Small RNA mediated repression of subtilisin production in *Bacillus licheniformis*

Robert Hertel¹, Sandra Meyerjürgens¹, Birgit Voigt²,³, Heiko Liesegang¹ & Sonja Volland¹

The species *Bacillus licheniformis* includes important strains that are used in industrial production processes. Currently the physiological model used to adapt these processes is based on the closely related model organism *B. subtilis*. In this study we found that both organisms reveal significant differences in the regulation of subtilisin, their main natural protease and a product of industrial fermentation processes. We identified and characterized a novel antisense sRNA AprAs, which represents an RNA based repressor of *apr*, the gene encoding for the industrial relevant subtilisin protease. Reduction of the AprAs level leads to an enhanced proteolytic activity and an increase of Apr protein expression in the mutant strain. A vector based complementation of the AprAs deficient mutant confirmed this effect and demonstrated the necessity of *cis* transcription for full efficiency. A comparative analysis of the corresponding genome loci from *B. licheniformis* and *B. subtilis* revealed the absence of an *aprAs* promoter in *B. subtilis* and indicates that AprAs is a *B. licheniformis* species specific phenomenon. The discovery of AprAs is of great biotechnological interest since subtilisin Carlsberg is one of the main products of industrial fermentation by *B. licheniformis.*

*Bacillus licheniformis* was originally isolated and described by Weigman in 1898¹. The species is a member of the *B. subtilis* species complex² and exhibits a saprophytic life style on organic materials³,⁴. The lifestyle results in three properties which promote *B. licheniformis* as a platform for productive fermentations. (I) *Bacilli* can use a broad spectrum of plant derived C- and N-sources⁵-⁷. (II) Since the organisms live on organic matter they are evolved for high concentrations of nutrient supply which enables them to reach high cell densities in fermentations⁸. Finally, (III) they secrete high amounts of many efficient bio-polymer hydrolases⁹ like lichenases⁹, lipases¹⁰, thermostable alpha-amyloses¹¹, and many more¹²-²⁰. Exoproteases of the subtilisin Carlsberg family are an industrial fermentation product of *B. licheniformis* and *B. subtilis* strains with an annual production rate of about 500 t²¹. Subtilisin is highly thermostable, active under alkaline conditions (pH 10-11) and, due to its low substrate specificity, an important additive for household detergents²¹,²².

In 2004 the type strain *B. licheniformis* DSM13 (also ATCC14580) was sequenced by Rey et al. (Davis, USA)²³ and Veith et al. (Göttingen, Germany)²⁴ revealing a plasmid free 4.2 Mbp genome. Almost a decade later, the RNA based regulatory network of *B. licheniformis* DSM13 was analysed by RNA-Seq using samples from different stages of an industrial fermentation²⁵. The transcriptome data revealed a considerable number of protein encoding RNAs and also 3,314 non-coding RNAs, divided in 2853 mRNA-bound and 461 small RNAs. In general, small RNAs (sRNA) are versatile regulators of gene expression, which can facilitate their function in *cis* or *trans*, controlling e.g. mRNA stability, degradation, termination and translation²⁶,²⁷. Three prominent classes of sRNAs have been described: (i) structured sRNAs with limited complementarity to their targets, (ii) sRNAs that bind regulatory proteins and (iii) antisense RNAs synthesized from the strand complementary to their target mRNA²⁵. In the transcriptome of *B. licheniformis* an antisense RNA element on the 3′ end of the *apr* gene, encoding the subtilisin protease, gained considerable attention, since the samples were taken from a subtilisin fermentation²⁷. Due to its association with *apr*, we called the RNA element AprAs. Its active region has a length of 144 nucleotides and shows complete sequence identity to the antisense 3′ coding region of the *apr* gene. The RNA-Seq data from Wiegand et al.²³ also indicated that the small RNA is transcribed by its own promoter. Its transcription rate exceeds the transcription rate of the *apr* mRNA by three orders of magnitude.

¹Department of Genomic and Applied Microbiology & Goettingen Genomics Laboratory, Institute of Microbiology & Genetics, University of Goettingen, Goettingen, Germany. ²Institute for Microbiology, Ernst-Moritz-Arndt University, Greifswald, Germany. ³Present address: Research Institute of Leather and Plastic Sheeting gGmbH, Freiberg, Germany. Correspondence and requests for materials should be addressed to S.V. (email: svollan@gwdg.de)
Asci10 promoter box was replaced with the GC-rich recognition pattern of AprAs, the AT-rich region of the respective genomic region and digested with the endonuclease I. The mutant strain exhibited the expected 1.3 kbp DNA band consisting of the degradation products of the 2.6 kbp PCR product (see Supplementary Figure S1). A slot blot experiment confirmed that no transcriptional activity of the aprAs gene was detectable in the mutant strain compared to the original strain. For the closely related organism B. subtilis no comparable antisense RNA associated with the -1 end of aprE was described, although extensive transcriptome analyses were performed by Rasmussen et al. in 2009 and Nicolas et al. in 2012. The subtilisin gene aprE in B. subtilis was found to be regulated by the interacting global transcriptional regulators CodY and ScoC as well as by AbrB31. Barbieri et al. could also show that the regulatory network in B. subtilis results in a repression of the aprE gene within the exponential growth phase and an exclusive expression of AprE within the stationary phase. The genome of B. licheniformis DSM13 also exhibits orthologues of aprE regulators and the transcriptome analysis of B. licheniformis DSM13 showed a corresponding expression pattern for aprE. However, the association of the apr gene with a highly expressed small antisense RNA indicates an additional regulatory layer in B. licheniformis.

The here presented work focuses on the impact of the newly discovered antisense RNA AprAs. By inactivation of the aprAs promoter and vector encoded transcription of AprAs we could show its regulatory effect on proteolytic activity and Apr expression.

**Results**

**Construction and characterization of an AprAs deficient mutant.** To investigate the impact of the 144 base large AprAs sequence was analyzed via the RNAfold WebServer, using “The Vienna RNA Website”55. The calculation of secondary structures was possible and the optimal structure with a minimum free energy of −51.9 kcal/mol is shown in dot-bracket notation.

For the closely related organism B. subtilis no comparable antisense RNA associated with the 3′-end of aprE was described, although extensive transcriptome analyses were performed by Rasmussen et al. in 2009 and Nicolas et al. in 2012. The subtilisin gene aprE in B. subtilis was found to be regulated by the interacting global transcriptional regulators CodY and ScoC as well as by AbrB. Barbieri et al. could also show that the regulatory network in B. subtilis results in a repression of the aprE gene within the exponential growth phase and an exclusive expression of AprE within the stationary phase. The genome of B. licheniformis DSM13 also exhibits orthologues of aprE regulators and the transcriptome analysis of B. licheniformis DSM13 showed a corresponding expression pattern for aprE. However, the association of the apr gene with a highly expressed small antisense RNA indicates an additional regulatory layer in B. licheniformis.

