

# Streptomycin-Induced Expression in *Bacillus subtilis* of YtnP, a Lactonase-Homologous Protein That Inhibits Development and Streptomycin Production in *Streptomyces griseus*

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***Bacillus subtilis* induces expression of the gene *ytnP* in the presence of the antimicrobial streptomycin, produced by the Gram-positive bacterium *Streptomyces griseus*. *ytnP* encodes a lactonase-homologous protein that is able to inhibit the signaling pathway required for the streptomycin production and development of aerial mycelium in *S. griseus*.**

Bacterial communities display diverse defensive strategies in response to the presence of secreted natural products from coexisting microorganisms. These molecules act as signals to trigger changes in gene expression that allow bacteria to adapt to particular conditions (7, 22, 46, 53). Adaptation sometimes involves the activation of a defensive strategy against the presence of these natural products. For instance, the soil-dwelling organism *Bacillus subtilis* forms a biofilm in response to certain antimicrobials (30). Alternatively, several *Bacillus* species produce hydrolytic enzymes that are able to degrade the quorum-sensing signals from competing bacteria, a phenomenon known as quorum quenching (10–12). Some of these hydrolytic enzymes are metallo-lactamases (4, 5), which are able to degrade the lactone ring of the acyl-homoserine lactone signaling molecules (28, 35). Metallo-lactamases share a similar molecular structure, and they conserve important residues in their active sites (5, 6, 50), despite the low protein identity among the different subgroups (approximately 25%).

Bioinformatic analysis carried out in the model organism *Bacillus subtilis* NCIB3610 led to the identification of YtnP, a protein that shares a similar deduced domain to proteins described as quorum-quenching lactonases (Fig. 1A). Using ClustalW, a comparative alignment of the following proteins was performed: YtnP and AiiA from *Bacillus* sp. (10–12); AiiA homologues from *Staphylococcus aureus* and *Listeria innocua* (6); AttM, AiiB, and AiiC from *Agrobacterium tumefaciens* (6, 17, 29); AhlD from *Arthrobacter* sp. (39); and glyoxalase II (YcbL) from *Salmonella enterica* (45). AiiM from *Microbacterium testaceum* (52) and AidH from *Ochrobactrum* sp. (33) were excluded from this comparative analysis because of their nature of unusual lactonases. Results of the alignment confirmed that the important residues in the active sites of metallohydrolases are present in YtnP (Fig. 1B) (12, 24, 26, 27). Subsequently, overexpression of a 6×His-tagged version of the YtnP protein was carried out with *Escherichia coli*. 6×His-YtnP was purified using Ni<sup>2+</sup> exchange chromatography (see Fig. S1 in the supplemental material). Inhibition of the quorum-sensing activity of YtnP was assayed based on the ability of *Pseudomonas aeruginosa* to form a biofilm in a quorum-sensing-dependent manner (3, 8, 32). Small concentrations of purified YtnP added to the biofilm formation assay of the cultures of *P. aeruginosa* inhibited biofilm formation, while similar concentrations of YtnP did not affect biofilm formation in cultures of *S. aureus* (Fig. 1D). This might be because the quorum-sensing molecule that controls bio-

film formation in *S. aureus* has a peptidic nature and is not affected by the lactonase activity of YtnP (36, 38, 49).

