RESEARCH LETTER

Cervimycin C resistance in *Bacillus subtilis* is due to a promoter up-mutation and increased mRNA stability of the constitutive ABC-transporter gene *bmrA*

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Abstract

Two independent cervimycin C (CmC)-resistant clones of *Bacillus subtilis* were identified, each carrying two mutations in the intergenic region preceding the ABC transporter gene *bmrA*. In the double mutant, real-time PCR revealed an increased amount of *bmrA* mRNA with increased stability. Accordingly, isolation of membrane proteins yielded a strong band at 64 kDa corresponding to BmrA. Analyses showed that one mutation optimized the −35 box sequence conferring resistance to 3 μM CmC, while the +6 mutation alone had no effect, but increased the potential of the strain harboring the −35 mutation to grow at 5 μM CmC. Transcriptional fusions revealed an elevated *bmrA* promoter activity for the double mutant. Electrophoretic mobility shift assays (EMSAs) confirmed a 30-fold higher binding affinity of RNA polymerase for this mutant compared with the wild type, and the effect was due to the −35 box alteration of the *bmrA* promoter. *In vitro* transcription experiments substantiated the results of the EMSA. EMSAs in the presence of heparin indicated that the mutations did not influence the formation and/or the stability of open complexes. Half-life measurements demonstrated that the +6 mutation stabilized *bmrA* mRNA ≈ 2-fold. Overall, we found that an ABC transporter confers antibiotic resistance by the cumulative effects of two mutations in the promoter region.

Introduction

Cervimycin C (CmC) belongs to a complex of compounds produced by *Streptomyces tendae* and consists of a tetracyclic polyketide decorated with trideoxysugar chains, solely active against Gram-positive bacteria (Herold *et al.*, 2005).

Generally, microorganisms are able to adapt to antibiotic stress by a variety of specific and unspecific mechanisms (Wright, 2000). A more general mechanism affecting hydrophobic drugs is exerted by exporters lowering intracellular drug concentrations either acting as antiporters or by ATP hydrolysis-driven export (Kerr *et al.*, 2005). Ohki & Tateno (2004) described the increased stability of the multidrug efflux transporter *bmr3* mRNA resulting in a multidrug-resistant phenotype in *Bacillus subtilis*. *Bacillus subtilis* possesses a number of genes belonging to the ABC exporters. One of these is BmrA, which was shown *in vitro* to export ethidium bromide and doxorubicin (Steinfels *et al.*, 2004). However, despite a detailed description of structural and functional features, so far, no biologically relevant substrate or function of BmrA has been identified (Chami *et al.*, 2002; Orelle *et al.*, 2003; Steinfels *et al.*, 2004; Ravaud *et al.*, 2006; Orelle *et al.*, 2008).

For analysis of the genetic changes, whole-genome sequencing can be applied. Andries *et al.* (2005) identified mutations in the *atpE* gene leading to diarylquinone resistance in *Mycobacterium tuberculosis* and *Mycobacterium*

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smegmatis. By whole-genome sequencing, base substitutions suppressing *relA* mutations were identified (Srivatsan *et al.*, 2008). In *B. subtilis*, a point mutation in the *yqiD* gene generated one type of L-form (Leaver *et al.*, 2009). Makarov *et al.* (2009) identified the arabinan pathway as a target for benzothiazinones in *M. tuberculosis*.

Here, we report the molecular basis for a mechanism circumventing the action of the new antibiotic CmC on *B. subtilis*.

Materials and methods

Enzymes

Taq, *Taq* native and *Pvu* DNA polymerases were purchased from Fermentas. DNase I and SuperScript^{TM}III reverse transcriptase were from Ambion and Invitrogene, respectively.

Strains, media and growth conditions

Escherichia coli strains DH5α and TG1 and *B. subtilis* strain 168 were used and grown in Luria–Bertani (LB) medium. According to Steinfels *et al.* (2004), mutant *B. subtilis* 8R was grown in the presence of CmC with or without the addition of 50 μM reserpine.