The here presented work focuses on the impact of the newly discovered antisense RNA AprAs. By inactivation of the aprAs promoter and vector encoded transcription of AprAs we could show its regulatory effect on proteolytic activity and Apr expression.

**Construction and characterization of an AprAs deficient mutant.** To investigate the impact of the 144 base large AprAs sequence was analyzed via the RNAfold WebServer, using “The Vienna RNA Website”55. The calculation of secondary structures was possible and the optimal structure with a minimum free energy of −51.9 kcal/mol is shown in dot-bracket notation.

**Figure 1.** AprAs transcription profile, promoter region and sequence analysis. (a) Transcription profile of AprAs in the early stage of an industrial subtilisin production. Transcriptome data were visualised in a logarithmic scale using TraV. The red graph represents the transcription activity of aprAs and the blue of apr. The black arrow on the complementary strand represents the coding region of apr and the yellow arrow of aprAs. The dark red arrows represent the identified promoters and green arrows predicted transcription terminators. (b) Promoter region of aprAs. The −10 box (TAGAAT) of the potential SigA promoter is highlighted in red and the exchanged sequence (Ascl pattern) is given. Predicted transcription terminators are framed with green arrows. Protein coding sequences are shown in black bold letters and the stop codons are underlined. The aprAs coding sequence is given in orange letters and the experimentally determined transcription start site of AprAs is framed in red. (c) AprAs sequence. The 144 base large AprAs sequence was analyzed via the RNAfold WebServer, using “The Vienna RNA Website”55. The calculation of secondary structures was possible and the optimal structure with a minimum free energy of −51.9 kcal/mol is shown in dot-bracket notation.

For the closely related organism B. subtilis no comparable antisense RNA associated with the 3′-end of aprE was described, although extensive transcriptome analyses were performed by Rasmussen et al. in 2009 and Nicolas et al. in 2012. The subtilisin gene aprE in B. subtilis was found to be regulated by the interacting global transcriptional regulators CodY and ScoC as well as by AbrB. Barbieri et al. could also show that the regulatory network in B. subtilis results in a repression of the aprE gene within the exponential growth phase and an exclusive expression of AprE within the stationary phase. The genome of B. licheniformis DSM13 also exhibits orthologues of aprE regulators and the transcriptome analysis of B. licheniformis DSM13 showed a corresponding expression pattern for aprE. However, the association of the apr gene with a highly expressed small antisense RNA indicates an additional regulatory layer in B. licheniformis.

The here presented work focuses on the impact of the newly discovered antisense RNA AprAs. By inactivation of the aprAs promoter and vector encoded transcription of AprAs we could show its regulatory effect on proteolytic activity and Apr expression.
The initial strain Bacillus licheniformis MW3 was used for allelic exchange to replace the aprAs gene with the vector-encoded aprAs gene. The strain was subjected to quantitative exoprotease assay to evaluate the proteolytic activity of the mutant strain in comparison to the original strain MW3. The results showed a reduction in exoprotease activity, indicating a correlation between the AprAs level and Apr expression.

**Verification of AprAs phenotype.** Bacillus licheniformis MW3 AprAs− was complemented with a vector encoded aprAs gene to restore the original phenotype and confirm the observed exoprotease phenotype of Bacillus licheniformis MW3 AprAs− is based on the inactivation of AprAs transcription. The aprAs gene with its native promoter was cloned into the Bacillus/E. coli shuttle vector pV233, creating pV2::aprAs. The vector was introduced into Bacillus licheniformis MW3 AprAs− and the original strain Bacillus licheniformis MW3. The vector pV2 was used as a control. The slot blot analysis (Fig. 2) confirmed the re-establishment of AprAs transcription in Bacillus licheniformis MW3 AprAs−pV2::aprAs. B. licheniformis MW3 pV2 (lane 3) exhibited AprAs transcription due to the chromosomal aprAs gene. B. licheniformis MW3 pV2::aprAs showed an increased AprAs transcription due to expression from the chromosomal aprAs gene in addition to the multi-copy vector encoded aprAs gene.

The qualitative evaluation of the protease activity on M9 skim milk agar plates (Fig. 3c) showed that the complemented mutant strain Bacillus licheniformis MW3 AprAs−pV2::aprAs could re-establish the wild type phenotype. Its halo formation was comparable to Bacillus licheniformis MW3 pV2 and reduced in comparison to Bacillus licheniformis MW3 AprAs−. These qualitative results were confirmed by the quantitative exoprotease activity evaluation (Fig. 3d), determined in the supernatant of 4 ml culture in liquid M9 skim milk media. The reduction of AprAs transcription in Bacillus licheniformis MW3 AprAs− resulted once more in an approximately 4 times increased exoprotease activity. This effect was reversed by the ectopic AprAs transcription in Bacillus licheniformis MW3 AprAs−pV2::aprAs. The in trans overexpression of AprAs in the original strain MW3 resulted in an additional reduction of exoprotease activity of approximately 50% (see Bacillus licheniformis MW3 pV2::aprAs).

