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The reduction in small ribosomal subunit abundance in ethanol-stressed cells of *Bacillus subtilis* is mediated by a SigB-dependent antisense RNA

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ABSTRACT

One of the best-characterized general stress responses in bacteria is the σ^B-mediated stress response of the Gram-positive soil bacterium *Bacillus subtilis*. The σ^B regulon contains approximately 200 protein-encoding genes and 136 putative regulatory RNAs. One of these σ^B-dependent RNAs, named S1136–S1134, was recently mapped as being transcribed from the S1136 promoter on the opposite strand of the essential *rpsD* gene, which encodes the ribosomal primary-binding protein S4. Accordingly, S1136–S1134 transcription results in an *rpsD*-overlapping antisense RNA (asRNA). Upon exposure of *B. subtilis* to ethanol, the S1136 promoter was found to be induced, while *rpsD* transcription was downregulated. By quantitative PCR, we show that the activation of transcription from the S1136 promoter is directly responsible for the downregulation of *rpsD* upon ethanol exposure. We also show that this downregulation of *rpsD* leads to a reduced level of the small (30S) ribosomal subunit upon ethanol stress. The activation of the S1136 promoter thus represents the first example of antisense transcription-mediated regulation in the general stress response of *B. subtilis* and implicates the reduction of ribosomal protein abundance as a new aspect in the σ^B-dependent stress response. We propose that the observed reduction in the level of the small ribosomal subunit, which contains the ribosome-decoding center, may protect *B. subtilis* cells against misreading and spurious translation of possibly toxic aberrant peptides under conditions of ethanol stress.

1. Introduction

During their lifetime, all organisms are challenged with a wide range of stresses. In bacteria these stresses can range from oxidative stress, heat or cold stress, hypoxia, starvation, osmotic stress, and antibiotic exposure, to ethanol stress (Refs. in [1,2]). To anticipate or combat such stresses, specific or general stress response pathways have evolved. These well-coordinated response pathways will sense a particular stress and subsequently activate specific genes, while deactivating others. One of the best-characterized general stress response pathways is the σ^B-mediated response of the Gram-positive soil bacterium *Bacillus subtilis* [3]. In case of acute physical stress, activation of σ^B takes place via a post-transcriptional sensing mechanism involving the stressosome [4]. The σ^B regulon has been uncovered by a multitude of large-scale experimental approaches and was found to consist of ~200 genes [3,5]. These genes take part in a wide variety of processes, such as oxidative stress resistance, control of protein folding and protein degradation, membrane transport, and the rewiring of metabolism [3,6,7]. A recent large-scale transcriptomics study expanded the σ^B regulon to include 136 putative regulatory RNAs [8]. Another interesting observation from this study as well as an earlier study was the global transcriptional downregulation of genes encoding components of RNA polymerase and ribosomes upon ethanol stress [8,9]. This downregulation may take place to limit translation errors, since biochemical studies have shown that purified *Escherichia coli* ribosomes are prone to misreading errors when treated with ethanol [10]. To date, it has however remained unclear whether the downregulation of RNA polymerase and ribosomal genes in living bacteria reflects indirect effects caused by the negative impact of ethanol on growth, or rather is an actively regulated stress responsive process.

Regulatory RNAs are appreciated as important post-transcriptional regulators in all organisms studied [11,12]. One class of regulatory RNAs are transcribed from the opposite strand of protein-encoding genes and this results in the production of complementary antisense RNAs (asRNAs). AsRNAs can regulate their sense RNAs by a variety of mechanisms (for reviews see [13,14]), which can be divided into context-independent and context-dependent mechanisms. An example

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of a context-independent mechanism is the triggering of mRNA degradation by base-pairing interactions. In this case, the asRNA can also exert its regulatory function *in trans*. Context-dependent asRNA regulation is exemplified by the promoter collision mechanism, where the asRNA-transcribing RNA polymerase pushes off the mRNA-transcribing RNA polymerase from the opposite strand [14]. Thus far, five experimentally confirmed sense-antisense interactions are known to occur in *B. subtilis*, among which are two so-called toxin-antitoxin modules [15,16]. The other three asRNA interactions have been shown to affect either the cognate mRNA levels or the respective protein levels, but despite considerable efforts, no clear biological functions have been reported for these asRNAs [17–19]. The limited understanding of asRNA regulation in *B. subtilis* is all the more intriguing as it was recently reported that 13% of all protein-encoding genes are overlapped by asRNAs [8]. One of these annotated asRNAs, S1136-S1134, is σ^B-dependently transcribed from the convergent P_{S1136} promoter, situated on the opposite DNA strand of the *rpsD* gene. Its transcription therefore results in an asRNA that is complementary to the *rpsD* transcript.

The *rpsD* gene encodes the essential *B. subtilis* ribosomal protein S4. Upon the initiation of ribosome assembly, S4 is one of six ribosomal primary-binding proteins that bind to the nascent 16S rRNA molecule to initiate the assembly of the small (30S) ribosomal subunit [20]. The small ribosomal subunit contains the ribosome decoding center where codon–anticodon pairing takes place to select the correct aminoacyl-tRNA. In *B. subtilis* and related bacteria, S4 expression is negatively autoregulated by its binding to the 5′ untranslated region (UTR) of the *rpsD* mRNA [21]. Remarkably, the respective RNA secondary structure to which S4 binds is very similar to S4's binding site on the 16S rRNA [21,22]. While ribosomal gene organization is highly conserved, early genetic analyses found that *B. subtilis rpsD* is independently transcribed [21], in contrast to *E. coli* where *rpsD* is the third gene of a five-gene operon [23]. Since the protein S4 also mediates autorepression of this operon in *E. coli*, there was no clear explanation for this altered genomic organization until now.