Preparation of total RNA

Total RNA was prepared as described (Heidrich *et al.*, 2006). RNA used for real-time PCR was treated with 3 μL DNase I (1 U μL^{-1}) in 50 μL in the presence of 0.5 μL RiboLock^{TM} RNase Inhibitor (40 U μL^{-1}) and DNase I buffer with $MgCl_2$ for 30 min at 37 °C, followed by 10 min at 80 °C to inactivate the enzyme. The RNA was further purified using the DNA-free RNA Kit from Zymo Research.

Quantitative real-time PCR (qRT-PCR)

For qRT-PCR, the Applied Biosystems StepOne real-time PCR system and the GeneAmp Fast SYBR Green Master Mix were used. The PCR conditions on the cDNA were optimized in the Applied Biosystems fast cycler 'Verity'. Ratios were calculated using the $\Delta\Delta C_T$ method (Pfaffl, 2002).

Membrane protein extraction

Membrane proteins were prepared using a protocol adapted from Steinfels *et al.* (2002).

Generation of a PCR fragment with the single upstream mutation

Primers pxyvcC-F and yvcC2MF_2 as well as pxyvcC-R1 and primer yvcC2MR_2 were used to generate PCR fragments. After annealing, the resulting chimera sequences were extended and amplified using primers pxyvcC-F and pxyvcC-R1 to give rise to a long fragment of 1289 bp. Similarly, using primers yvcC1 MF_2 and yvcC1MR_2, a PCR fragment containing only the +6 mutation was generated. These fragments were used to transform *B. subtilis* 168 and select for growth in the presence of different CmC concentrations.

Preparation of *B. subtilis* RNA polymerase (RNAP) and *in vitro* transcription experiments

Preparation of *B. subtilis* RNAP and *in vitro* transcription experiments were performed as described previously (Licht *et al.*, 2008). Gels were dried and subjected to Phosphoimaging (Fujix BAS 1000). PC BAS 2.0E software was used for the quantification of the bands.

Results and discussion

Increasing concentrations of cervimycin selected two resistant mutants of *B. subtilis* 168

Bacillus subtilis 168 grown till the late log-phase was inoculated 1 : 100 in 10 mL LB medium without an antibiotic and LB with 0.25 μM CmC [0.5 × minimal inhibitory concentration (MIC)], with 0.5 μM CmC (1 × MIC) and with 1 μM CmC (2 × MIC) and incubated for 24 h at 37 °C and 200 r.p.m., yielding turbid growth only in the 1 μM CmC culture. From this culture, 1/100 aliquots were inoculated into LB medium with either 1, 2 or 5 μM CmC and incubated for another 24 h, yielding a culture growing at 2 μM CmC. Dilution aliquots were plated in parallel on LB agar and LB agar containing 5 μM CmC. From the 5 μM CmC plate, 24 clones were isolated and found to be tryptophane auxotroph, proving by this genetic marker their descent from strain 168. Two independently isolated resistant clones designated 8R and IR revealed no cross-resistance against most antibiotics tested, either structurally related or different (summarized in Supporting Information, Table S2). However, they were more resistant, in particular, against doxorubicin, a hydrophobic polyketide cancer antibiotic (Table S2). Both mutants grew in the presence of 5 μM CmC, and this resistance was unchanged after propagation for > 20 generations in the absence of the drug. In contrast, Ohki & Tatenu (2004) obtained their spontaneous multidrug-resistant mutant on the *bmr3* gene in a one-step procedure.

Characterization of the mutants

The genomes of the resistant clones 8R and IR as well as the parent strain *B. subtilis* 168 were sequenced to near completion and compared with the reference GenBank: AL009126.3 database (Srivatsan *et al.*, 2008; Barbe *et al.*, 2009). Two of the mutations could be confirmed by PCR amplification and

