest of cell growth, which was rescued by induction of RelB2Spn antitoxin but only within a time-frame window: long-time exposure to the toxin led to cultures unable to reach growth [33]. We report here on the role of the pneumococcal relBE2Spn TAS in the bacteria lifestyle. We have compared the behaviour of two pneumococcal isogenic strains, wild type (wt) R6 and a relBE2Spn mutant derivative (R6ΔrelBE2Spn) [33] under different growth conditions, and differences were found when cells were subjected to stress conditions that impaired protein synthesis. The RelE2Spn toxin could act as a modulator of protein synthesis under stress, but it could also induce cell death when the level of protein synthesis was dramatically reduced. Further, if relBE2Spn played a role in bacterial fitness, then it should contribute to colonization and survival after infection (an important part of the switch from commensal to infective for a bacterium like S. pneumoniae). If this was the case, the relBE2Spn genes should be ubiquitous in the S. pneumoniae population. Thus, the presence and integrity of the relBE2Spn locus was tested in 100 strains from different sources. Unlike E. coli, where several strains lacked the relBE operon [1,14], all pneumococcal strains analyzed retained this locus in their chromosome. Although the relBE2Spn operon exhibited various degrees of polymorphisms in the different isolates, none of the changes impaired the functionality of the relBE2Spn locus. A molecular model of the pneumococcal RelE2Spn protein was constructed based on the three dimensional structure of the RelBE complexes from Pyrococcus horikoshii (PhRelE) [10] and compared with that of Methanococcus jannaschii (MjRelE) [11]. The modelled RelE2Spn kept several residues related to the catalytic activity of ribonucleases, which are also present in MjRelE. However, these residues are missing in EoRelE and PhRelE proteins [10,35,36], raising the possibility that the two former RelE proteins, albeit being ribonucleases, could use a mechanism of action different than the one proposed for EoRelE [12].

Results

Mutation of the relBE2Spn operon has no effect on pneumococcal cell viability under either regular conditions of growth or carbon starvation

We first tested the differences in growth (optical density at 650 nm, OD650) and viability (measured by determination of the colony-forming units, cfu) between the two strains, R6 wt and its relBE2Spn mutant derivative, R6ΔrelBE2Spn (Table 1). The mutant strain contains two mutated copies of the relB2 gene, and has the relE2 gene placed away from its promoter, also disrupting the translational coupling that appears to exist in this pneumococcal operon [33]. RT-PCR assays showed that in the mutant strain there was not detectable synthesis of relE2 mRNA (not shown). To perform the experiments, cultures of both strains were grown 24 h, and OD650 and cfu were determined at time intervals. No differences were found between the strains during the entire period in which the cultures passed through exponential (0–2 hrs), stationary (2–8 hrs), and autolysis (8–24 hrs) phases of growth (Figure S1 A, B). Autolysis is a distinctive property of S. pneumoniae whose cells show a tendency to spontaneously lyse when the culture reaches the stationary phase [37]. Autolysis plays an important role in the bacterial infection by the release of virulence factors which may modulate the inflammatory response [38]. Glucose starvation activates relBE transcription in E. coli, probably because of degradation of RelB by the protease Lon, an event that would lead to an increase in free RelE toxin and a reduction in the number of cfu [39]. In the case of S. pneumoniae, the sugars routinely used as a carbon source are di-saccharides (sucrose or maltose) rather than glucose because of poorer utilization of the latter [40]. Carbon-starved cultures of either wt or ΔrelBE2Spn mutant cells showed no differences although cessation of growth was observed for either strain as compared to sucrose-grown cultures, and no decrease in viability was observed in either sucrose-depleted culture (Figure S1 C, D).

The relBE2Spn operon modulates pneumococcal growth under amino acid starvation

Serine hydroxamate (SHT) induces amino acid starvation because it blocks incorporation of Ser residues into proteins by interfering with the load of seryl-tRNA [41,42]. In E. coli, addition of SHT (similarly to carbon starvation) resulted in an increase of relE transcription leading to the increase of free RelE due to RelB Lon-dependent proteolysis [39]. Thus, we

| Bacteria | Genotype | Reference/ source |
|----------|----------|-------------------|
| E. coli | TOP10 | F-mcrA, A(mrr-hsdRMS-mcrBC), φ80lacZ1M15, lacX74, relA1, deoR, araD139, araC-leu7697, galU, galK, rpoD157, endA1, nupG | Invitrogen |
| S. pneumoniae | R6Wild type | [40] |
| S. pneumoniae | R6ΔrelBE2Spn | [33] |

| Plasmid | Features | Reference/ source |
|---------|----------|-------------------|
| pFU52 | pMB1, P שא, araC, neo | [50] |
| pNM220 | mini-R1, pA1/4D/03, bla, lacF | [51] |
| pE2wt | pFU52, relBE2Spnwt | This work |
| pE71 | pFU52, relBE2Spn7153 | This work |
| pE81 | pFU52, relBE2Spn8651 | This work |
| pE600 | pFU52, relBE2SpnK-600 | This work |
| pEter | pFU52, relBE2Spn7153ter | This work |
| pP2wt | pNM220, relBE2Spnwt | This work |