The recent finding that the S1136–S1134 RNA segment emanating from the P_{S1136} promoter overlaps with *rpsD* [8] suggested that the expression of *rpsD* might be subject to asRNA-mediated regulation. In addition, we observed a highly significant negative correlation between the expression of *rpsD* and S1136–S1134, which was related at least in part to the σ^B-dependent induction of S1136–S1134 upon exposure to ethanol and the simultaneous downregulation of the *rpsD* transcript level [8]. Therefore, the present study was aimed at assessing the possible function of the activation of the P_{S1136} promoter in antisense transcription-mediated regulation of *rpsD*. To this end, a marker-less deletion mutant of the S1136 promoter region was constructed, and the effects of this mutation on the *rpsD* mRNA levels were tested by quantitative PCR (qPCR). Indeed, we observed that activation of the promoter driving S1136–S1134 transcription is largely responsible for the *rpsD* downregulation upon exposure of the cells to ethanol. Since *rpsD* encodes the ribosomal primary binding protein S4, we subsequently tested the effect of *rpsD* regulation on ribosomal subunit abundance by sucrose density gradient centrifugation. These experiments showed that the decrease in small ribosomal subunit abundance upon ethanol stress was reduced in the S1136 promoter mutant. Altogether, our present observations imply that the reduction of the small ribosomal subunit abundance upon ethanol stress is the consequence of an actively regulated stress response that depends on σ^B and is mediated by the activation of the S1136 promoter.

2. Materials and methods

2.1. Bacterial strain construction

E. coli and *B. subtilis* strains and plasmids used in this study are listed in Supplementary Table S1. *E. coli* TG1 was used for all cloning procedures. Oligonucleotides used in this study are listed in Supplementary Table S2. The ΔP_{S1136} 'pop-out' strain was constructed following the approach described by Tanaka et al. [24]. *B. subtilis* transformations were performed as described previously [25]. Reintroduction of the S1136–S1134 construct under control of its native promoter in *B. subtilis* ΔP_{S1136}, resulting in strain ΔP_{S1136} *amyE*::S1136–S1134, was achieved in several steps. S1136–S1134 was first introduced by Ligation-Independent Cloning [26] into pRMC, a plasmid that allows the incorporation of genes via double cross-over recombination into the chromosomal *amyE* gene of *B. subtilis* [27]. Primer annealing sites for this construction were chosen to include the native promoter mapped by Nicolas et al. [8] and to not overlap with the translation initiation site of the *rpsD* gene on the opposite strand from S1136–S1134. The correct introduction of S1136–S1134 in pRMC was subsequently verified by sequencing. Next, *B. subtilis* was transformed with pRMC bearing S1136–S1134, and the correct integration of S1136–S1134 into the *amyE* locus was confirmed by growing transformants on starch-containing plates and testing the absence of α-amylase secretion by staining of the plates with iodine as described previously [28].

2.2. Media and growth conditions

Bacteria were grown in Lysogeny Broth (LB) at 37 °C supplemented with the appropriate antibiotics. For *E. coli* this was ampicillin (100 μg ml^{-1}), and for *B. subtilis* either phleomycin (4 μg ml^{-1}), neomycin (15 μg ml^{-1}), tetracycline (5 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), or combinations thereof were used.

Ethanol stress RNA sampling was performed with cells grown on Belitsky Minimal Medium (BMM) [8]. For these experiments, an overnight *B. subtilis* culture in LB with antibiotics was diluted >1:50 in fresh LB medium and grown for approximately 2.5 h. These cells were subsequently pre-cultured by 1:20 dilution in pre-warmed BMM medium and incubation for approximately 2 h, which corresponds to mid-exponential growth. Next, the pre-culture was again diluted 1:20 in BMM. At an OD_{500nm} of ~0.4, 96% ethanol was added to a final concentration of 4%. Cells for RNA extraction and Western blotting were sampled as described previously [8] immediately before and 10 min after ethanol addition.

2.3. Western blotting

Western blot analysis was performed as described [29] using crude whole cell lysates. To prepare lysates, cell pellets were resuspended in LDS-sample buffer with reducing agent (Life Technologies), and disrupted with glass beads in a bead beater (3 × 30 s at 6500 rpm with 30 s intermittences). Samples were boiled for 10 min and centrifuged to pellet the glass beads and cell debris. Proteins in sample aliquots corresponding to equal OD units were separated on NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell) by semi-dry blotting. The CitZ and σ^B proteins were detected with polyclonal antibodies raised in rabbits kindly provided by Abraham Sonenshein and Ulf Gerth, respectively. Bound antibodies were then further detected by fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and visualized at 700 and 800 nm with the Odyssey Infrared Imaging System (LiCor Biosciences). Relative amounts of σ^B and CitZ were quantified with ImageJ.