**Impact of AprAs on Apr expression.** In order to investigate the correlation between the AprAs level and the expression of the protease Apr, the extracellular proteomes of the original strain MW3, the AprAs− mutant and the complemented strain were analysed by 2D-gel electrophoresis and MALDI-TOF-MS/MS. All strains were grown in 400 ml liquid M9 skim milk medium and supernatants were harvested after 12 h, 24 h, 36 h and 48 h. The quantitative exoprotease assay was performed using the supernatants of three experiments (Fig. 4a). The overall proteolytic activity of the strains increased constantly from 12 h to 48 h. The AprAs deficient mutant strain Bacillus licheniformis MW3 AprAs− showed the highest activity levels with approximately 0.8 U/ml after 48 h. The initial strain Bacillus licheniformis MW3 and the vector complemented strain MW3 AprAs−pV2::aprAs showed both lower activity levels with the highest level of approximately 0.5 U/ml. The samples with the highest activity levels (48 h) were analysed in triplicates by 2D-gel electrophoresis. Three Apr spots were identified in the supernatants of the cultures. Figure 4b exemplarily shows the dual channel images of 2D-gels from the strain Bacillus licheniformis MW3 compared to the mutant strains Bacillus licheniformis MW3 AprAs− and Bacillus licheniformis MW3 AprAs−pV2::aprAs respectively. The first image demonstrates a strong increase of Apr protein expression in the extracellular proteome of the AprAs− mutant compared to MW3. This effect is reversed after vector complementation of the mutant strain: Bacillus licheniformis MW3 AprAs−pV2::aprAs shows only a slight increase in Apr expression compared to the original strain Bacillus licheniformis MW3. To quantify the differences in Apr protein expression,
The sequence comparison showed that the aligned concerning regions of 17 AprAs −
extracellular proteases did not change in relation to the AprAs level. Thus, the observed protease phenotype of the initial strain and the complemented strain revealed an increased exoprotease activity visible through a stronger halo formation compared to MW3. (b) The four fold increased exoprotease activity of B. licheniformis MW3 AprAs− confirms the exoprotease phenotype observed in Fig. 3a. (c) B. licheniformis MW3 pV2::aprAs shows the smallest halo formation. The halo of the complemented B. licheniformis MW3 AprAs− pV2::aprAs is comparable to MW3 pV2. B. licheniformis MW3 AprAs− pV2 shows the strongest proteolytic activity indicated by the largest halo formation. The ectopic transcription of aprAs results in a reduction of exoprotease activity in both MW3 and mutant AprAs−. (d) The protease activity of the complemented strain B. licheniformis MW3 AprAs− pV2::aprAs is strongly decreased compared to B. licheniformis MW3 AprAs− and comparable to B. licheniformis MW3 pV2. B. licheniformis MW3 pV2::aprAs shows an approximately 50% reduced exoprotease activity.

the spot volumes of the Apr protein spots of all three replicates were compared. Figure 4c shows that the Apr expression in the extracellular proteome of B. licheniformis MW3 AprAs− was three times increased compared to the original strain B. licheniformis MW3. After vector complementation of the AprAs− mutant, a reduction of the Apr protein level was visible (see B. licheniformis MW3 AprAs− pV2::aprAs), but was still two times increased in comparison to the original strain MW3.

The complete dual channel images of the compared strains (Supplementary Figure S2) showed no differential expression for protein spots of other known extracellular B. licheniformis proteases, such as the metalloprotease Mpr and the minor extracellular protease Vpr (Vpr_1–Vpr_6), as well as the extracellular bacillopeptidase Bpr (Bpr_1, Bpr_2).

Sequence comparison of the AprAs promoter region. Orthologues of the subtilisin gene are found in B. licheniformis and B. subtilis strains. Yet, to our knowledge no orthologous aprAs genes associated with apr genes were described in the transcriptome analyses of B. subtilis 168 from Rasmussen et al. in 200930 and Nicolas et al. in 201235. To find out if aprAs and especially its promoter region are conserved, we performed a comparative sequence analysis. The comparison (Fig. 5) comprised the intergenic region between apr, with a special focus on the −10 promoter box (green coloration), which was experimentally verified to be responsible for the transcription of AprAs in B. licheniformis DSM13 in the present investigation (see also Fig. 1). The comparison of B. licheniformis DSM13 and B. subtilis 168 showed that the aprAs promoter box (green coloration) is missing in B. subtilis 168. (Fig. 5a). To clarify if AprAs is a strain or species specific phenomenon, we also aligned the concerning regions of 17 B. licheniformis strains (see Fig. 5b) and 35 B. subtilis strains (see Fig. 5c). The sequence comparison showed that the aprAs promoter is present in all investigated B. licheniformis strains but not in the compared B. subtilis strains.

Discussion
Our results clearly reveal the small RNA AprAs as a repressor of the protease Apr. The protease activity measurements also demonstrated the necessity of cis transcription of AprAs in relation to apr for highly efficient repression. The observed protease phenotypes of the initial strain and the complemented strain B. licheniformis MW3 AprAs− pV2::aprAs were on the same level, showing that the transcription of one aprAs gene in cis amounts to approximately 50 copies in trans. The combination of the single cis encoded aprAs gene and the multi-copy vector in B. licheniformis MW3 pV2::aprAs reduced the exoprotease activity even 50% below the initial level (Fig. 3c,d).

The exoproteome analysis (Fig. 4) showed the direct correlation between the observed protease related phenotype and the expression of the protease Apr. The comparison of MW3 and mutant proteomes revealed Apr as the only differentially expressed extracellular protease (Supplementary Figure S2). Protein spots of other known extracellular proteases did not change in relation to the AprAs level. Thus, the observed protease phenotype...
was facilitated by the negative regulation of AprAs on Apr expression. Although the Apr protein expression levels in *B. licheniformis* MW3 and MW3 AprAs−pV2::aprAs did not exactly correspond to the levels of the proteolytic activity, both analyses clearly indicate AprAs to be a repressor of Apr expression. Antisense RNA regulation on protein expression in *Bacilli* has been described before. However, this is, to our knowledge, the first time that an RNA repressor of a biotechnologically relevant product has been reported.

A requirement of the proteome analysis was the scale up of our experiment from 4 ml test tubes to 400 ml flask culture. Interestingly, after scaling up, we observed only a doubling of the proteolytic activity (Fig. 4a) in contrast to the four fold increased activity observed in the 400 ml 4 ml volume experiment (Fig. 3b and d). Most likely, these differences result from the adaption of the experimental design, which can have an impact on productivity, but might also reflect additional regulatory layers. Investigations on the orthologous gene aprE from *B. subtilis* 168 revealed a complex regulatory network of the subtilisin protease including, as mentioned before, regulators such as ScoC, SinR, AbrB, DegU and further associated proteins. The genome of *B. licheniformis* DSM13 encodes orthologues of these regulators, thus a regulatory network of similar complexity can be assumed.

In case of AprAs, no orthologous sRNA was identified in *B. subtilis* 168. Our sequence comparison of the promoter box of aprAs showed its presence in all investigated *B. licheniformis* strains and its absence in all investigated *B. subtilis* strains. Therefore we assume that AprAs is a species specific sRNA and thus, our results demonstrate a clear difference on subtilisin protease regulation between *B. licheniformis* and *B. subtilis*. It remains unclear if a similar regulatory layer on the subtilisin protease exists in other *B. subtilis* species complex members or if it is unique for *B. licheniformis*.

Transcriptome analysis in *B. subtilis* and *S. aureus* revealed that antisense activities can arise from inefficient termination of the sense transcription or from spurious initiation by alternative sigma factors. However, the strong transcriptional activity and the distinct transcription start site as well as the presence of a conserved SigA promoter pattern support the hypothesis that AprAs is a real non-coding RNA and therefore a relevant regulatory layer of Apr expression.