Table 1. Bacterial strains and plasmids.
followed growth and viability of the wt and the mutant strains of *S. pneumoniae* under a SHT-mediated amino acid starvation. The results (Figure 1A) showed that the growth of both cultures almost stopped, quickly and in a similar manner, indicating that cells entered into stasis. In contrast to sucrose starvation, differences in viability between both strains were observed (Figure 1B). In the mutant strain, a 50%-reduction in cfu was detected during the first 90 min of SHT treatment, followed by a slight recovery at longer times. Such a recovery was not detected for the wt strain, in which a continuous reduction in cfu was seen; these differences were more evident when the cfu were plotted on a linear, rather than logarithmic scale (Figure 1C). After the 180 min starvation period, SHT was removed, cells were resuspended in fresh pre-warmed medium, and incubation was continued for additional 180 min (Figures 2A and 2B). SHT withdrawal allowed resumption of bacterial growth. However, the mutant cells recovered and entered into stationary phase faster than the wt, whereas the latter showed a more prolonged exponential phase (Figure 2B, inset). These results suggested to us that i) the mutant strain could recover faster because it lacked toxin RelE, and ii) the wt cells could have saved more efficiently their physiological resources during stasis, allowing a full recovery after amino acid starvation.

### Blocking protein synthesis by erythromycin or streptomycin treatment leads to antibiotic tolerance in the *relBE2Spn* mutant

In addition to carbon- and amino acid-induced starvation, treatment with inhibitors of protein synthesis also caused a *relBE* transcriptional induction in *E. coli* [39]. We employed erythromycin (Erm) which inhibits protein synthesis by binding to the 23S rRNA, interfering with the amino acyl translocation [43]. Erm is an effective agent against streptococcal infections and its minimal inhibitory concentration (MIC) is low, since for selection for pneumococcal Erm-resistant transformants, the concentration 1 μg.ml⁻¹ is sufficient [44] and MIC for the majority of wild-type isolates fall in the range of 0.032–0.125 μg.ml⁻¹ (http://www.eucast.org/mic_distributions/). To test whether the mutation of *relBE2Spn* had any effect on cell-response to blocking protein synthesis, pneumococcal cultures (wt and Δ*relB2Spn* strains) were challenged with two dosages of Erm, 0.1 and 1 μg.ml⁻¹ (that is 10 and 100 fold MIC for R6, respectively), followed by OD₆₅₀ and cfu determination at different times. After the 20 min treatment, growth of both cultures was stopped, concomitantly with a progressive reduction in cfu (Figures 3A and 3B). At the end of the incubation period (180 min), a 10 to 1000-fold reduction in cfu was observed. Interestingly, the reduction in cfu was much more pronounced (10- to 100-fold difference) in the wt than in the

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**Figure 1. Changes in the growth profile of *S. pneumoniae* cells of the R6 or the R6 Δ*relB2Spn* mutant strains after inhibition of protein synthesis by SHT.** *S. pneumoniae* cultures, wt (○) and mutant (●) cells, were grown exponentially until an OD₆₅₀ = 0.2. Then, SHT (1.5 mg.ml⁻¹) was added, and incubation proceeded. Growth was monitored by determination of the OD₆₅₀ of the cultures treated (filled symbols) or untreated (open symbols) with SHT (A). At the times indicated, the numbers of cfu were determined by plating appropriate cell dilutions on SHT-free medium, and incubation for 36 h at 37 C (B). Percentages of viable cells from wt (x) or mutant (*) strains upon SHT-treatment (C) were calculated considering the number of cfu at time zero of the treatment as 100%. doi:10.1371/journal.pone.0011289.g001
mutant strain. These findings indicate that activation of RelE after antibiotic treatment would induce a complete shut-off in protein synthesis leading to cell stasis or even cell death. Then, lack of the \textit{relBE2Spn} operon would lead to antibiotic tolerance.