To determine whether the expression of *ytnP* in *B. subtilis* serves as a defensive strategy against competing bacteria, an experiment was carried out to test the activation of *ytnP* expression in the presence of several acylhomoserine lactone (AHL) molecules. To do this, *B. subtilis* strain 3610 was labeled with a transcriptional fusion, P<sub>ytnP</sub>-yfp (*yfp* encodes the yellow fluorescence protein [YFP]), and the emission of fluorescence was monitored when small concentrations of AHL (purchased from Sigma-Aldrich) were added to cultures of *B. subtilis* growing in MSgg (2). We did not observe any change in *ytnP* expression associated with the addition of AHL molecules to the cultures of *B. subtilis*. Then, other natural products were tested for their abilities to induce *ytnP* expression in *B. subtilis*. This idea was based on the results published in a previous report that describes an increase in the expression of a protein similar to YtnP in *S. aureus* when certain antimicrobials were added to the cultures (1). Consequently, a battery of antibiotics was collected, and they were tested for their abilities to induce the expression of P<sub>ytnP</sub>-YFP when added at sub-growth-inhibitory concentrations (<50 nM) (see Table S1 in the supplemental material). We found that streptomycin, an antimicrobial produced by the soil-dwelling bacterium *Streptomyces griseus*, increased the expression of the reporter P<sub>ytnP</sub>-YFP approximately 5-fold when added at sub-growth-inhibitory concentrations to *B. subtilis* cultures (Fig. 2A) (41, 51). Reverse transcription-PCR (RT-PCR) experiments confirmed that cultures that were previously treated with sub-growth-inhibitory concentrations of streptomycin showed an increase in the expression of the *ytnP* gene (Fig. 2B).

A search in the DBTBS software was performed to study the mechanisms responsible for the activation of *ytnP* expression in response to streptomycin. This search identified the CodY repressor as a regulatory protein involved in the regulation on *ytnP*

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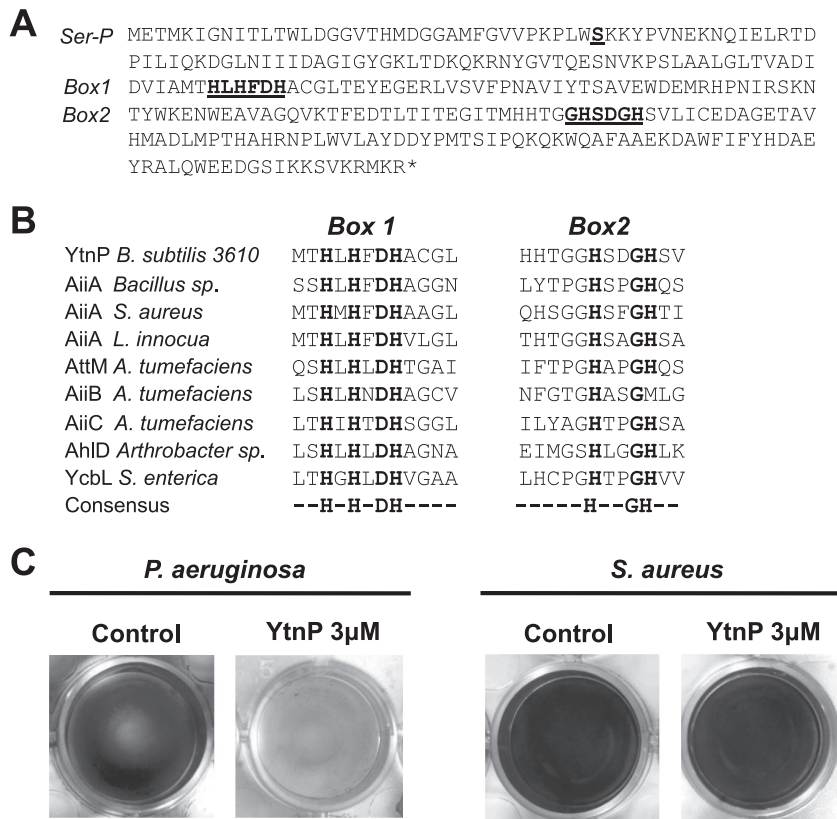
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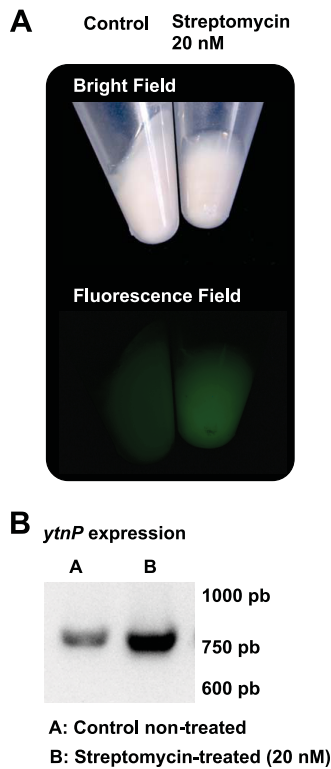