2.4. RNA isolation, qPCR and data analysis

RNA was isolated by phenol-chloroform extraction as described previously [8]. After checking the RNA integrity on a 1% agarose gel, DNA was removed from 2 μg of RNA with an RQ-1 DNase kit according to the protocol of the manufacturer (Promega, Madison, WI, USA). cDNA was produced from this DNase-treated RNA by Taqman Reverse Transcription according to the manufacturer's instructions (Applied Biosystems, New Jersey, USA). Actinomycin D was added to a final

concentration of 5 μg/ml to prevent antisense artifacts [30]. Quantitative PCR on an Applied Biosystems 7500 machine was performed with 5 μl diluted cDNA in a total volume of 25 μl using the GoTaq qPCR Master Mix (Promega, Madison, WI, USA).

qPCR data was analyzed according to the method described by Pfaffl [31]. For all primer sets (*recF*, *rpsD*, and S1136) a standard curve analysis on serially diluted genomic DNA was performed to determine the validity of the linear range of the qPCR, and the obtained $R^2 + 1$ was used as the real-time PCR efficiency in the Pfaffl formula. In all cases, measurements for *rpsD* and S1136 were normalized for the presence of *recF*.

2.5. Ribosome subunit profiling

For the profiling of ribosomal subunits, cells were cultured in BMM and stressed with a final concentration of 4% ethanol as was described above. 50 ml of culture was sampled, cooled on ice for 5 min, and centrifuged for 5 min at 6000 g at 4 °C. Cell pellets were frozen and processed further within 24 h after freezing. For disruption, cell pellets were resuspended in 400 μl gradient buffer supplemented with Complete™ protease inhibitor (Roche). The gradient buffer recipe was from Hase et al. [32] and contained 10 mM Tris–HCL (pH 7.8), 10 mM $MgCl_2$, 60 mM NH_4Cl and 1 mM DTT. DTT was added freshly on the day of the experiment. The cell suspension was divided over two bead-beating tubes containing 90 μl glass beads and disrupted in a bead-beater for 3 × 30 s with 30 s intermissions. The tubes were subsequently centrifuged for 20 min at 14,000 rpm at 4 °C. The supernatant (cell free extract; CFE) was transferred using a gel-loading pipette tip to avoid carry-over of glass beads, and stored at −80 °C until the day of gradient centrifugation. An 11.5 ml 5–20% w/v sucrose density gradient in gradient buffer was prepared using a gradient mixer in a 12 ml ultracentrifuge tube (Beckman Coulter). The absorbance at 260 nm (A260) was used as a measure of protein concentration and was determined with a Nanodrop™ spectrophotometer. Equal A260 units for CFEs from cells incubated with or without ethanol (250–350 μl cell extract of approximately 7.5 mg/ml with A260 = 1 corresponds to 1 mg/ml settings) were loaded onto the sucrose density gradient and centrifuged for 4 h and 10 min in a Beckman Coulter SW41 swing out rotor at 35,000 rpm and 4 °C. Sucrose density gradients were fractionated in 50–110 fractions into low-absorbance UV-Star® 96-well plates (Greiner Bio-One) starting at the bottom of the tube. Absorbance at 260 nm was measured in a BioTek® microplate reader. Peak areas from ribosomal subunit profiles were quantified with ImageJ taking the smallest A260 measurement of that respective profile as the base of the peak.

3. Results

3.1. Annotation of S1136–S1134 as an antisense RNA of rpsD

One of the new putative regulatory RNAs reported in the tiling array study by Nicolas et al. [8] is transcribed from a convergent promoter on the opposite strand of the essential *rpsD* gene, and downstream of the essential *tyrS* gene, encoding the major tyrosyl-tRNA synthetase (Fig. 1A; Supplementary Fig. S1). This RNA segment was designated as S1136. Since S1136 contains its own promoter and is not attached to a coding sequence (CDS), it was annotated as an independent RNA segment (*indep*). Furthermore, transcription from the S1136 promoter seems to extend further downstream of S1136 as a result of incomplete termination. The RNA segment resulting from this incomplete termination was annotated as S1134. While S1136 overlaps *rpsD* by 23 nucleotides, S1134 was shown to span the entire length of the *rpsD* gene. This implies that the S1136–S1134 RNA is de facto an asRNA (hereafter referred to as S1136–S1134). Importantly, the study by Nicolas et al. [8] addressed the *B. subtilis* transcriptome across 104 different conditions, and an inspection of the expression of both *rpsD* and S1136–S1134 under all these conditions revealed a strong negative correlation between the *rpsD* mRNA levels and those of S1136–S1134 (−0.59 for S1136 and −0.48 for S1134; P-values < 0.0001). This negative correlation is due to the induction of S1136 –S1134 under conditions of sporulation and ethanol stress, and the concomitant downregulation of *rpsD* under these conditions [8]. Interestingly, most ribosomal genes were found to be transcriptionally downregulated upon ethanol stress, in