To confirm these observations, a test of bacterial viability was performed by employment of the LIVE/DEAD Baclight (Invitrogen) stain method. The pneumococcal cultures were stained and examined under the microscope: living cells were stained in green, whereas dead cells were stained in red. Micrographs of the pneumococcal cultures were taken at 0 and after 180 min of Erm treatment. The results showed no significant differences between both strains when the cultures were challenged with 0.1 \(\mu\text{g.ml}^{-1}\) of Erm (Figure 3C upper panel). When Erm concentration was raised to 1 \(\mu\text{g.ml}^{-1}\), a drastic loss of viability in the wt strain was found, which was not observed in the mutant cells (Figure 3C, lower panel). We performed a similar experiment using streptomycin (Sm), another protein synthesis inhibitor, at 20 \(\mu\text{g.ml}^{-1}\) (selection for transformants to Sm-resistance is 100 \(\mu\text{g.ml}^{-1}\)). In this case, we observed, again, that the mutant cells were more tolerant to Sm-treatment than the wild type strain (Figure S2). We conclude that the \textit{relBE2Spn} operon seems to be activated when protein synthesis is inhibited, so that under these unfavourable

Figure 2. Recovery of cell growth after removal of SHT. SHT-amino acid starved pneumococcal cells (180 minutes of starvation) were washed twice and suspended into pre-warmed fresh medium, and incubation proceeded for the indicated times. Recovery of starvations was followed by turbidity of the cultures (OD_{650}) of wt (○) and mutant (△) strains (A). The number of cfu was determined by plating appropriate dilutions of the cells at the times indicated (B). Inset: Linear plot showing cell viability evolution after SHT removal in wt (○) or mutant (△) cultures.

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conditions this TAS could contribute to modulate the survival response through stasis.

The relBE2Spn locus is conserved among S. pneumoniae clinical isolates

We reasoned that if the expression of relBE2Spn could confer a selective advantage to the pneumococcus, then a conservation of this locus in the bacterial chromosome of most, if not all, isolates should be expected, in spite of hyper-recombination typical for this species [45]. A preliminary analysis was performed in a small set of five Spanish clinical isolates, for which the presence of the relBE2Spn and the yefM-yoeBSpn loci (another pneumococcal TAS that was used as a control), was tested by PCR using the oligonucleotide pairs relB2p/relE2c and yefM N/yoeBC, respectively. The first pair was previously used to amplify the relBE2Spn locus of R6 strain [33], the relE2c primer annealing partially into the region encoding RelE2Spn toxin (Figure 4A). In these five strains, amplification of the yefM-yoeBSpn locus was feasible, in contrast to relBE2Spn (Figure 4B, left panel). However, when oligonucleotide relE2tga (fully complementary to the 3′ region encoding the toxin gene) was used instead of primer relE2c, a PCR product was detected (Figure 4B, right panel). The size of

Figure 3. Inhibition of protein synthesis by Erm treatment. Cultures of R6 (○) or R6ΔrelB2Spn mutant (△) cells were treated with two concentrations of Erm, 1 µg.ml⁻¹ (black symbols) or 0.1 µg.ml⁻¹ (grey symbols). Growth was followed by absorbance at OD₆₅₀ (A) of untreated (open symbols) and treated (filled symbols) cultures. At the indicated times, appropriate dilutions of cells were plated and incubated as above (B). All experiments were performed at least three times. LIVE/DEAD-staining of wt and the R6ΔrelBE2Spn cells (C). The cultures were harvested 180 min after addition of Erm and stained with BacLight Bacterial viability Kit (Invitrogen). Live cells show green fluorescence, whereas dead cells fluoresce red. Percentages of viable cells in every assay, calculated from the results of Figure 3B are indicated in parentheses.
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one of the PCR products was bigger than expected (around 2000 bp instead 650 bp), due to the presence of the IS1167 sequence (see below). The results obtained for the Spanish isolates demonstrated that the five strains analyzed contained the relBE2Spn locus and all but one (strain CipR-25) exhibited changes compared to strain R6 in the chromosomal regions flanking the relBE2Spn operon, whereas the region around the yefM-yoeBSpn locus was kept intact.

These initial polymorphisms prompted us to perform a similar search in a variety of clinical isolates. To this end, we chose more strains from well-characterized collections of clinical isolates from Spain and Poland [46–48].

Spanish strains were isolated from blood and sputum in the years 2002 and 2006. The Polish clinical isolates proceeded from the National Medicines Institute pneumococcal collection, and amounted to 58 isolates representing 37 serotypes and 52 different STs [48].

Figure 4. Presence of the relBE2Spn operon in the chromosome of isolates of S. pneumoniae. Genetic organization of the relBE2Spn and yefM-yoeBSpn loci in the R6 strain; the position of promoters P_rel and P_yoe, respectively, are shown (A). The primers used in the PCR amplifications are drawn as arrows, and the expected sizes of the corresponding PCR fragments are shown below. The PCR products detected using the oligonucleotide pairs relB2_p/relE2_c and yefM/yoeB_c (left panel) or the oligonucleotide pair relB2_p/relE2_tga (right panel) were separated on agarose gels (B). DNAs from loci relBE2Spn (r) or from yefM-yoeBSpn (y) were isolated from different S. pneumoniae macrolide-resistant strains, as follows: CipR-67 (1); CipR-25 (2); CipR-22 (3); CipR-14 (4); CipR-23 (5); R6wt (C); Mr. Molecular weight standard, Hyper ladder I (BIOLINE).