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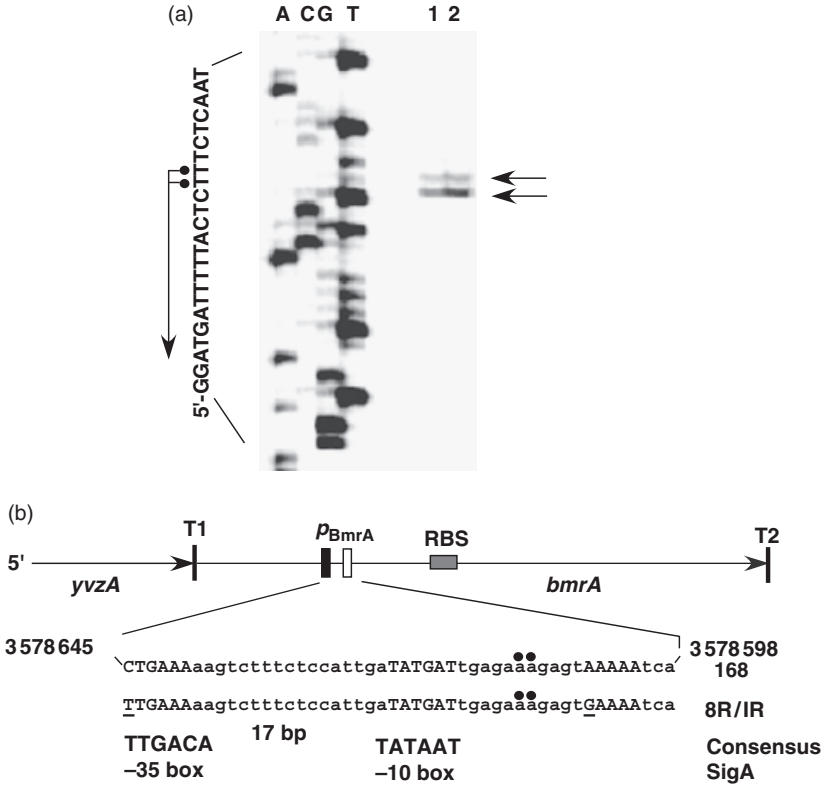

Fig. 1. Mapping of the 5′ end of *bmrA*-mRNA and location of the *bmrA* gene. Reverse transcription with specific IRD800-labelled primers (primer extension) was performed using SuperScript^{TM}III (200 U μL^{-1}) according to the manufacturer's instructions (Invitrogen). (a) On the left four lanes, a sequencing reaction with a PCR fragment and primer c-DNA-1 is shown. 1 and 2, Primer extension reaction on total RNA from *Bacillus subtilis* 168 and 8R, respectively, obtained from exponentially grown cultures. The arrows on the right side indicate the transcriptional start sites. On the left side, part of the sequence is shown. Dots and arrow show the mapped transcriptional start site and the direction of transcription. (b) Schematic representation of the location of the *bmrA* gene on the *B. subtilis* chromosome. The direction of transcription is indicated by arrows. *yvzA*, gene localized upstream of *bmrA*. T1, transcription terminator of *yvzA*; RBS, ribosome-binding site of *bmrA*; T2, transcription terminator of *bmrA*. Black and white rectangles indicate the − 35 and − 10 boxes of the *bmrA* promoter. Below: sequence of the 5′ noncoding region of the *bmrA* gene in the wild-type and double mutant strains. The sigma A consensus sequence is shown below. Nucleotides altered in mutant 8R are underlined. The dots show the mapped transcriptional start site. Small numbers indicate the coordinates on the *B. subtilis* chromosome (−) strand.

sequencing. Sequence comparison localized these two mutations 40 bp apart in the 5′ noncoding region of the *yvcC* = *bmrA* gene (Fig. 1). In *S. tendae*, the producer of the cervimycin complex, self-resistance is facilitated by a member of the MFS superfamily, possibly extruding cervimycin (M. Unger & C. Hertweck, unpublished data). BmrA (Steinfels *et al.*, 2002; Orelle *et al.*, 2003) belongs to the largest gene class of ATP-dependent ABC exporters in *B. subtilis* and was found to be expressed constitutively (Steinfels *et al.*, 2004). Previously, the *in vitro* transport of Hoechst 33342, doxorubicin, 7-aminoactinomycin and EtBr by BmrA was shown using membrane vesicles, whereas reserpine inhibited the EtBr-efflux (Steinfels *et al.*, 2004). The *bmrA* knockout mutant (Steinfels *et al.*, 2002) had a twofold reduced resistance to CmC compared with *B. subtilis* 168. This supports the conclusion that this gene is responsible for CmC resistance, but also indicates the involvement of more factors contributing to the basal resistance.