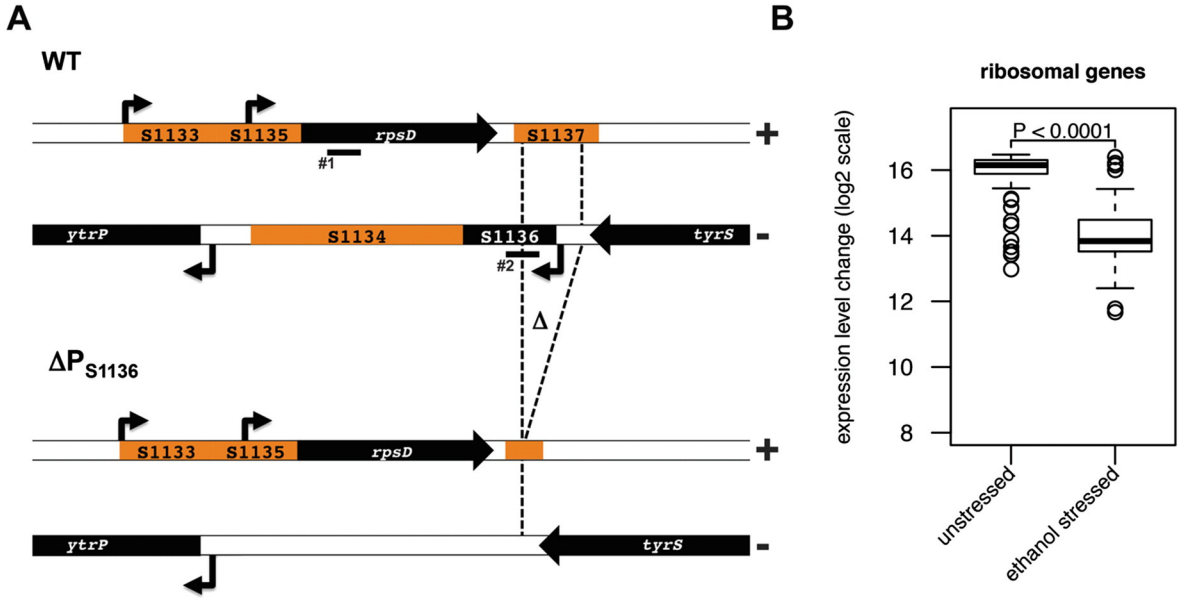

Fig. 1. *rpsD* genomic organization and ribosomal gene expression upon ethanol stress. A) Schematic representation of the genomic region encoding S1136–S1134 and *rpsD* in the wild-type (WT) *B. subtilis* 168 strain and its ΔP_{S1136} mutant derivative. Arrows indicate previously mapped promoters, and regions marked in orange represent UTRs mapped by Nicolas et al. [8]. Plus and minus symbols indicate the forward and reverse DNA strands. Black bars and numbers below *rpsD* (#1) and S1136 (#2) indicate the positions of the employed qPCR probes. The lower part of panel A shows the genomic organization of S1136–S1134 and *rpsD* in the ΔP_{S1136} strain where the promoter region of S1136–S1134 has been deleted. B) Ethanol stress results in a global downregulation of ribosomal genes. Expression data from Nicolas et al. [8] of unstressed and ethanol-stressed cells were plotted for all genes with the SubtiWiki annotation "*ribosomal proteins*" (http://subtiwiki.uni-goettingen.de).

some cases to a similar extent as *rpsD* [8] (Fig. 1B, Supplementary Fig. S2). Of note, expression of S1136–S1134 was less prominent under other SigB-inducing stress conditions (e.g. osmotic or oxidative stress), and the S1134 moiety was only detectable upon ethanol stress and sporulation [8]. This implies that ethanol stress is the most relevant stress condition to study S1136–S1134 expression. These observations were in accordance with the notion that S1136–S1134 could be a functional asRNA regulating *rpsD* under conditions of sporulation and ethanol stress.

3.2. S1136 expression depends on the stress sigma factor σ^B

Before testing the possibility of S1136–S1134-mediated regulation of *rpsD*, we determined which factor could be responsible for the induction of the S1136 promoter upon exposure to ethanol. The most logical candidate for regulation of stress-induced genes in *B. subtilis* is the RNA polymerase subunit σ^B, which is activated specifically upon exposure to several types of stress including ethanol stress. This is supported by the promoter clustering analysis by Nicolas et al., which predicted σ^B regulation of the S1136 promoter with a posterior possibility of 0.965 (Supplementary Table S4 in Nicolas et al. [8]). Accordingly, the −35 and −10 sequences for a putative SigB-dependent promoter can be discerned in the S1136 promoter region (Supplementary Fig. S3). In fact, Nicolas et al. predicted σ^B as the only sigma factor recognizing the S1136 promoter. However, it should be noted that a large part of the variation in the expression levels of S1136 (0.687) could not be explained solely by this RNA segment being a member of the σ^B cluster [8]. To determine whether σ^B is responsible for induction of the S1136 promoter upon ethanol exposure, we compared the S1136 levels in a Δ*sigB* strain and the isogenic wild-type strain 168 by quantitative PCR (qPCR). Indeed, σ^B was responsible for S1136 induction upon ethanol stress, as shown by the absence of S1136 induction in the Δ*sigB* strain. In contrast, S1136 was on average ~35-fold induced in the wild-type strain (Fig. 2).