The regulation by AprAs could involve a pairing of the small RNA and its mRNA target leading to a guided degradation. As has been discussed for antisense regulators in Thomason and Storz and in Desnoyers et al., the transcription of AprAs is three orders of magnitude stronger than the transcription of its target RNA, which resembles viral toxin/antitoxin type I systems, where the sense/antisense duplex is guided to degradation by RNaseIII. Apparently, a quantitative titration of the target mRNA by the small RNA is involved in the regulatory mechanism. This observation is supported by the fact that in Real-time PCR analysis the apr mRNA level varies in

---

**Figure 4.** Protease activity and Apr protein expression in *B. licheniformis* MW3, MW3 AprAs− and MW3 AprAs−pV2::aprAs. The strains were grown in 400 ml M9 skim milk medium for 48 h. (a) The cell culture supernatants of samples from time points 12 h, 24 h, 36 h and 48 h were used for quantitative exoprotease activity determination. The absolute value of protease activity is shown as the average of three independent experiments, each with triplicate measurements. The exoprotease activity increased from 12 h to 48 h and is clearly stronger in *B. licheniformis* MW3 AprAs− in all experiments compared to *B. licheniformis* MW3 and MW3 AprAs−pV2::aprAs. The error bars display the standard deviation and the colour legend is shown in the figure. (b) Extracellular proteins were isolated from supernatants of 400 ml M9 skim milk cultures after 48 h of growth. The protein fractions were separated by 2D-gelelectrophoresis. A gel image of *B. licheniformis* MW3 (green) was overlaid with the respective image of MW3 AprAs−. The volume of the three identified Apr protein spots is strongly increased in *B. licheniformis* MW3 AprAs− (red) compared to the original strain *B. licheniformis* MW3 (green). In addition, the gel image of *B. licheniformis* MW3 (green) was overlaid with the respective image of MW3 AprAs−pV2::aprAs (red). After vector complementation, the effect of the mutation is reversed. *B. licheniformis* MW3 AprAs−pV2::aprAs shows only a slight increase in Apr protease expression compared to *B. licheniformis* MW3. (c) To quantify the Apr expression the spot volumes (in percentage of the whole protein spot volume) were calculated. The average of the normalized spot volumes of three replicates is shown and the standard deviation is given. The Apr protein expression in the extracellular proteome of *B. licheniformis* MW3 AprAs− is approximately three times increased compared to the original strain *B. licheniformis* MW3. The Apr expression of *B. licheniformis* MW3 AprAs−pV2::aprAs is reduced compared to the precursor strain *B. licheniformis* MW3 AprAs−, but still higher than in *B. licheniformis* MW3.
relation to the AprAs level in the investigated mutant strains (Supplementary Figure S3). However, since a cis encoded aprAs is more effective than multiple vector encoded gene copies, an additional regulatory layer is possible. Figure 1c shows that AprAs might form a secondary structure which could delay the interaction of the sRNA and target mRNA. Hence, the transcription of AprAs close to its target mRNA could be an advantage that may explain the differences in efficiency between cis and vector encoded AprAs.

It has been shown that the production of subtilisin in B. subtilis starts at the beginning of the stationary phase31. In contrast, Wiegand et al.25 observed a delay between the beginning of the transcriptional activity of the apr gene at the end of the exponential growth phase and the actual occurrence of the Apr protease spot in the proteome of B. licheniformis. It is tempting to assume that an AprAs guided degradation of the apr mRNA may be responsible for this delay between transcription and expression of the apr gene. However, life time evaluations

---

**Figure 5.** Alignment of AprAs promoter sequences from B. licheniformis and B. subtilis strains. Protein coding sequences are marked in grey. Transcription terminator sequences are marked in green and the promoter regions (−10 box) in red. The alignment was performed using MUSCLE53. (a) Sequence alignment of B. licheniformis DSM13 (NC_006270) and B. subtilis 168 (NC_000964). B. subtilis 168 lacks the −10 box upstream of the aprAs gene of B. licheniformis DSM13. (b) Sequence alignment of 17 B. licheniformis strains. The AprAs −10 box is present in all strains. (c) Sequence alignment of 35 B. subtilis strains. The AprAs −10 box is absent in all strains.

---
of small RNAs in a complex industrial high density medium are heavily challenged by the background RNase activity within the fermentation process.

The production of subtilisin proteases in Bacilli is of great biotechnological relevance since these production systems are extensively used to produce the main enzymatic components of household detergents. Thus, a four-fold increase of the subtilisin activity, as has been achieved with the B. licheniformis wild type subtilisin gene in the AprAs− mutant, is very promising. Although apr orthologues have been identified in most genomes of members of the B. subtilis species complex, the presence of AprAs-like RNA repressors seems less ubiquitous. AprAs was not identified in B. subtilis 168, or in the 35 investigated B. subtilis genomes (Fig. 5). However, the possibility to achieve an increased subtilisin production by an AprAs− mutation in B. licheniformis has been demonstrated in our study and should be evaluated for B. licheniformis based fermentations of subtilisin-like enzymes.

### Conclusion and outlook

The newly identified antisense RNA AprAs was shown to be a negative regulator of the biotechnologically important subtilisin family protease Apr in the type strain Bacillus licheniformis DSM13. The prevention of AprAs transcription led to an up to 4-fold increase of exoprotease activity as a result of the increased Apr protein expression. This effect was reversed by ectopic overexpression of AprAs and correspondingly, the native AprAs transcription led to an up to 4-fold increase of exoprotease activity as a result of the increased Apr protein expression.

Further analyses should focus on revealing the detailed mechanism of AprAs regulation and the search for AprAs homologues in other Bacilli and industrially used apr gene loci.

### Material and Methods

#### Bacterial strains and culture conditions.

The bacterial strains used in this study are listed in Table 1. If not stated otherwise, the strains were grown in NB medium at 37 °C. M9 medium was prepared as described by Sambrook and Russell. 1 L of M9 medium was additionally supplemented with 100 µl of SL-8 trace element solution as described by Atlas and 100 µl vitamin solution. The vitamin solution contained per litre 50 mg pantothenic acid, 50 mg riboflavin, 10 mg pyridoxamine-HCl, 20 mg biotin, 20 µg folic acid, 25 µg niacin, 25 µg nicotinamide, 50 µg α-aminobenzoic acid, 50 µg thiamine–HCl and 50 µg cobalamin dissolved in H2O. M9 medium was supplemented with a final concentration of 0.1% (w/v) sterile skim milk to generate M9 skim milk medium. For medium solidification agar with a final concentration of 1.5% (w/v) was added prior to sterilisation. Medium for solidification agar with a final concentration of 1.5% (w/v) was added prior to sterilisation by autoclaving.