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These strains were mainly isolated from cerebral spinal fluid during the years 1997 to 2002. Spanish and Polish isolates were tested for presence of the relBE2Spn locus and its flanking regions by amplification with different oligonucleotides spanning the appropriate regions (Figure 5A). In addition to those, we checked, through bioinformatics procedures, the presence of the relBE2Spn operon in another 31 strains whose sequences are available at the NCBI Genome Project (http://www.ncbi.nlm.nih.gov/) and at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_pneumoniae/), thus making a total of 100 strains analyzed (Table S1). In addition, STs of sequenced pneumococcal strains for which MLST data was not available were determined in silico.

The results of the global PCR analyses of the chromosomal structure of S. pneumoniae around the relBE2Spn locus allowed us to classify the 100 isolates tested into three categories (Figure 5B and Table S1): Type I shared the genetic organization found in known strains (T1GR4, D39, and R6). Type II lacked the open reading frames (orf) SP1222 and SP1221, which were replaced by an orf homologous with the cation channel protein family. The genetic organization of Type III was similar to Type II but, in addition, it contained a 1200 bp sequence insertion located upstream the promoter of the relBE2Spn operon. The nucleotide sequence of this insertion corresponded to the IS1167 transposon sequence, which includes inverted repeats flanking the transposase [49]. There was no association of the TAS types with pneumococcal serotypes. However, a very good concordance was observed in all cases when more than a single representative of a given ST was analyzed. Altogether, the latter observation was made for 15 STs that included 44 isolates. Determination of the nucleotide sequence of several of the clinical isolates belonging to the three pneumococcal relBE Types (Table S1) showed no sequence changes in the antitoxin-encoding relBE2Spn gene. However, in the region encoding the RelE2Spn toxin, several nucleotide changes were detected. Some of them corresponded to silent mutations (GTC to GTT at V74; ATC to ATT at I65; GAC to GAT at D39, and TGA to TAA at the stop codon of the gene). Two other polymorphisms in which minor amino acid changes occurred were also found (T34I, and D39G), whereas some strains, like CipR-25 (Figure 5B), exhibited changes in the region spanning the -35 and -10 region of the relBE2Spn promoter, a region probably involved in the transcriptional self-regulation of the operon (I. Moreno, C. Nieto and M. Espinosa, unpublished).

Even though the nucleotide sequence of the relBE2Spn promoter region in the different types was not essentially modified, synthesis of the relBE2Spn mRNA (and hence the expression of these two proteins) could be affected. To detect the relBE2Spn mRNA in several clinical isolates, primer extension analyses were carried out. Total RNA was isolated from selected strains belonging to the three genomic types: i) from Type I, strains R6, and CipR-25, the latter containing the A/G change at the position -28 in the relBE2Spn proposed regulatory region; ii) from Type II, strains CipR-31, CipR-67, and 2115, the latter harbouring also the same change in the proposed regulatory region, and iii) from Type III, CipR-51 and CipR-14. In all strains, a primer extension product was detected (Figure S3) and its size was the same as the one detected previously for the laboratory R6 strain [33].

Taken together, we can conclude that all strains analyzed retained the relBE2Spn module, but exhibited three different genetic arrangements: 21.5%, 61.3% and 17.2% of the analyzed strains exhibited a genetic organization of the type I, II and III, respectively; 36% of the sequenced strains bore mutations in the gene encoding the RelE2Spn toxin. Furthermore, the operon organization, and thus co-transcription of both genes, was maintained in all strains tested.

**Attenuated toxicity in relE2Spn mutants**

As shown above, most of the nucleotide changes identified in the sequence of the relBE2Spn locus were located within the relE2Spn gene, two of them being missense mutations that affected the RelE2Spn toxin: change D39G was found in three strains (Polish 7153, and the NCBI genome project P1031 and JJA), whereas change T34I was relatively frequent (around 35% of the sequenced isolates). To verify whether these changes affected the toxic activity of RelE2Spn, and to elucidate their possible physiological consequences, we tested toxicity on E. coli cells based on two criteria previously used for the pneumococcal relM-yeed operon [34]: i) inhibition of cell growth after expression of either wild type or mutant RelE2Spn, and ii) ability of RelE2Spn to interact with the cognate RelB2Spn antitoxin in cultures harbouring uncleaved genes (relB2Spn and relE2Spn) cloned in two different plasmids under inducible promoters. In the latter conditions, induction of the antitoxin should neutralize the toxic effect of RelE2Spn thus permitting bacterial growth. To this end, DNA fragments containing genes relE2Spn, wt or mutants harbouring the D39G, T34I, and, as a control, relE2ter (encoding a truncated and inactive RelE2Spn protein) were cloned into plasmid pUFUS [50] under the control of the araBAD promoter (P araBAD), which is inducible by arabinose and repressed by glucose.