Confirmation of a link between genotype and phenotype

A PCR fragment of *bmrA* obtained from mutant 8R genomic DNA with primers px yvcC-F/-R1 transformed *B. subtilis* 168 to 5 $\mu g\,mL^{-1}$ CmC resistance. In accordance with published data (Steinfels *et al.*, 2004), we found additionally that in the presence of 50 $mg\,L^{-1}$ reserpine, the mutant was unable to grow in the presence of CmC. From these data, we could conclude that the ABC transporter BmrA is responsible for the CmC resistance of the mutants. So far, reports on spontaneous constitutively resistant mutants in Gram-positive bacteria revealing overexpression due to promoter mutations are rare (Piddock, 2006). One case was reported after growing *B. subtilis* 168 in the presence of high concentrations of puromycin and lincomycin where a transcription repressor (LmrA) was involved (Mwangi *et al.*, 2007). Ohki & Tateno (2004) described the increased expression of the *bmr3* efflux

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transporter due to a double mutation at positions −18 and +4 from the transcription start site.

Reporter gene fusions and primer extension identified the transcriptional start site of *bmrA*

Transcriptional *lacZ* reporter gene fusions with a region upstream of the *bmrA* SD sequence were constructed and integrated by double crossing over into the *amyE* locus of the *B. subtilis* 168 chromosome. Measurements of β-galactosidase activity determined the putative promoter region (Fig. 2). Subsequently, primer extension was used to identify the transcription start downstream of a potential promoter (Fig. 2a). The wild-type promoter shows a nearly perfect −10 box with TATGAT, a 17-bp spacer, but a weak −35 box with CTGAAA. In mutant 8R, the C of the −35 box was altered to T, making it more similar to the consensus σ^A −35 box TTGACA (Fig. 2b). The second point mutation was located six bases downstream from the transcription start site (+6) altering an A_5 stretch to GAAAA (Fig. 1b).