#### PCR, gel electrophoresis and vector construction.

Primers used in this study are listed in Table 2. Primers (50 µl) consisted of 200 µM deoxynucleotides, 100 ng of template DNA, 5 pmol of each primer and 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Darmstadt, Germany). PCR products were purified directly using the PCR Purification Kit (Qiagen, Hilden, Germany) or after gel electrophoresis using a Qiaquick Gel extraction Kit (Qiagen, Hilden, Germany). DNA was analysed using a TAE agarose gel system as described elsewhere and stained with Ethidium bromide (1 µg/ml) for 10 min. Vectors were constructed as described previously using Escherichia coli TOP10 (Invitrogen, Carlsbad, USA) and isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). For sequence verification Sanger-sequencing was performed on an ABI3730XL capillary sequencer (BigDye 3.1 chemistry; Applied Biosystems, Darmstadt, Germany).

#### Table 1. Bacterial strains und plasmids.

| Strain            | Genotype                                                                 | Source/Ref. |
|-------------------|--------------------------------------------------------------------------|-------------|
| E. coli TOP10     | F. mcrA, Δ(merr–hidBMS–menBC), Δ801acZ, ΔM15, ΔlauX74, mpr1, recA1, araD139, Δ[ara-leu]7697, galE15, galK16, rpsL(St3), endA1, λ− | Invitrogen  |
| E. coli S17−1     | Sm4, Tp4, modI, recA, thi, pro, recA, RP4-Tc-Mu-Km:Ti7                   | Lab strain collection |
| B. licheniformis MW3 | B. licheniformis DSM13 (ΔhisR1, ΔmcrA, ΔhisR2)                          | Prof. Dr. Friedhelm Meinhardt, University Münster |
| B. licheniformis MW3 AprAs− | B. licheniformis DSM13 (ΔhisR1, ΔmcrA, ΔhisR2, AprAs−)                  | this study   |
| B. licheniformis MW3 pV2::aprAs | B. licheniformis MW3 with pV2::aprAs                                        | this study   |
| B. licheniformis MW3 AprA− pV2::aprAs | B. licheniformis MW3 AprA− with pV2::aprAs                                    | this study   |
| B. licheniformis MW3 pV2 | B. licheniformis MW3 with pV2                                           | this study   |
| B. licheniformis MW3 AprA− pV2 | B. licheniformis MW3 AprA− with pV2                                        | this study   |
| Plasmid           | Genotype                                                                 | Host        | Source/Ref. |
| pKVM2             | bla tet5 (PclpB–hgaB) oriT                                               | E. coli, Bacillus | 47         |
| pKVM2 AprAs−      | AprAs deletion vector                                                    | E. coli, Bacillus | this study |
| pV2               | bla, kan, oriT                                                           | E. coli, Bacillus | 33         |
| pV2::aprAs        | bla, kan, oriT, aprAs                                                    | E. coli, Bacillus | this study |
CTAATACGACTCACTATAGGGAGA

Scientific Reports | 7: DOI:10.1038/s41598-017-05628-y

Manufacturer’s instructions and detection was accomplished via the ChemoCam Imager (Intes, Göttingen, Germany). Hybridization was performed using the DIG Northern Starter Kit (Roche, Basel, Switzerland) following the manufacturer’s instructions. Each sample contained one blank as reference.

B. licheniformis-negative derivative of B. licheniformis chromosomal DNA. The used primers included the Ascl restriction site instead of the −10 box of the putative AprAs promoter. The flanks were fused via SOE-PCR and cloned into the temperature-sensitive vector pKVM2 using BamHI and Ncol restriction sites (BamHI, Ncol, FastAP and T4-DNA-ligase (Thermo Scientific, Darmstadt, Germany)). Escherichia coli S17-149 was used for conjugal transfer of the resulting pKVM2 AprAs− deletion vector. The introduced deletion was confirmed by restriction analysis. For this purpose, the region of interest was amplified via PCR using primer HLHR306/307 and digested with the endonuclease AscI restriction site instead of the BamHI restriction site, which was flanked by the restriction-modification (RM) system of B. licheniformis MW3 AprAs−. The genotytype of B. licheniformis MW3 AprAs− was also confirmed by sequencing using primer pair HLHR306/307 and the primers HLHR304/305/306/307.

The complementation vector pV2::aprAs was constructed by amplification of aprAs from B. licheniformis chromosomal DNA using primers HLHR320/321 and cloning of the PCR product into the Bacillus shuttle vector pV2 using Ascl and MdiI restriction sites.

For construction of the B. licheniformis plasmid harbouring strains, pV2::aprAs or the empty pV2 were transferred via conjugation using Escherichia coli S17-149. The generated strains were controlled via plasmid re-isolation and restriction analysis.

Cells were collected at different time points and disrupted with a Mikrodismembrator U (B. Braun Biotech International GmbH, Melsungen, Germany). Total RNA was prepared using the RNaseasy Mini Kit (Qiagen, Hilden, Germany). The RNA quality was analysed using the Agilent RNA 6000 Nano ladder (Agilent Technologies, Waldbronn). Digoxigenin-labelled RNA probes were prepared by in vitro transcription with T7 RNA polymerase using the DIG Northern Starter Kit (Roche, Basel, Switzerland) and templates for in vitro transcription were generated by PCR using the primer pair HLHR306/307. Total RNA was diluted in 10x SSC to a concentration of 0.5 μg/100 μl and blotted on to a positively charged Nylon membrane (Roche, Basel, Switzerland) using the Bio-Dot SF microfiltration unit (BIO-RAD Laboratories GmbH, Munich, Germany). Subsequently, the RNA was covalently bound by exposing the membrane to UV-light (302 nm) for 120 sec on a UV-light table (Image Quant 100, GE Healthcare, Little Chalfont, UK). RNA probe hybridization was performed using the DIG Northern Starter Kit (Roche, Basel, Switzerland) following the manufacturer’s instructions and detection was accomplished via the ChemoCam Imager (Image Quant 100, GE Healthcare, Little Chalfont, UK).

Real-time PCR. Reverse transcription of 100 ng total RNA from each sample was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Darmstadt, Germany). For quantification of the apr cDNA the Real-time PCR Thermal Cycler iQ5 (Biorad, Munich, Germany) was used in combination with the QuantiTect SYBR® Green PCR Kit (Qiagen, Hilden, Germany) and the apr specific primer pair apr fwd/apr rev. For absolute quantification of apr transcripts a standard curve was calculated using vector pV2::aprAs with concentrations of 10⁸ to 10⁶ copies/μl.