The resulting plasmids (Table 1) were termed pE2wt (wt RelE2Spn), pE71(D39G RelE2Spn), pE81(T34I) (from strain 8651) or pE600 (from strain k-600), both harbouring the T34I RelE2Spn mutation and pE2ter (truncated RelE2 protein). In addition, the relE2Spn wt gene was cloned in plasmid pNM220 [51], which allows IPTG-inducible expression of the antitoxin from the P araBAD promoter; the resulting plasmid was termed pB2wt (Table 1). As expected, no significant difference in growth rate was observed for E. coli cells with the control pE2ter upon induction of P araBAD. However, a total growth arrest was observed for E. coli harbouring the plasmids pE2wt, pE71(D39G), pE81(T34I), or pE600(T34I) (Figure 6A). Additionally, a severe decrease in the number of viable cells compared to cultures containing the pE2ter plasmid was seen (Figure 6B). This reduction in cfu occurred in the following order: relE2wt (almost four orders of magnitude) > relE2T34I (nearly two orders) > relE2D39G (twofold). The toxicity of the RelE2Spn toxin could be counteracted by its cognate antitoxin, encoded in the pB2wt plasmid. Cells, containing the different pairs of plasmids were streaked on plates supplemented with 0.4% arabinose (induction of toxin synthesis) with or without 2 mM IPTG (induction of antitoxin synthesis). Transformants containing, in addition to pB2wt, plasmids encoding the toxin (totally or partially functional) were able to grow only on plates supplemented with IPTG while control cells harbouring pB2wt and either pUFUS or pE2ter did not show growth differences in the presence or absence of IPTG (Figure 6C).

**Discussion**

The human upper respiratory tract is a natural environment for S. pneumoniae from which these bacteria spread to other body parts and to new hosts; hence an increase in pneumococcal persistence during colonization may influence its virulence and epidemicity [52]. Persistence may be one of the roles performed by the bacterial TAS by allowing bacteria to survive under nutrient limited conditions, thereby improving adaptability to selective pressures and permitting the bacteria to retain their capacity to colonize humans without reduction in virulence. The pneumococcal relBE2Spn was identified in the chromosome of S. pneumoniae and shown to be functional, in contrast to the pneumococcal homologue termed relBE1Spn [16,33]. Cells lacking the relBE2Spn
operon exhibited the same growth profile and response to sugar starvation as the wt bacteria did (Figure S1). Differences were found, however, when cells were subjected to protein synthesis inhibition either by amino acid starvation or antibiotic treatment. In the case of the E. coli RelBE, the toxin was activated because of antitoxin RelB degradation subsequent to inhibition of protein synthesis [39]. Toxicity of EooRelE protein is due to cleavage of translating mRNA at the ribosomal A site [12,53]; other RelE homologues, including RelE2Spn seem to cleave RNA in a similar manner, and thus cells exposed to the toxin showed a drastic growth arrest [16,33]. Similarly, activation of RelE2Spn by SH1 treatment, led to reduction in the number of cfu (Figures 1 and 2), but this reduction was not due to cell death as detected by the LIVE/DEAD BacLight bacterial viability method (not shown), but rather to a slower rate of cell growth. After SH1 removal, cells returned to normal growth, although two major differences were observed between the wt and the mutant strains (Figure 1 and 2): i) the wt cells recovered more slowly than the mutants, most likely because recovery of cell growth of the former required prior antitoxin synthesis to neutralize the RelE2Spn toxin, and ii) the wt strain exhibited an exponential growth period after recovery which was longer than that of the mutant. These results indicated that the pneumococcal relBE2 system, under amino acid starvation, could help the bacteria to divert the scarce resources to essential processes, thus improving its survival potential. Treatment with Erm (or with Sm) also resulted in a different response in the two pneumococcal strains used. The wt showed a higher and quicker reduction in cfu than the mutant (Figures 3B and S2) although, at 0.1 μg ml⁻¹ of Erm, it was not due to diminished cell viability. However, at 1 μg ml⁻¹ of Erm, a clear loss in viability was observed for the wt, which was not the case for the mutant (Figure 3C). The higher sensitivity of the wt strain to a low dosage of Erm can be explained as the result of RelE2Spn activation subsequent to protein synthesis inhibition. When higher concentrations of the antibiotic were used, cell lysis was observed in the wt after 180 minutes of treatment and, during the recovery period, only the mutant cells were able to resume growth but only after 24 hours of Erm removal (not shown). Thus, activation of the pneumococcal relBE2Spn operon subsequent to antibiotic treatment appeared to induce an extreme interruption of the protein synthesis, leaving the bacteria unable to recover viability or even inducing cell death. Then, lack of the relBE2Spn operon in S. pneumoniae would lead to antibiotic tolerance a role that coincides with the one proposed for the E. coli mazEF TAS [21].