3.3. Deletion of the S1136 promoter region reduces rpsD downregulation in ethanol-stressed cells

Our next step was to determine whether the activation of the S1136 promoter has a role in downregulating the level of the *rpsD* mRNA. Experimental verification of the regulatory mechanisms of asRNA can be challenging simply because deleting a sequence stretch on one strand inherently also deletes information on the opposite strand. Another challenge is to construct deletion mutants of asRNAs that have their promoter embedded within a CDS running antisense to it. In the case of S1136–S1134 it was possible to delete the promoter region of S1136 without interfering with the *rpsD* transcription unit. To this end, we constructed a 'clean' marker-less deletion of the S1136 promoter region (Fig. 1A, lower part). This effectively abolished S1136 transcription in the respective mutant strain, which is hereafter referred to as ΔP_{S1136}. We subsequently exposed the ΔP_{S1136} mutant and the isogenic wild-type strain to ethanol stress, withdrawing samples for RNA isolations immediately before and 10 min after the addition of 4% ethanol. To this end, we applied exactly the same experimental conditions as described by Nicolas et al. [8], where ethanol was added to a final concentration of 4%. This ethanol concentration was used because it had been shown previously that it leads to a clear induction of the σ^B-dependent stress response while growth of *B. subtilis* is only slightly affected as evidenced by an increase in generation time from 22 to 35 min [33,34]. Induction of a σ^B-dependent stress response in either of the two strains was verified by Western blotting with σ^B-specific antibodies, using aliquots of the same samples that were collected for RNA extraction (Supplementary Fig. S4). qPCR analysis on the RNA samples from the wild-type strain confirmed that S1136 is strongly induced upon ethanol stress (Fig. 3, left panel), and that *rpsD* is concomitantly downregulated (Fig. 3, right panel). Importantly, the qPCR analysis on RNA extracted from the ΔP_{S1136} mutant showed that S1136 induction upon ethanol stress was abolished by the promoter deletion and that the downregulation of *rpsD* was strongly and significantly alleviated in this strain (Fig. 3). Combined, these data show that the σ^B-dependent promoter driving the transcription of S1136 also controls the downregulation of *rpsD* upon exposure to ethanol. In turn, this implies that

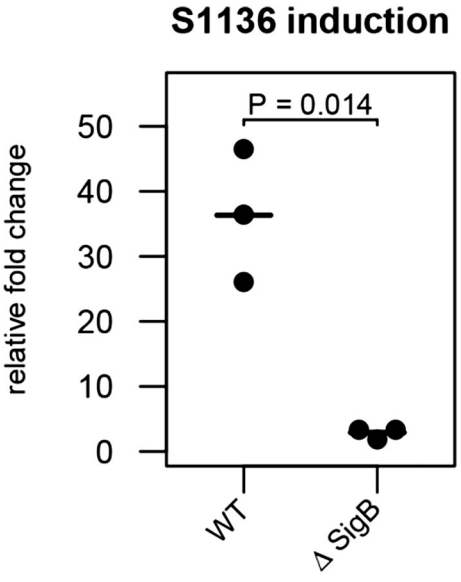

Fig. 2. Ethanol-induced S1136 expression depends on σ^B. The abundance of S1136 in a Δ*sigB B. subtilis* mutant and the isogenic wild-type (WT) strain 168 before and after ethanol stress was determined by qPCR. Relative fold changes in the expression of S1136 upon ethanol stress were calculated by setting the transcript levels before ethanol induction at one. Statistical analysis was performed with a one-sided Welch two-sample *t*-test (H1: relative fold change WT > Δ*sigB*). The respective p-value is indicated.

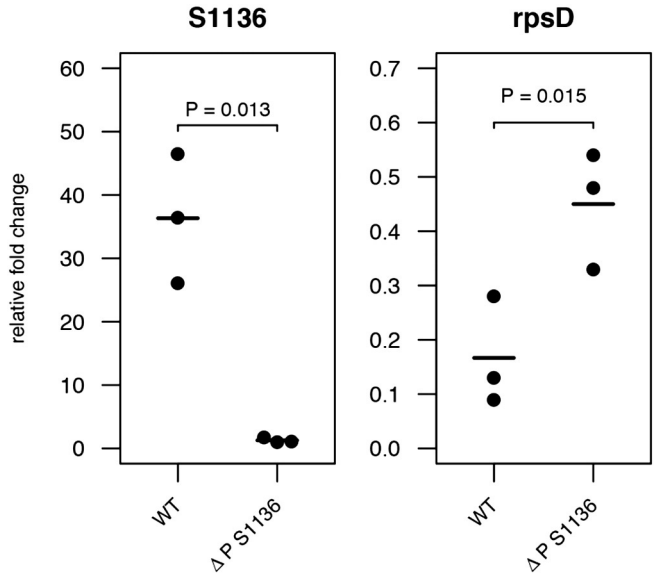

Fig. 3. *rpsD* downregulation upon ethanol stress is dependent on S1136 promoter activity. A qPCR analysis of the relative S1136 and *rpsD* mRNA levels was performed using cells of the wild-type (WT) *B. subtilis* strain 168 and the ΔP_{S1136} mutant. Relative fold changes in the abundance of the respective RNA levels upon ethanol stress were determined by setting transcript levels before ethanol induction as one. Statistical analyses were performed with a one-sided Welch two-sample t-tests, and p-values thus obtained are indicated.