Protease assay. For exoprotease activity determination cells were cultivated 12–48 h in M9 skim milk medium and pelleted for 2 min at 16,200 x g at 4 °C. The supernatants containing the exoprotease were analysed with Sigma’s non-specific protease assay. The protease activity for each sample was determined in triplicates using 2 mL Eppendorf tubes. Each sample contained one blank as reference.

| Name     | Sequence                  |
|----------|---------------------------|
| HLRH300  | AAGGATCCATTGGCGGAGACGAGCTCAAGCG  |
| HLRH301  | CTGAAATCAGGCGGCGCCACAATTCGAGATAAGAAACGGCTTTGATCTTG  |
| HLRH302  | GCCATTTGTTTGCCTGTATGTG  |
| HLRH303  | TTTCCCTGAGCAGTGAAGGCGGCAGCAGCCTTTGAGCAGCTTCGACATTG  |
| HLRH304  | GGGCGGTCGGGCGGACAGGAGCGAGCGAGCAGCGGACGTCCTGAGCCTTTGAGCAGCTTCGACATTG  |
| HLRH305  | GCCATTTGT  |
| HLRH306  | GCCATTTGT  |
| HLRH307  | TCTTTCCCTGAGCAGTGAAGGCGGCAGCAGCCTTTGAGCAGCTTCGACATTG  |
| HLRH308  | TTTCCCTGAGCAGTGAAGGCGGCAGCAGCCTTTGAGCAGCTTCGACATTG  |
| HLRH309  | CTAATACGACTCACTATAGGGAGA  |
| apr fwd  | CACCAACGCTTTCTTTCTATATCATACATTG  |
| apr rev  | ACGGCGACTTTATTGGAGAAGC  |
| HLRH320  | CGGGCGGTCGGGCGGACAGGAGCGAGCGAGCAGCGGACGTCCTGAGCCTTTGAGCAGCTTCGACATTG  |
| HLRH321  | TGCCCCACGGGGTGAGATTGTGCGTCGTCGGGATACACC  |

Table 2. Primers. Primer extensions are presented in italics and restriction sites in bold letters. The T7 RNA polymerase promoter is underlined and presented in italics.

Mutant construction. For construction of B. licheniformis MW3 AprAs−, the markerless deletion protocol of Rachinger et al.47 was applied to the strain B. licheniformis MW348, a restriction-modification (RM) system-negative derivative of B. licheniformis DSM1323, 24. The deletion cassette consisted of flank A (primer pair HLHR300/301) and flank B (primer pair HLHR302/303), which were amplified from B. licheniformis chromosomal DNA. The used primers included the Ascl restriction site instead of the −10 box of the putative AprAs promoter. The flanks were fused via SOE-PCR and cloned into the temperature-sensitive vector pKVM2 using BamHI and Ncol restriction sites (BamHI, Ncol, FastAP and T4-DNA-ligase (Thermo Scientific, Darmstadt, Germany)). Escherichia coli S17-149 was used for conjugal transfer of the resulting pKVM2 AprAs− deletion vector. The introduced deletion was confirmed by restriction analysis. For this purpose, the region of interest was amplified via PCR using primer HLHR306/307 and digested with the endonuclease Ascl. The introduced Ascl restriction site in the mutant strains results in the degradation of the 2.6 kbp PCR product to 1.3 kbp. The genotype of B. licheniformis MW3 AprAs− was also confirmed by sequencing using primer pair HLHR306/307 and the primers HLHR304/305/306/307.

The complementation vector pV2::aprAs was constructed by amplification of aprAs from B. licheniformis chromosomal DNA using primers HLHR320/321 and cloning of the PCR product into the Bacillus shuttle vector pV2 using Ascl and MdiI restriction sites.

For construction of the B. licheniformis plasmid harbouring strains, pV2::aprAs or the empty pV2 were transferred via conjugation using Escherichia coli S17-149. The generated strains were controlled via plasmid re-isolation and restriction analysis.

Slot Blot analysis. Cells were collected at different time points and disrupted with a Mikrodismembrator U (B. Braun Biotech International GmbH, Melsungen, Germany). Total RNA was prepared using the RNaseasy Mini Kit (Qiagen, Hilden, Germany). The RNA quality was analysed using the Agilent 2100 Bioanalyser and the Agilent RNA 6000 Nano ladder (Agilent Technologies, Waldbronn). Digoxigenin-labelled RNA probes were prepared by in vitro transcription with T7 RNA polymerase using the DIG Northern Starter Kit (Roche, Basel, Switzerland) and templates for in vitro transcription were generated by PCR using the primer pair HLHR306/307. Total RNA was diluted in 10x SSC to a concentration of 0.5 μg/100 μl and blotted on to a positively charged Nylon membrane (Roche, Basel, Switzerland) using the Bio-Dot SF microfiltration unit (BIO-RAD Laboratories GmbH, Munich, Germany). Subsequently, the RNA was covalently bound by exposing the membrane to UV-light (302 nm) for 120 sec on a UV-light table (Image Quant 100, GE Healthcare, Little Chalfont, UK). RNA probe hybridization was performed using the DIG Northern Starter Kit (Roche, Basel, Switzerland) following the manufacturer’s instructions and detection was accomplished via the ChemoCam Imager (Image Quant 100, GE Healthcare, Little Chalfont, UK).

Real-time PCR. Reverse transcription of 100 ng total RNA from each sample was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Darmstadt, Germany). For quantification of the apr cDNA the Real-time PCR Thermal Cycler iQ5 (Biorad, Munich, Germany) was used in combination with the QuantiTect SYBR® Green PCR Kit (Qiagen, Hilden, Germany) and the apr specific primer pair apr fwd/apr rev. For absolute quantification of apr transcripts a standard curve was calculated using vector pV2::aprAs with concentrations of 10⁸ to 10⁶ copies/μl.

Protease assay. For exoprotease activity determination cells were cultivated 12–48 h in M9 skim milk medium and pelleted for 2 min at 16,200 x g at 4 °C. The supernatants containing the exoprotease were analysed with Sigma’s non-specific protease assay. The protease activity for each sample was determined in triplicates using 2 mL Eppendorf tubes. Each sample contained one blank as reference.
Preparation of extracellular protein extracts for 2D-PAGE.  
* B. licheniformis* cells were grown in M9 skim milk medium for 48 h and supernatants were harvested by centrifugation at 360 mL cell culture at 10,000 rpm and 4 °C for 10 min. Extracellular proteins were prepared according to Voigt et al. Briefly, TCA was added to the supernatant to a final concentration of 10% of the initial culture volume and proteins were precipitated at 7 °C overnight. The proteins were collected by centrifugation at 10,000 rpm and 4 °C for 10 min and washed 8 times with 96% ethanol, and two times with 70% ethanol. After drying, the pellets were dissolved in a solution containing 8 M urea and 2 M thiourea, and centrifuged at 15,000 rpm and 4 °C for 30 min. The protein concentration of the samples was determined with RotiNanoquant (Carl Roth, Karlsruhe, Germany).