The pneumococcal relBE2Spn operon is not essential, at least under the laboratory growth conditions used (S3) and Figure S1), but it showed a functional conservation in all the strains tested (see below). This was unlike the two other TAS characterized in S. pneumoniae, namely pezAT and yoeB-yefMSpn. The former was found to be absent in several clinical isolates of S. pneumoniae [7], whereas a search for the presence of yoeB-yefMSpn in 31 pneumococcal strains sequenced (NCBI project or Sanger institute) showed that more than 40% of them lacked this TAS (not shown). In E. coli, the homologous reIB TAS have been lost in several strains [14]. In addition, analyses of 395 E. coli strains showed decay in the chromosomally-encoded ccdAB TAS and a molecular evolution analysis of these data suggests that this TAS does not seem to retain any role in E. coli [54]. A recent study on comparative metagenomic analyses of plasmid-encoded functions in the human gut microbiome showed that the RelBE TAS, as compared to other TAS, is relatively abundant and retains a broad phylogenetic distribution in the human gut microbiome, suggesting that prevalence of RelBE could be related to fitness of the bacterial host [55]. Our analysis of the relBE2Spn locus showed the existence of three different genetic organizations, although transcription of the operon was not affected by these rearrangements (Figure S3). Type II seems to be more divergent and most prevalent (Table S1). This may suggest this is an ancestral type. It is easy to imagine how types I and type III are made by single genetic events from II. Type III looks homogenous but all the isolates of type III that were sequenced originated from the same clone so this could have been expected.

In addition to the above arrangements, various isolates showed several more polymorphisms at the relBE2Spn gene, some of them affecting the amino acid sequence of the protein. Two of them were found to be relevant, namely changes T34H and D39G, since toxin activation in either of the two mutants led to growth arrest, although their toxic effect was lower than the wt toxin (Figure 6), indicating that these amino acid changes could affect critical residues of the toxin. We constructed a molecular model of the pneumococcal RelE2Spn based on the crystal structures of the RelBE protein complexes of P. horikoshii (PrRelBE) [10] and the recently published structure of the RelE protein from Methanobrevibacter smithii (MjRelBE) [11]. Amino acid sequence alignment and the structural model (Figure 7) indicated that all R residues that were previously related to PrRelE toxicity (R10, R58, R65, and R85) were conserved in RelE2Spn (R41, R56, R63, and R83). Curiously, this R-distribution was not fully conserved in the pneumococcal RelE1Spn (also present in the R6 strain; Figure 7) and RelE2Spn (Figure 7), residues T34 and D39 (changed in the RelE2Spn low toxicity mutants to I and G, respectively) appeared to be located close to the toxicity-related R residues. According to the structure of toxins Kd and MazF [56–58], we can postulate that: i) mutation T34H would allow the toxin to retain its RNAse activity but with slight changes in its substrate binding capacity, and ii) D39, together with E38, are acidic residues that could act in the catalysis of RNA in the active site of the toxin so that mutation D39G would reduce the RelE2Spn mRNA cleavage activity, thereby diminishing RelE2Spn toxicity (Figure 6). The model of RelE2Spn also sheds light on some possible structural peculiarities of the toxin: unlike its E. coli and P. horikoshii homologues, RelE2Spn would include in its catalytic site (besides the conserved R residues), residues H43, Y57, and two acidic residues (E38 and D39) (Figure 7 and Figure S4). These residues are present in the catalytic site of toxins with ribosome-independent RNase activity, such as YoeB [6], Kd [57] and MazF [58]. The presence of these additional residues allow us to speculate that RelE2Spn could mediate the cleavage of translating...
mRNA [16] but also could have an intrinsic RNase activity able to cleave untranslated mRNA, as shown for Ecoli YoeB [6].

**Concluding remarks**

Our results indicate that the relBE2Spn locus could provide a mechanism for S. pneumoniae to cope with unfavourable conditions, allowing the bacteria to efficiently survive and colonize humans. Further, the results show the importance of TAS as targets for designing new antimicrobials, which is especially true for bacteria like S. pneumoniae because of their high recombination rates and, being naturally competent, horizontal transfer. As a consequence, the appearance of new polymorphic and antibiotic resistant strains poses a serious threat for infection management.

**Materials and Methods**

**Bacterial strains, growth conditions and plasmid constructions**

Strains and plasmids used in this study are listed in Table 1. E.coli cultures were grown in TY medium [59] with selection for ampicillin resistance (AmpR, 150 μg.ml⁻¹), or kanamycin resistance (KmR, 50 μg.ml⁻¹). S. pneumoniae cells were grown in AGCH medium [40].
supplemented with 0.3% sucrose and 0.2% yeast extract (complete AGCH) medium, with or without selection for resistance to chloramphenicol (CmR, 2 μg/ml). All cultures were grown at 37°C. The agar plates were incubated at 37°C in air. SHT (used at 1.5 mg/ml; a compound which specifically blocks charging of seryl-tRNA, thus inhibiting protein synthesis), Erm (used at 0.1 or 1 μg/ml) and Sm (used at 20 μg/ml) both of them blocking bacterial ribosomes, were purchased from Sigma. We used Erm instead of Cm because the mutant strain, R6 \( \text{D relB2Spn} \), harbours a chromosomally-integrated copy of the \text{cat} gene (Table 1).