**FIG 1** YtnP is a lactonase-homologous protein. (A) Amino acid sequence of YtnP from *B. subtilis* strain 3610. Conserved motifs present in other metallo-lactamases are marked as Box 1 and Box 2 and are underlined and bold. Also an additional serine (Ser36) important for YtnP activity is marked as Ser-P. (B) Alignment of the conserved motifs of the active site of the following: YtnP and other metallo-lactamases; AiiA from *Bacillus* sp. and homologues from *S. aureus* and *L. innocua*; AttM, AiiB, and AiiC from *A. tumefaciens*; AhID from *Arthrobacter* sp.; and glyoxalase II (YcbL) from *S. enterica*. The last lane of the alignment shows the consensus sequence. (C) Addition of YtnP to the biofilm formation assays inhibited biofilm formation in *P. aeruginosa* but not in *S. aureus*. Biofilm formation is observed as a pellicle attached to the submerged surfaces (at the bottom of the well plate). Crystal violet staining was used in the assay for better visualization, according to the protocol described by O'Toole and Kolter (37). For the quantification of biofilm formation, the crystal violet associated with biofilms was dissolved in acetic acid (33%) and was measured spectrophotometrically with an optical density at 595 nm (37).

expression (43). CodY loses activity and target genes are transcribed when the growth rate of *B. subtilis* slows down in response to adverse conditions (42, 44). Given that antimicrobials affect bacterial growth rate, *B. subtilis* probably responds to a decrease in growth rate with an induction of *ytnP* expression. Hence, a  $\Delta codY$  strain labeled with the  $P_{ytnP}$ -*yfp* reporter was constructed and the expression of the reporter in this strain was compared to the expression observed in the wild-type strain. The absence of *codY* increased the expression of *ytnP* 3- to 5-fold in *B. subtilis* cultures, similar to the addition of streptomycin (Fig. 2D). Activation of *ytnP* in response to cellular stress is consistent with results from a previous publication, which shows that the expression of AttM in *A. tumefaciens* increases in response to starvation and to other cellular stresses (54).

Although the aminoglycoside antibiotic streptomycin is not a lactone, the quorum-sensing signal that positively regulates streptomycin production and development of aerial hyphae in *S. griseus* is a  $\gamma$ -butyrolactone (termed A factor) (15, 18, 19, 25). We predicted that this molecule could be a putative target for YtnP. Thus, experiments were conducted to elucidate whether YtnP interferes with the development of *S. griseus* by assaying the ability of YtnP to inhibit the formation of aerial hyphae during *S. griseus* development. The 6 $\times$ His-YtnP-tagged protein was overexpressed