2D-PAGE, gel image analysis and protein identification.  
Commercially available IPG strips (18 cm long, Serva, Heidelberg, Germany) in the pH range 3–10 were used for isoelectric focusing (IEF). 100 µg protein was adjusted to 306 µL with a solution containing 2 M thiourea and 8 M urea. CHAPS solution (20 mM DTT, 1% w/v CHAPS, 0.5% w/v Phamlyte, pH 3–10) was added (34 µL for each sample). IEF strips were rehydrated with this solution over night. IEF was performed with the following program: step 1, 150 V for 150 Vh, step 2, 300 V for 300 Vh, step 3, 600 V for 600 Vh, step 4, 1500 V for 1500 Vh, step 5, 3000 V for 37.5 kVh. Following IEF, strips were equilibrated as described by Görg et al. Flat top gels (2D HPE) Large Gel NF-12.5%; Serva, Heidelberg, Germany) were used for protein separation in the second dimension. Gels were stained with Lava Purple (Fluorotechnics, Sydney, Australia) according to the instructions of the manufacturer. Gel images were analyzed with the Delta2D software (Decodon, Greifswald, Germany). Spot quantification was done according to Wolf et al. Briefly, gel images (three replicates) from the wild type samples were overlaid with the gels from the mutant samples. Fusion gels created with the image fusion function of the Delta2D software set to union fussion were used for spot detection. Spots were edited and transferred to the single gels. For spot quantification the % volume of each spot was calculated representing the relative portion of an individual spot of the total protein present on the gel.

Proteins were excised from the gels using the Etta Spot Picker (GE Healthcare, Little Chalfont, UK). Digestion and spotting onto MALDI targets were performed in the Etta Spot Handling Workstation (GE Healthcare, Little Chalfont, UK). MALDI-TOF-MS/MS with the Proteome Analyzer 4800 (Applied Biosystems, Darmstadt, Germany) was performed as described by Wolf et al. Peak lists were searched with the MASCOT search engine version 2.1.0.4 (Matrix Science, London, UK) and search parameters as in Wolf et al. against a *B. subtilis* data base.

Sequence analysis.  
For identification of the putative aprAs promoter region in 17 *B. licheniformis* strains and *B. subtilis* 168, the intergenic region between yhfN and apr from *B. licheniformis* DSM13 was used to perform a blastn search. Correspondingly, the intergenic region between yhfN and apr from *B. subtilis* 168 was used as query for a blastn search on 35 *B. subtilis* genomes. The identified regions were extracted from the respective genomes and used for a comparative sequence alignment performed with MUSCLE using default parameters. Transcriptional terminators were predicted with the program TransTermHF. Secondary structure predictions were performed using “The Vienna RNA Websuite”.