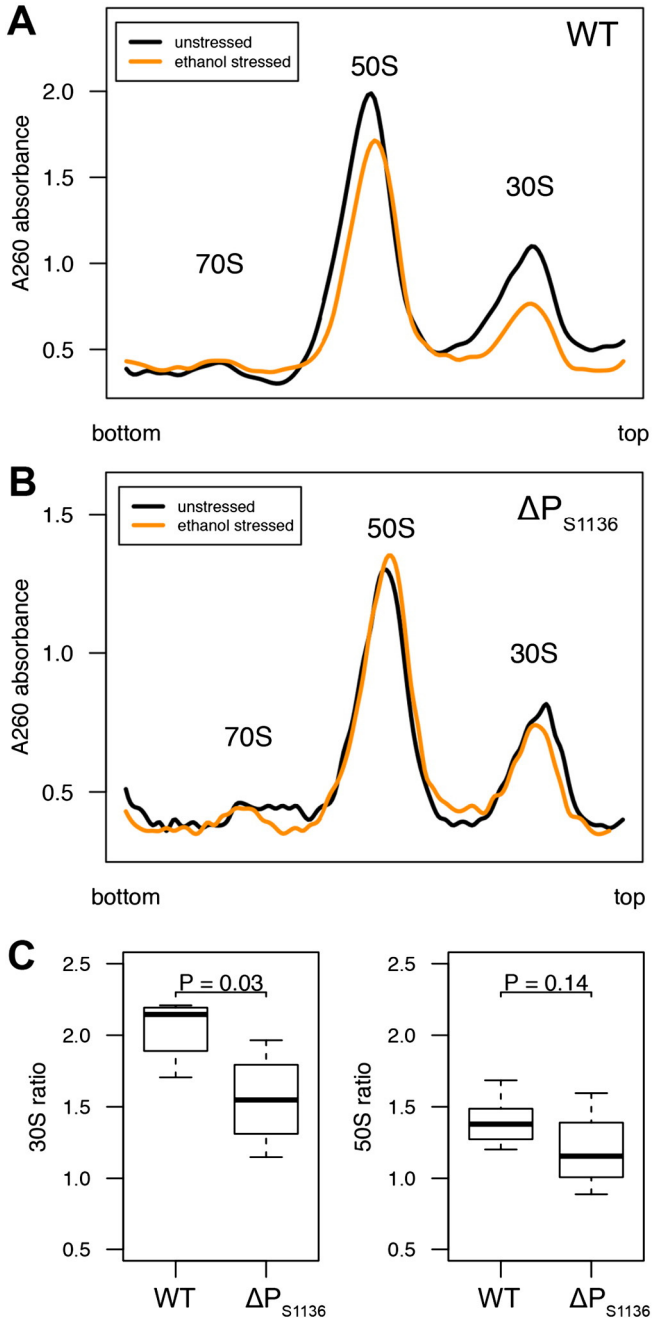

Fig. 4. S1136 promoter activity has a negative impact on the 30S ribosomal subunit abundance upon ethanol stress. Ribosomal subunit profiles were determined for the ethanol-stressed or unstressed wild-type (WT) strain 168 (A) or the ΔP_{S1136} strain (B). Peak fractions of 50S and 30S ribosomal subunits, and the position of translationally active 70S ribosomes are marked. The y-axis scale difference between A and B is caused by a differing number of collected fractions between these two experiments. C) Box plots of quantifications of peak area ratios from unstressed/ethanol stressed cells for 30S subunits (left panel) and 50S subunits (right panel). The data is based on four independent experiments (i.e. biological replicates) with the ΔP_{S1136} strain and five with the WT strain 168. Statistical data analysis was performed using a one-sided Welch two-sample *t*-test (H_1: unstressed/ethanol ratio for ΔP_{S1136} < WT strain), and the respective p-values are indicated.

the act of synthesizing the asRNA S1136 serves to downregulate the expression of *rpsD* upon ethanol stress. To investigate whether S1136–S1134 could function *in trans*, we cloned S1136–S1134 under the control of its native σ^B-dependent promoter in the *amyE* locus of the ΔP_{S1136} strain. The resulting strain was named ΔP_{S1136} *amyE*::S1136–S1134. To preclude transcription of *rpsD* from this construct, we only included the antisense sequence up to the *rpsD* start codon at the end of the S1134 sequence. As assessed by qPCR, S1136 expression upon ethanol exposure was restored in the ΔP_{S1136} *amyE*::S1136–S1134 strain, but *rpsD* regulation was not restored (data not shown). This might suggest that *rpsD* regulation by the S1136 promoter activation is dependent on the genomic context, but it could also mean that the S1134 sequence up until the ribosome-binding site of *rpsD* or even further is needed for effective antisense regulation.