and purified in *Bacillus subtilis* PY79. A sample of purified YtnP was spotted on an agar plate that had previously been planted with *S. griseus* spores. The aerial mycelium of *S. griseus* arose from the surfaces of the colonies, giving a white, hairy appearance to the agar plate. After 3 days of growth, the area subjected to the action of YtnP did not develop any aerial mycelium, lacking the characteristic fuzzy colony morphology of the wild type (Fig. 3A). Interestingly, YtnP did not inhibit the development of aerial hyphae on *S. coelicolor* (see Fig. S2 in the supplemental material). This might be due to the fact that the projection of the aerial mycelium in *S. coelicolor* is not regulated by the A factor (14, 16, 20, 47). Development of the aerial mycelium in *S. griseus* is intimately related to streptomycin production, since both processes are positively regulated by the A factor. Consequently, the inhibition of streptomycin production was monitored in cultures of *S. griseus* that were previously exposed to the activity of YtnP. Detection of streptomycin produced by *S. griseus* was based on the inhibitory halo that the secreted streptomycin caused in the soft agar that was previously planted with *B. subtilis* strain ATCC 6633 (9, 18, 21, 34). An agar section from a plate of *S. griseus* that was exposed to the activity of YtnP was excised and placed on the surface of the soft agar previously inoculated with *B. subtilis* ATCC 6633. After 24 h of incubation at 37°C, the agar section from YtnP-treated cultures

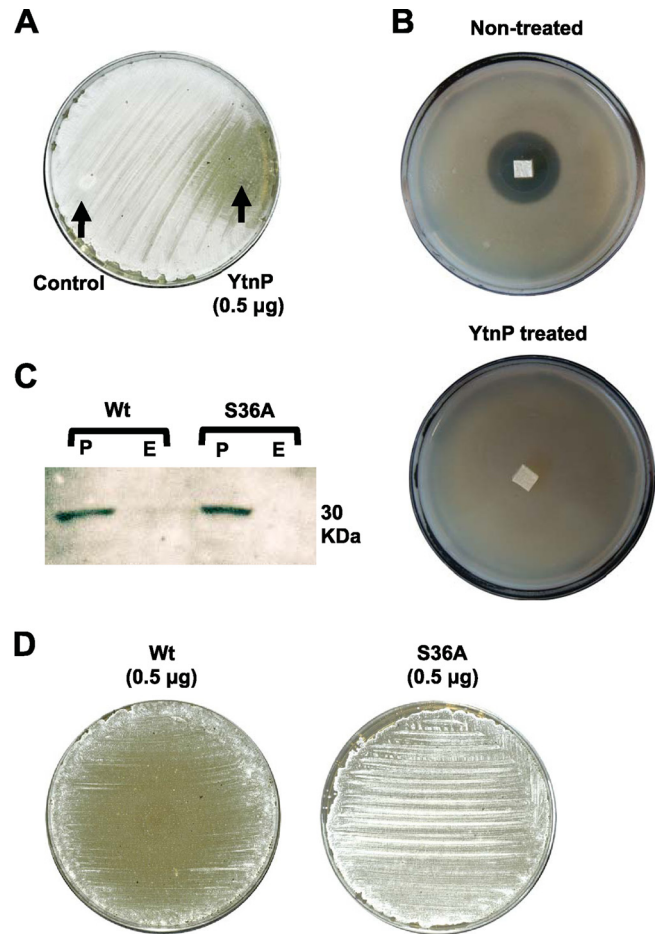


**FIG 2** Streptomycin induces the expression of *ytnP*. (A) Fluorescence emitted by the expression of the reporter  $P_{ytnP}$ -*yfp* in *B. subtilis* when incubated in the presence or absence of streptomycin. Pellets of nontreated (control) and YtnP-treated (20 nM) cultures of *B. subtilis*  $P_{ytnP}$ -*yfp* are presented in bright field (top) and fluorescence field (bottom). (B) RT-PCR analysis of *ytnP* expression from nontreated and streptomycin-treated (20 nM) cultures of *B. subtilis*.

of *S. griseus* showed no growth-inhibitory halo on *B. subtilis* ATCC 6633 (Fig. 3B), indicating that YtnP-treated cultures of *S. griseus* has decreased in streptomycin production.