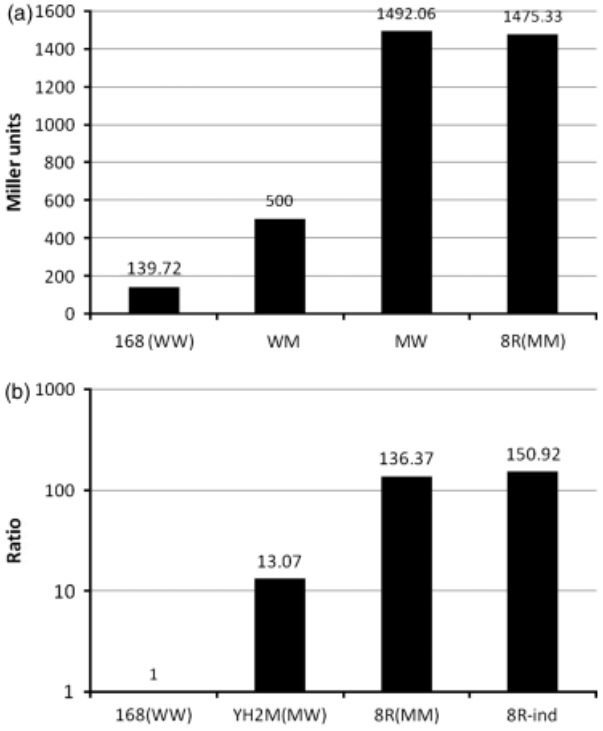

Fig. 2. Promoter activity and amounts of *bmrA* mRNA. To construct plasmids pACWW157 and pACMM157 containing the *bmrA-lacZ* transcriptional fusions, a PCR was performed on chromosomal DNA of *Bacillus subtilis* strain 168 (WW) or double mutant *B. subtilis* 8R (MM). The single-mutation fragments were obtained by a two-step PCR. First, two PCR reactions with primer pairs pyvccE3/pyvcc2MR and pyvccG3/pyvcc2MF were performed on chromosomal DNA of *B. subtilis* 168, PCR fragments were isolated and subjected to a joint PCR with outer primers pyvccE3 and pyvccG3 and inserted into pAC6 vector (Stülke *et al.*, 1997). All pAC6 derivatives were integrated into the *amyE* locus of the *B. subtilis* 168 chromosome. β-Galactosidase activities of the resulting strains were determined as described (Licht *et al.*, 2005). (a) pAC6 derivatives pACWW157, pACMM157, pACMW157 and pAWM157 integrated into the chromosomal *amyE* locus include a fragment of 157 bp from position −90 till +67 relative to the *bmrA* transcription start. The resulting *B. subtilis* 168 strains were grown in LB media till $OD_{595\,nm} = 2.0$, samples were withdrawn and β-galactosidase activities were measured. The averages of three independent measurements are shown. Column diagrams show the amount of Miller units measured in the wild-type/wild-type (WW), wild-type/mutant (WM), mutant/wild-type (MW) and double mutant (MM) strains, corresponding to −35 and +6 mutations, respectively. (b) Amounts of *bmrA* RNA in *B. subtilis* strains 168, 8R-ind and MW determined by qRT-PCR. Averages of up to three biological repeats and several technical duplicates, normalized to *gyrB*-mRNA.

LacZ-reporter gene assays allowed dissecting the impact of each single mutation on altered *bmrA* expression

To dissect the contribution of each single mutation on the elevated expression of *bmrA*, plasmids carrying transcriptional *bmrA–lacZ* fusions with fragments of different sizes were constructed designated pACMM (double mutant), pACWW (wild type), pACMW (−35 mutation) and pAWM (+6 mutation) (Fig. 1a). All pAC6 derivatives were integrated into the *amyE* locus and the β-galactosidase activities measured (see Fig. 1a). Increased β-galactosidase activities compared with the wild type were found in the double mutant and in the single mutant affecting the −35 box, whereas only marginally different β-galactosidase activities were measured for the +6 mutation (3.5-fold increased). The 157-bp upstream region increased β-galactosidase 10–11-fold in both the double and the MW mutant compared with the wild type.

Real-time PCR revealed significantly increased amounts of *bmrA* mRNA in the mutant 8R

To investigate the impact of the mutations on *bmrA* expression, total RNA from wild-type strain 168 and mutant 8R was isolated, DNase treated and assayed using real-time PCR. The amount of *bmrA* mRNA in mutant 8R with the −35 and +6 mutations was 135-fold increased, whereas in strain YH2M with the −35 mutation alone, the amount of *bmrA* mRNA was about 13-fold increased (Fig. 2(b). 8R-ind). Real-time PCR on total RNA isolated from *B. subtilis* 8R propagated in the presence of CmC (0.5 μM) corroborated the results of the Jault laboratory on the constitutive expression of *bmrA* (Steinfels *et al.*, 2004).

Electrophoretic mobility shift assay (EMSA) demonstrated an increased affinity of the RNAP to the mutant promoters

To analyze the binding of the RNAP to the *bmrA* promoter region, EMSAs were performed. The four 157-bp fragments

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used for the *lacZ*-reporter gene fusions were radioactively labelled and incubated with increasing concentrations of *B. subtilis* RNAP. As shown in Fig. 3a and b, the −35/+6 mutant MM and the single −35 mutant MW displayed a 30-fold increased affinity for RNAP. Interestingly, the single mutant WM carrying only the +6 mutation behaved like the wild type. The addition of heparin (Fig. 3c) did not decrease the amount of the formed complex, thus proving the lack of influence of any of the mutations on the formation and/or the stability of the open complex. From these results, we can conclude that the effect of mutant 8R on transcription is exclusively due to the alteration in the −35 box, whereas the