References
1. Weigmann, H. Über zwei an der Käsereifung beteiligte Bakterien. *Zentralbl.Bakteriol Hyg II* 4, 820–834 (1898).
2. Rooney, A. P., Price, N. P. J., Ehrhardt, C., Swezey, J. L. & Bannan, J. D. Phylogeny and molecular taxonomy of the *Bacillus subtilis* species. *Appl. Environ. Microbiol.* 73, 589–596 (2007).
3. Logun, N. A. & De Vos, P. Systematic Bacteriology. doi:10.1007/978-0-387-68489-5 (Springer New York, 2009).
4. Sonenshein, A. L. Control of key metabolic intersections in *Bacillus subtilis*. *Nat. Rev. Microbiol.* 5, 917–927 (2007).
5. Jürgen, B. et al. Global expression profiling of *Bacillus subtilis* cells during industrial-close fed-batch fermentations with different nitrogen sources. *Bacillus. Bioeng.* 92, 277–298 (2005).
6. Oztürk, S., Çalış, P. & Ozdamar, T. H. Fed-Batch Biomolecule Production by Bacillus subtilis: A State of the Art Review. *Trends Biotechnol.* 34, 329–345 (2016).
7. Schallmein, M., Singh, A. & Ward, O. P. Developments in the use of Bacillus species for industrial production. *Can. J. Microbiol.* 50, 1–17 (2004).
8. Gao, Z. Purification and characterization of a novel lichenase from *Bacillus licheniformis* GZ-2. *Biotechnol. Appl. Biochem.* 63, 249–256 (2016).
9. Anbu, P. & Har, B. K. Isolation of an organic solvent-tolerant bacterium *Bacillus licheniformis* PAL05 that is able to secrete solvent-stable lipase. *Biotecnol. Appl. Biochem.* 61, 528–534 (2014).
10. Khairnar, R. S., Mahadele, M. P. & Pathak, A. P. Nanoactivator mediated modifications in thermostable amylase from *Bacillus licheniformis*. *Indian J. Biochem. Biophys.* 49, 468–71 (2012).
11. Fasimoye, F. O., Olajuyigbe, F. M. & Sanni, M. D. Purification and characterization of a thermostable extracellular phytase from *Bacillus licheniformis* PFBL-03. *J. Biogeogr. Appl. Microbiol. Biotechnol.* 44, 193–205 (2014).
12. Lehman, H. U. et al. Degradation of complex carbohydrate: Immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. *Food Chem.* 139, 1081–1086 (2013).
13. Anbu, P. & Har, B. K. Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in Pichia pastoris and its characterization. *Bioresour. Technol.* 134, 81–86 (2013).
14. Liu, B. et al. Expression and characterization of extreme alkaline, oxidation-resistant keratinase from *Bacillus licheniformis* in recombinant *Bacillus subtilis* WB600 expression system and its application in wool fiber processing. *World J. Microbiol. Biotechnol.* 29, 825–832 (2013).
15. Sarker, P., Talukdar, S., Deb, P., Sayem, S. & Mohsina, K. Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003. *Springerplus* 2, 506 (2013).
18. Tang, X.-M. et al. Purification and characterisation of an alkaline protease used in tannery industry from Bacillus licheniformis. Biotechnol. Lett. 26, 1421–1424 (2004).
19. Rachadech, W., Navacharoen, A., Ruangwit, W., Pongtharangkul, T. & Vangnai, A.S. An organic solvent-, detergent-, and thermostable alkaline protease from the mesophilic, organic solvent-tolerant Bacillus licheniformis 3C5. Microbiology 79, 620–629 (2010).
20. Borgi, M. A. et al. The attractive recombinant phytase from Bacillus licheniformis: biochemical and molecular characterization. Appl. Microbiol. Biotechnol. 98, 5937–5947 (2014).
21. Rao, M. B., Tankale, A. M., Ghatge, M. S. & Deshpande, V. V. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62, 897–635 (1998).
22. Sellami-Kamoun, A. et al. Stability of thermostable alkaline protease from Bacillus licheniformis RP1 in commercial solid laundry detergent formulations. Microbiol. Res. 163, 299–306 (2008).
23. Reym. W. et al. Complete genome sequence of the industrial bacterium Bacillus licheniformis and comparisons with closely related Bacillus species. Genome Biol. 5, R77 (2004).
24. Veith, B. et al. The complete genome sequence of Bacillus licheniformis DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. 7, 204–11 (2004).
25. Wiegand, S. et al. RNA-Seq of Bacillus licheniformis: active regulatory RNA features expressed within a productive fermentation. BMC Genomics 14, 667 (2013).
26. Brantl, S. & Brückner, R. Small regulatory RNAs from low-GC Gram-positive bacteria. RNA Biol. 11, 443–56 (2014).
27. Mars, R. A. T., Nicolas, P., Denham, E. L. & van Dijl, J. M. Regulatory RNAs in Bacillus subtilis: a Gram-Positive Perspective on Bacterial RNA-Mediated Regulation of Gene Expression. Microbiol. Mol. Biol. Rev. 80, 1029–1057 (2016).
28. Gottesman, S. & Storz, G. Bacterial Small RNA Regulators: Versatile Roles and Rapidly Evolving Variations. Cold Spring Harb. Perspect. Biol. 3, a003798–a003798 (2011).
29. Rasmussen, S., Nielsen, H. B. & Jarmer, H. The transcriptionally active regions in the genome of Bacillus subtilis. Mol. Microbiol. 73, 1043–1057 (2009).
30. Nicolas, P. et al. Condition-Dependent Transcripome Reveals High-Level Regulatory Architecture in Bacillus subtilis. Science (80-) 335, 1103–1106 (2012).
31. Barbieri, G., Albertini, A. M., Ferrari, E., Sonenshein, A. L. & Belitsky, B. B. Interplay of CodY and ScoC in the Regulation of Major Extracellular Protease Genes of Bacillus subtilis. J. Bacteriol. 198, 907–920 (2016).
32. Sharma, C. M. et al. The primary transcripome of the major human pathogen Helicobacter pylori. Nature 464, 250–5 (2010).
33. Hertel, R., Volland, S. & Liesegang, H. Conjugative reporter system for the use in Bacillus licheniformis and closely related Bacilli. Lett. Appl. Microbiol. 60, 162–17 (2015).
34. Wölf, C. et al. Proteonomic analysis of antioxidative strategies of Staphylococcus aureus: Diverse responses to different oxidants. Proteomics 8, 3139–3153 (2008).
35. Gryczan, T. J., Contente, S. & Dubnau, D. Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. J. Bacteriol. 134, 318–29 (1978).
36. Voigt, B. et al. Cell physiology and protein secretion of Bacillus licheniformis compared to Bacillus subtilis. J. Mol. Microbiol. Biotechnol. 16, 53–68 (2009).
37. Voigt, B. et al. High-resolution proteome maps of Bacillus licheniformis cells growing in minimal medium. Proteomics 15, 2629–2633 (2015).
38. Gupta, R. K. et al. Scale-up of an alkaline protease from Bacillus pumilus MTCC 7514 utilizing fish meal as a sole source of nutrients. J. Microbiol. Biotechnol. 22, 1230–6 (2012).
39. Abe, S., Yasumura, A. & Tanaka, T. Regulation of Bacillus subtilis aprE expression by glnA through inhibition of scoC and sigma(D)-dependent degR expression. J. Bacteriol. 191, 3050–8 (2009).
40. Mader, U. et al. Staphylococcus aureus Transcripome Architecture: From Laboratory to Infection-Mimicking Conditions. PLOS Genet. 12, e1005962 (2016).
41. Thomason, M. K. & Storz, G. Bacterial Antisense RNAs: How Many Are There, and What Are They Doing?*. Annu. Rev. Genet. 44, 167–188 (2010).
42. De moyers, G., Bouchard, M.-P. & Massé, E. New insights into small RNA-dependent translational regulation in prokaryotes. Trends Genet. 29, 92–98 (2013).
43. Jahn, N., Preis, H., Wiedemann, C. & Brantl, S. BsrG/SR4 from Bacillus subtilis—the first temperature-dependent type I toxin-antitoxin system. Mol. Microbiol. 83, 579–98 (2012).
44. Durand, S., Gillet, L. & Condon, C. The essential function of B subtilis RNase III is to silence foreign toxin genes. PLoS Genet. 8, e1003181 (2012).
45. Sambrook, J. & Russell, D. W. Molecular Cloning: A Laboratory Manual, Third Edition (3 volume set) (Cold Spring Harbor Laboratory Press, 2001).
46. Atlas, R. M. Handbook of Microbiological Media, Fourth Edition (CRC Press, 2010).
47. Rachinger, M. et al. Complete genome sequence of the industrial bacterium Bacillus licheniformis and comparisons with closely related Bacillus species. Microbiol. Mol. Biol. Rev. 78, 181–8 (2008).
48. Simon, R., Priefer, U. & Pühler, A. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Bio/Technology 1, 784–791 (1983).
49. Capp, E. & Ford, C. Sigma’s Non-Specific Protease Activity Assay—Casein as a Substrate. J. Vis. Exp., doi:10.3791/899 (2008).
50. Voigt, B. et al. The extracellular proteome of Bacillus licheniformis grown in different media and under different nutrient starvation conditions. Proteomics 6, 268–281 (2006).
51. Gorg, A., Boguth, G., O’bermaier, C., Posch, A. & Weiss, W. Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimensin (IPG-Dalt): The state of the art and the controversy of verticalversus horizontal systems. Electrophoresis 16, 1079–1086 (1995).
52. Edgar, R. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–7 (2004).
53. Kingsford, C. L., Ayanbule, K. & Salzberg, S. L. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. Genome Biol. 8, R22 (2007).
54. Gruber, A. R., Lorenz, R., Bernhart, S. H., Neuböck, R. & Hofacker, I. L. The Vienna RNA website. Nucleic Acids Res 36, W70–4 (2008).
55. Dietrich, S., Wiegand, S. & Liesegang, H. TraV: A Genome Context Sensitive Transcripome Browser. PLoS One 9, e93677 (2014).
Author Contributions
R.H., H.L. and S.V. conceived and designed the research. S.V. supervised the experiments and performed the slot blot analysis. B.V. performed the proteome analysis. R.H. and S.M. designed and performed all not otherwise stated experiments. R.H., H.L. and S.V. wrote the manuscript and all authors analysed data and reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-05628-y

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017