3.4. P_{S1136}-mediated rpsD downregulation results in lower levels of the small ribosomal subunit upon ethanol stress

After defining a role for the σ^B-dependent S1136 promoter activity in downregulation of the *rpsD* mRNA, we wondered whether this regulation might impact on ribosome assembly. This is a relevant question, since *rpsD* is one of the six primary binding proteins of the small ribosomal subunit [20]. The levels of the small (30S) and large (50S) ribosomal subunits, and the translationally active 70S ribosomes (i.e. monosomes) can be determined through sucrose density gradient centrifugation, and subsequent determination of the UV absorption in different fractions of the sucrose density gradient. Indeed, the application of this approach to *B. subtilis* cells resulted in clear ribosomal profiles (Fig. 4, panels A and B). We next determined which ribosomal subunits were present in the different fractions by analyzing the ribosomal RNA (rRNA) content. This showed that the two major peak fractions represented the small and large ribosomal subunits, and that hardly any 70S monosomes were isolated (Supplementary Fig. S5). The apparent lack of 70S monosomes is most likely due to the shear forces employed for the preparation of cell-free extracts. This unforeseen consequence of the applied experimental approach was, however, advantageous for answering the question whether the abundance of the 30S and 50S ribosomal subunits would change upon ethanol stress. Specifically, we investigated whether the abundance of these ribosomal subunits would decrease upon *rpsD* downregulation by ethanol stress and, if so, whether the S1136 promoter would have a role in this process. To this end, we compared ribosomal subunits from ethanol-stressed cells and non-stressed cells of the ΔP_{S1136} strain and the isogenic wild-type strain. In the wild-type strain we consistently observed a ~2.2-fold lowered abundance of the small 30S ribosomal subunit after ethanol stress (Figs. 4A and C, left panel). Remarkably, this decrease in abundance of the small ribosomal subunit was largely abolished in the ΔP_{S1136} strain (Figs. 4B and C, left panel). Importantly, quantification of the ribosome subunit abundances by determination of the area under the respective peaks revealed that the observed decrease in the small ribosomal subunit abundance upon ethanol stress was indeed significantly reduced in the ΔP_{S1136} strain (~1.5-fold) as compared to the wild-type strain (~2.2-fold; Fig. 4C, left panel). In contrast, the levels of the large 50S ribosomal subunit were in both the ΔP_{S1136} strain and the wild-type strain only mildly reduced upon ethanol stress (~1.2-fold and ~1.4-fold, respectively), and there was no statistically significant influence of the S1136 promoter deletion on the relative abundance of the 50S subunit (Fig. 4C, right panel). The ethanol-induced reduction in the small ribosomal subunit abundance thus depends on the activity of the S1136 promoter, and accordingly on *rpsD* downregulation. This reduction thus seems to reflect a reduced *de novo* assembly of 30S subunits within the 10 min of exposure to ethanol. Altogether, our findings show that a decrease in the small ribosomal subunit abundance is part of an active σ^B-dependent cellular response to ethanol stress.

4. Discussion

In the present study we show that the *rpsD* gene, which encodes the essential small ribosomal subunit primary binding protein S4, is regulated by the σ^B-dependent S1136 promoter that lies antisense to *rpsD*. Furthermore, we report that the *rpsD* downregulation upon ethanol stress

is important for the concomitant decrease in the small ribosomal subunit abundance, which seems to be a thus far overlooked aspect of the σ^B response. The specific downregulation of at least one ribosomal gene also shows that *B. subtilis* actively reduces its translational capacity in the presence of ethanol rather than this being an indirect effect of ethanol toxicity on growth.

Consistent with the expression profile cluster analysis by Nicolas et al. [8], we found that σ^B is responsible for the majority of S1136 induction upon ethanol stress. The resulting *rpsD* downregulation could contribute to two previously documented observations on σ^B. Firstly, expression of σ^B in the absence of its anti-sigma factor was reported to be deleterious for growth [35], and secondly, σ^B induction in cells growing at low growth rates was also found to be deleterious for growth [36]. Both these observations could relate to the fact that σ^B induction may lead to a strong downregulation of *rpsD*, whereas *rpsD* is essential for growth.

In addition to the induction of S1136–S1134 in response to ethanol stress, this asRNA is also highly expressed in sporulation-inducing conditions [8]. This suggests that it may be under control of additional factors besides σ^B. This idea would be supported by the identification of −35 and −10 sequences for a putative sporulation-specific SigF promoter in the S1136 promoter region, which actually overlaps with the putative SigB promoter (Supplementary Fig. S3). Furthermore, it cannot be excluded that this sporulation-induced expression could correspond to additional functions of S1136–S1134 besides *rpsD* downregulation. Intriguingly, no downregulation of *rpsD* was observed under S1136-inducing conditions other than ethanol stress and sporulation, which are in fact the only conditions where the S1134 segment was detectable [8]. This suggests that the S1134 segment could contribute to the antisense regulation of *rpsD* and, potentially, this even requires the moiety of S1134 that overlaps with the ribosome-binding site and promoter of *rpsD*.

It is noteworthy that, as indicated in Fig. 1A, the deleted S1136 promoter region corresponds to a part of the *rpsD* downstream region that encodes the RNA segment S1137. Judged by the location of the S1137-encoding sequences, the S1137 RNA segment could potentially represent a 3′UTR of *rpsD* or an independently expressed RNA molecule. Notably, there are two lines of evidence to suggest that S1137 does not play any significant role in the regulation of *rpsD* as described in our present study. Firstly, judged by the previously published tiling array data (Nicolas et al. [8]), the level of S1137 is very low in ethanol-stressed cells. In fact, the correlation in the abundance of the *rpsD* mRNA and the S1137 segment across the experimental condition space addressed by Nicolas et al. is low (0.388). These findings suggest that S1137 is not a genuine 3′UTR of *rpsD*, certainly not in ethanol-stressed cells. Secondly, *rpsD* was shown to be downregulated in an ethanol stress-dependent manner. If the stability of the *rpsD* mRNA would have generally been influenced by deletion of S1137-encoding sequences, this should have been the case also in non-stressed cells. However, we only observed an effect of the S1136 promoter deletion on the *rpsD* transcript level upon ethanol stress. Altogether, we consider the possibility that S1137 may have a role in the σ^B-dependent downregulation of *rpsD* under conditions of ethanol stress highly unlikely. Instead, we propose that this *rpsD* downregulation is due to σ^B-dependent S1136 antisense promoter activity.