Plasmids used in this work were constructed as follows: pE2wt, pE71, pE81, and pE600: The \text{relE2Spn} gene with its own ribosome binding site was amplified by PCR from chromosomal DNAs of strains R6 (pE2wt), 7153 (pE71), 8151 (pE81), or K-600 (pE600) and amplified with primers \text{relE2N} (5′-CGCG GATCCGATG-CATGATTTAGGCTTGAAGGATGAATA-3′) and \text{relE2tga} (5′-CGTGGTACCTCAATAAATATCTCTCCGATGACCAACT-TC-3′). The resulting 290-bp PCR products were digested with EcoRI and KpnI before ligation into the equivalent sites of pFUS2. Plasmid pE2ter was randomly isolated during construction of pE71 and contains a mutation in the \text{relE2} sequence changing the E38 residue for a termination codon, yielding a truncated \text{RelE2} protein. Plasmid pB2wt was constructed by amplification of a chromosomal DNA fragment encoding gene \text{relB2Spn} with its own ribosome binding site using the primers \text{relB2 BamHI} (5′-CGGGATCCTGTTACCTCAATAAATATCTCTCCGATGACCAACT-TC-3′) and \text{relLC C} (5′-CGGGGTACCATCGCGAATTC-TAAAACGTCTTGATGACACATTTATAC-3′). The resulting 310-bp DNA fragment was digested with BamHI and EcoRI before ligation into the equivalent sites of pNM220. All plasmids were rescued by transformation of competent \text{E. coli} cells.

Growth and recovery tests in \textit{S. pneumoniae} cells

Cultures of \textit{S. pneumoniae} R6 and R6\textit{relB2Spn} were exponentially grown in AGCH complete medium to OD\textsubscript{650} = 0.2 at 37°C. Then, half of each culture was exposed to the different experimental conditions: carbon starvation, or addition of SHT or Erm (or Sm). Growth of treated and untreated cultures was followed by OD\textsubscript{650} and the viability (number of cfu) was measured by plating serial dilutions of each culture on AGCH plates. After SHT-, Erm, or Sm-treatment, cultures were washed twice with pre-warmed AGCH and suspended in the same volume of complete AGCH drug-free medium. OD\textsubscript{650} and viability was then tested for at least 180 minutes. In the carbon starvation experiments exponentially growing cultures (to OD\textsubscript{650} = 0.2) were washed twice in pre-warmed AGCH medium and finally suspended in the same volume of AGCH medium supplemented with 0.2% yeast extract and with or without sucrose. Cell growth and viability were measured as above. All the experiments were performed at least three times.

Fluorescence microscopy

Cultures of R6 and R6\textit{relB2Spn} were exponentially grown at OD\textsubscript{650} = 0.25 in AGCH complete medium at 37°C and Erm was added at two concentrations (0.1 μg/ml or 1 μg/ml). After 180 min, cells were harvested by centrifugation, washed twice with
buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 8 mM MgSO4) and stained with the LIVE/DEAD BacLight Bacterial viability kit (Invitrogen) according to the manufacturer’s instructions. Cells were visualized using a multidimensional AF6000 LX LEICA microscope and filter cube L5 for green fluorescence or N3 to detect red fluorescence.

PCR-based gene detection in S. pneumoniae and MLST
Chromosomal DNAs were extracted using the Bacterial Genomic DNA Isolation Kit (NORGEN) and chromosomal DNA (50–100 ng) was added to PCR reactions performed using Phusion high fidelity DNA polymerase (Finzymes) and, as primers, the following oligonucleotides: yeMxC: 5′-CGGCGATCCGCTGTAACAAGGTTCCAATCGACATTTG-3′
yoeBx: 5′-CTGGAATTCCGGTAGAGACTTGAAGAAAGGCCCTA-3′
rel2p5: 5′-CGGAGATCCGATCGTTTCTGCTGTAACAAGGTTCCAATCGACATTTG-3′
relB2p: 5′-CAGATACTCGCAACACCACTTGACAG-3′
relB2N: 5′-TGCTTCCTGGGCTATTACATTAAAGATTTCTGAAAGGCTAATTTG-3′
relB2x: 5′-CGGAGATCCGATCGTTTCTGCTGTAACAAGGTTCCAATCGACATTTG-3′
relE2N: 5′-GAAAAGCGATCAAACAACTCATGTCATAGTAAGGACTAAACATA-3′
relE2p: 5′-GAAAAGCGATCAAACAACTCATGTCATAGTAAGGACTAAACATA-3′
relB2C: 5′-GAAAAGCGATCAAACAACTCATGTCATAGTAAGGACTAAACATA-3′
relE2C: 5′-GAAAAGCGATCAAACAACTCATGTCATAGTAAGGACTAAACATA-3′

MLST based on sequencing of fragments of seven housekeeping genes, _aroE_, _gldh_, _gki_, _relP_, _spi_, _spt_ and _ddl_ was performed as described by others [60]. The database http://spneumoniae.mlst.net was used to assign allele numbers and STs.