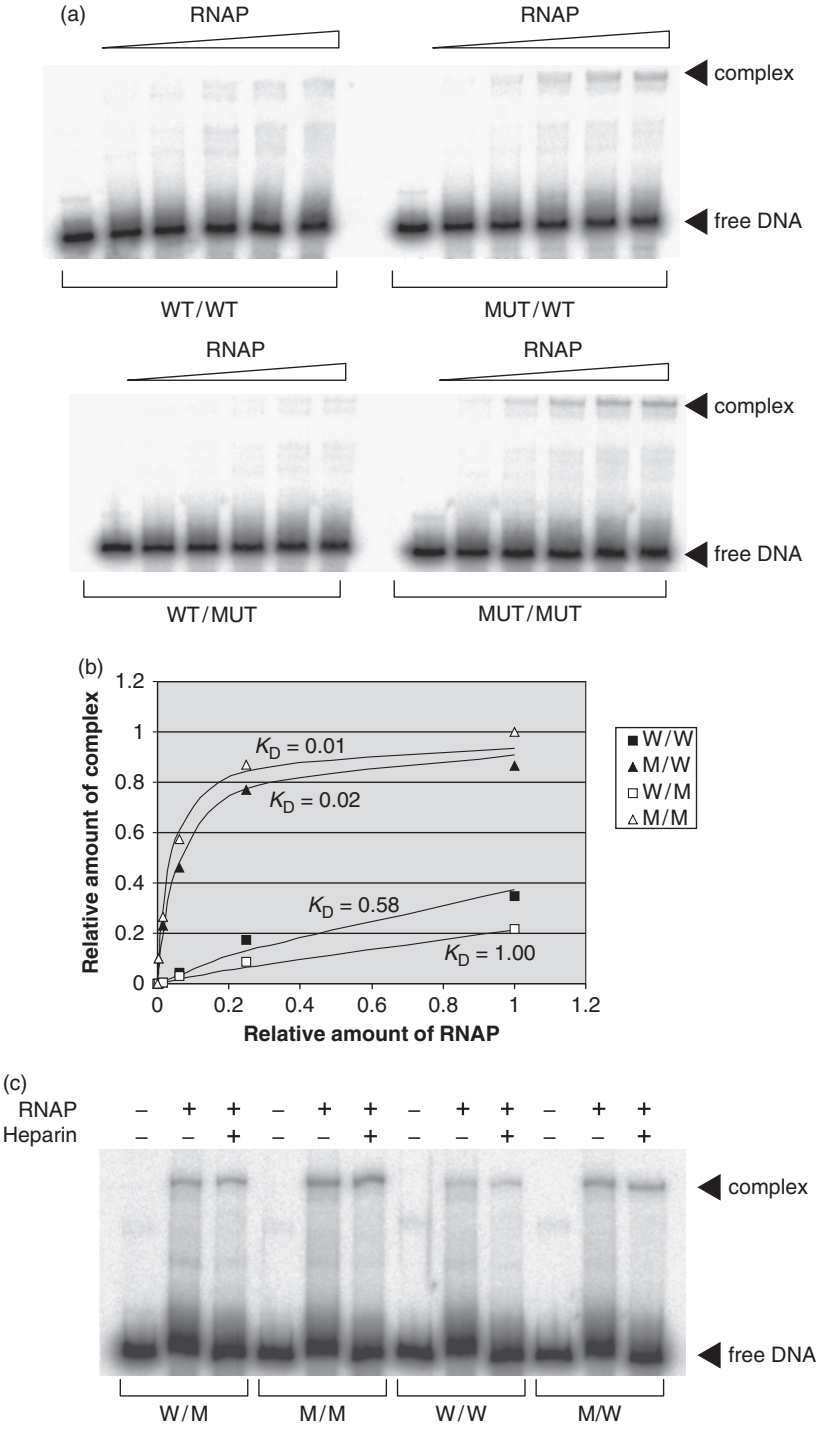

Fig. 3. RNA polymerase binding to the promoter region. (a) EMSAs using increasing amounts of *Bacillus subtilis* RNAP and 157-bp DNA fragments carrying the wild-type *bmrA* promoter or different mutations. Binding reactions were performed in a final volume of 10 μL containing *in vitro* transcription buffer (see Fig. 4), 0.05 g L^{-1} herring sperm DNA as a nonspecific competitor and 1 nM of end-labelled DNA fragment. RNAP dilutions were made in storage buffer and the same volume of diluted protein was used in each sample. After incubation at 37 °C for 15 min, the reaction mixtures were separated on 8% native polyacrylamide gels run at room temperature for 1 h at 200 V. Bands corresponding to free DNA and RNAP-DNA complex are indicated. (b) Binding curves deduced from the EMSAs in (a). Apparent relative K_D values designated next to the corresponding binding curve were calculated for each RNAP–DNA complex as follows: visualization and quantification of the bands were from dried gels performed using a FUJI-PHOSPHORIMAGER and the PCBAS 2.09E quantification software (Raytest). The image data generated by scanning the gel are linear proportionally to the radiation intensity of the sample. The amount of RNAP–DNA complex relative to the RNAP concentration was fitted with the nonlinear regression program SOLVER (included in Microsoft® EXCEL) to the following equation:

$$[C] = \frac{[D] \cdot [P]}{K_D + [P]}$$

where [*C*], [*D*] and [*P*] represent the total concentrations of formed complex, DNA and protein, respectively, and K_D is the apparent equilibrium dissociation constant. (c) Effect of the mutations on open complex formation. Heparin at a 0.1 g L^{-1} final concentration was added where indicated.

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downstream mutation does not contribute to the ability of the RNAP to bind the *bmrA* promoter. Most probably, the upstream mutation improves the initial binding of the RNAP.

In vitro transcription experiments confirmed an effect of the −35 mutation on the transcription efficiency