Inhibition of the aerial mycelium in *S. griseus* was used to assay the activity of YtnP. A previous study of the phosphoproteome of *B. subtilis* identified a serine phosphorylation (Ser36) in YtnP, a widely spread mechanism of posttranslational regulation (31, 40). To investigate the effect that phosphorylation of Ser36 causes on the activity of YtnP, an allele of YtnP-6XHis was constructed harboring a Ser36Ala replacement (YtnPS36A). The YtnPS36A allele was overexpressed and purified in *B. subtilis* PY79. Immunological studies (via Western blot analysis) using antibodies against the 6×His tag showed similar expression of YtnPS36A and wild-type alleles (Fig. 3C). Purified YtnPS36A was assayed for inhibition of aerial mycelium of *S. griseus* by spotting 0.5 μg YtnPS36A on a plate previously planted with spores of *S. griseus*. After 3 days of growth, the purified YtnPS36A protein did not abrogate the development of aerial mycelium in *S. griseus* (Fig. 3D). This might indicate the importance of the phosphorylation of Ser36 for the activity of YtnP, albeit other indirect effects that the substitution of Ser36 might cause in the structure and functional activity of the enzyme YtnP should not be discarded.

Taken altogether, data from this report suggest that the expression of YtnP from *B. subtilis* is induced in the presence of streptomycin and other cellular stresses. The prior expression of YtnP interferes with the signaling pathway that leads to the develop-



**FIG 3** YtnP inhibits development of aerial mycelium in *Streptomyces griseus*. (A) Inhibition of the formation of the aerial mycelium in *S. griseus* when grown on R2YE agar medium (23). Purified YtnP was spotted in the agar (spotted on the right side of the plate). Negative control was solution buffer with bovine serum albumin (BSA) (spotted on the left side of the plate). The plate was incubated for 3 days at 30°C. (B) Quantification of streptomycin production from nontreated and YtnP-treated agar cultures of *S. griseus* by the formation of an inhibitory halo on a lawn of *B. subtilis* ATCC 6633 grown on soft agar. (C) Detection of YtnP (Wt) and YtnPS36A (S36A) alleles by Western blot analysis. Anti-6×His antibodies were used to detect the proteins. Signals exclusively occurred in the cell pellet (P), and no signal was found in the extracellular protein fraction (E). The band has a molecular mass of 30 kDa. (D) Inhibition of formation of the aerial mycelium in *S. griseus* caused by the two alleles YtnP (Wt) and YtnPS36A (S36A). An agar plate of R2YE medium was planted with *S. griseus* (23). Purified Wt and S36A alleles were spotted in the center of the agar. Plates were incubated for 3 days at 30°C. Development of the aerial mycelium could be observed by the white, hairy appearance that the formation of the aerial mycelium gives to the lawn of *S. griseus* growing on the plate. Development of the aerial mycelium occurred only in the plate complemented with the S36A allele.

ment of the aerial mycelium and streptomycin production in *S. griseus*. *B. subtilis* might activate *ytnP* expression in response to the presence of certain antimicrobials, serving this as a defensive strategy against threatening bacteria because it would allow *B. subtilis* to selectively inhibit the quorum-sensing system of harmful microbial communities. The selective inhibition of quorum sensing would not occur if *B. subtilis* triggered the expression of *ytnP* in response to the presence of lactone molecules. Lactones are signaling molecules broadly found among prokaryotes, and the acti-



vation of YtnP expression in response to the presence of these signaling molecules will not allow *B. subtilis* to discriminate between threatening or potentially benign microbial communities that can generate beneficial interactions to *B. subtilis*. To ensure that the quorum-quenching activity of YtnP acts only in the presence of antimicrobials or other stressing factors that compromise the physiology of *B. subtilis* cells, YtnP is expressed as a cytoplasmic enzyme, as well as the rest of the quorum-quenching enzymes (11–13). Hence, the release of YtnP to the extracellular space occurs concomitantly with cell lysis. This might resemble other cases in microbiology, in which the release of proteins with extracellular activities requires cell lysis, as is the case with the secretion of TcdA and TcdB toxins in *Clostridium difficile* facilitated by TcdE, a holin-like protein responsible for cell lysis (48).

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