Primer extension analysis
Total RNA was isolated from R6 and from different pneumococcal isolates with Aurum total RNA minikit (BIO-RAD). For RNA extraction, 1.5–3 ml of the bacterial cultures in suspension was incubated 10 min at 30°C, resuspended in 100 μl of lysis buffer (50 mM Tris-HCl pH 7.6, 1 mM EDTA, 50 mM NaCl, 0.1% sodium deoxycholate). The cell suspension was incubated 10 min at 30°C and further preparation was done according to the manufacturer’s instructions. Primer extension assays were performed as described [61] using either a radiolabelled relB2Spn specific primer (relRNA, 5′-GAAGACTCTCCATAAACTTAGGC-3′) [33] or a malX specific primer mal1, 5′-GTTGTAACAGTCTCAGACACC-3′. The 3′-ends of primers were located 56 nt or 48 nt from the nucleotides A of the ATG initiation codon of the relB2Spn gene or in the malX ATG initiation codon, respectively [61].

Model construction
The three-dimensional model of RelE2Spn toxin was constructed using Geno3D molecular modelling program (pBIL.icp.fr.Genom3D, http://geno3d-pbil.ibcp.fr) and the 2.3 Å resolution X-ray crystallographic structure of the _P. horikoshii_ OT3 aRelBE complex [10] (PDB ID:1wmi), and the 2.1 Å resolution X-ray crystallographic structure of the _M. jannaschii_ MalRelBE complex [11], (PDB ID:3BPQ) as templates. The graphic display was performed with PyMOL program (DeLano Scientific LLC, http://www.pymol.org).

Supporting Information

Figure S1 Growth profile of _S. pneumoniae_ cells wt and mutant R6(capital delta)relB2Spn under normal or carbon-starvation conditions. Pneumococcal strains R6 (triangles) or (capital delta)relB2Spn (circles) were grown in AGCH complete medium (A, B). In carbon-starvation conditions (panels C and D), cells were exponentially grown in AGCH complete medium to an OD650 = 0.2, twice washed, and suspended in the same medium with (open symbols) or without sucrose (filled symbols). Growth of the cultures was followed by measurement of OD650 nm (A, C) or by determination of the number of cfu (B, D). Found at: doi:10.1371/journal.pone.0011289.s001 (1.35 MB EPS)

Figure S2 Inhibition of protein synthesis mediated by Sm treatment. _S. pneumoniae_ cells from strains R6 (circles) or R6(capital delta)relB2Spn (triangles) were grown exponentially in complete AGCH medium to an OD650 = 0.1–0.14. Then, Sm (20 (mu)g.ml−1) was added, and incubation was continued for 180 min more. Growth was followed by measurement of OD650 nm of the cultures untreated (open symbols) or treated (closed symbols) with Sm (A). At indicated times appropriate dilutions of cells were plated and incubated as in Figure 1 (B). Found at: doi:10.1371/journal.pone.0011289.s002 (1.11 MB EPS)

Figure S3 Primer extension analysis using total RNA from different _S. pneumoniae_ clinical isolates. RNA samples from R6 (1) and the following clinical isolates: CipR25 (2), 2115 (3), CipR67 (4), CipR31 (5); CipR14 (6), and CipR51 (7) were annealed with [32P]-labelled specific primers 11 (x: as a control) or with the relRNA oligonucleotide (v) to detect relB2Spn mRNA. Ct Indicates a G+A Maxam and Gilbert sequencing reaction, used as DNA size marker. Found at: doi:10.1371/journal.pone.0011289.s003 (0.53 MB EPS)

Figure S4 RelE2Spn three dimensional structural model. Location of R41, R56, R63, R83 and D39 residues is depicted. Other residues (Y31 and Y57, H43 and E38) supposedly involved in the catalytic mechanism are displayed in cyan. Found at: doi:10.1371/journal.pone.0011289.s004 (1.06 MB EPS)

Table S1
Found at: doi:10.1371/journal.pone.0011289.s005 (0.04 MB XLS)

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Author Contributions
Conceived and designed the experiments: CN ES ME. Performed the experiments: CN ES. Analyzed the data: CN ES. AGdC WH ME. Wrote the paper: CN ES ME. Corrected the manuscript: AGdC WH.

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