The majority of ribosomal genes of *B. subtilis* (51 out of 57) are transcribed from a large polycistronic operon, and *rpsD* represents one of the six exceptions to this together with *ctc* [37], *rpsF*/S6, *rpsB*/S2, *rplU*/L21, and *rpsR*/S18. In this respect, the monocistronic organization of the universally conserved *rpsD* gene in *B. subtilis* is particularly remarkable as it is part of ribosomal operon structures in other bacteria. For instance, *rpsD* is the third gene of a 5-gene operon in *E. coli* [21,23]. A physiological relevance of the identified asRNA-mediated *rpsD* downregulation under conditions of ethanol stress and sporulation would provide an explanation for this different genomic location of *rpsD* in *B. subtilis*. Specifically, two possible reasons are conceivable. Firstly, a downregulation of other ribosomal genes to the same extent as *rpsD* might be undesired in *B. subtilis*. Secondly, the evolution of mechanisms to downregulate the *rpsD* gene from its promoter might be difficult to achieve due to the post-transcriptional negative autoregulation of *rpsD* and the corresponding complicated promoter architecture of the *rpsD* 5′UTR region [8,21,22]. The present study would thus suggest a possible evolutionary rationale for the observed P_{S1136}-mediated antisense regulation of *rpsD*. In this context, it should be noted that the *rpsD* expression is still about 2-fold downregulated upon ethanol stress in the ΔP_{S1136} mutant. Thus, the observed downregulation of *rpsD* in the wild-type strain is not exclusively σ^B-dependent. Whether there is an additional regulatory mechanism that causes downregulation of *rpsD* upon ethanol stress, or whether the downregulation observed in cells of the ΔP_{S1136} mutant is due to indirect effects caused by ethanol remains to be shown.

The regulation of *rpsD* by activation of the S1136 promoter will mainly impact on de novo assembly of the small ribosomal subunit. Thus, it is well conceivable that the effect of this antisense regulation on the levels of the 30S ribosomal subunit is obscured to some extent by the ribosomes that are already present in the cells at the moment when they are exposed to ethanol. However, we would like to point out that growth is only moderately impaired by the 4% ethanol used [33,34]. Accordingly, within the 10 min exposure to ethanol (prior to cell disruption), both the wild-type cells and the ΔP_{S1136} mutant cells undergo about one-third of a generation time. We consider this sufficient time for the generation of a substantial pool of de novo-synthesized ribosomes and this idea is consistent with the ~2.2-fold reduction in the level of the 30S ribosomal subunits in WT cells. Of note, this decrease was smaller in the ΔP_{S1136} mutant cells (~1.5-fold), which is in full agreement with the qPCR data.

Lastly, a crucially important question that follows from the observed decrease in the small ribosomal subunit abundance upon ethanol stress is what could be the physiological relevance of this phenomenon? One answer to this question may be found in the observation from the large-scale transcriptome study by Nicolas et al. that ethanol stress results in an impaired Rho-dependent transcriptional termination of many genes [8]. In fact, it was observed that the length of mRNA 3′ extensions was negatively correlated with the level of *rho* expression; *rho* expression was particularly low under ethanol stress and during sporulation, which led to long mRNA 3′ extensions. While the molecular basis for the downregulation of *rho* expression under these conditions is currently not known, it is well conceivable that the presently observed P_{S1136}-mediated downregulation of *rpsD* serves to counteract any potentially detrimental consequences of *rho* downregulation. For instance, the decrease in the number of small ribosomal subunits thus achieved might protect against a possible spurious translation of potentially toxic aberrant peptides encoded by long mRNA 3′ extensions. In this respect, it is important to bear in mind that the small ribosomal subunit contains the ribosome-decoding center. Furthermore, there are multiple clues in the scientific literature that ribosomes are involved in stress-responses, albeit without clear mechanistic explanations. For instance, heat-shock proteins are induced by ribosomally-targeted antibiotics [38], inhibiting ribosome maturation or ribosome function increases salt resistance [32], and *B. subtilis* stress proteins form structural parts of the ribosome [39], or are required for σ^B activation [40]. Because of the higher structural instability of the small ribosomal subunit compared to the large subunit [41], the small subunit will be more sensitive to disturbances and thus more suitable for involvement in possible stress-sensing mechanisms. More directly related to ethanol stress are the general or specific effects that ethanol could have on the ribosome. Firstly, a general effect of ethanol is the destabilization of protein structure and this could explain the induction of the heat-shock response by ethanol stress [42]. Secondly, a specific role of ethanol on ribosomes was recently suggested in that it changes the conformation of the decoding center of the small ribosomal subunit [42], and this is in line with the observation that ethanol causes ribosome misreading

in vitro [10]. The importance of the decoding center is underscored by reports that ethanol tolerance is increased by mutations or methylation of the proteins or 23S RNA in close proximity to the ribosomal decoding center ([42] and references therein). In the latter large-scale systems biology approach to unravel the effects of ethanol toxicity, it was also proposed that ethanol disrupts the natural conformation of the ribosomal decoding center, thereby allowing accommodation of non-cognate aminoacyl-tRNAs [42]. This would thus result in potentially toxic translation errors and thereby provide an additional rationale to actively reduce the level of translation via a decrease in small ribosomal subunit abundance as observed in our present study.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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