In vitro transcription experiments were carried out using *B. subtilis* RNAP and wild type and the three mutated template DNAs covering the *bmrA* promoter and a region downstream from the transcription start site. Figure 4 shows the formation of a visible band only in lanes 2 (MW) and 4 (MM), which is in accordance with the data obtained by real-time PCR on the amount of mRNA in the wild type and double mutant strain as well as the results of the *lacZ* reporter gene assays. Furthermore, the *in vitro* transcription data substantiate the results of the EMSA.

BmrA can be visualized as a membrane protein in the double mutant

To confirm that the increased levels of *bmrA* mRNA correspond to an increase in the corresponding protein level, membrane protein fractions were prepared from wild type and double mutant 8R and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. As shown in Fig. 5, a new band of ≈ 64 kDa is visible in the mutant fraction that is hardly detectable in the wild-type extract from *B. subtilis* 168. Elution of the band, its digestion with trypsin and subsequent analysis confirmed that this band consists of BmrA.

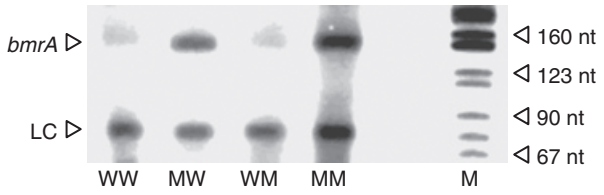

Fig. 4. *In vitro* transcription experiments with *Bacillus subtilis* RNAP. Multiround *in vitro* transcription assay using templates carrying different *bmrA* promoter sequences and *B. subtilis* RNAP. Reactions were performed in a final volume of 10 μL *in vitro* transcription buffer (40 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM EDTA, 50 mM KCl, 0.05 g L^{-1} bovine serum albumin, 2.5% glycerol, 2 mM dithiothreitol) in the presence of 1 mM ATP, 0.1 mM CTP and GTP, 0.01 mM UTP and 0.011 μM [α-^{32}P]UTP. Double-stranded DNA template (100 nM) was added, followed by 100 nM of RNAP. The reaction was gently mixed and incubated for 30 min at 37 °C. One volume of formamide loading dye was added, followed by denaturation for 5 min at 90 °C, quick cooling on ice and analysis on a 6% denaturing polyacrylamide gel. Electrophoresis was performed at 300 V/25 mA for 50 min. The following templates were used: WW, wild type; MW, single mutant in −35 region; WM, single downstream mutant; MM, double mutant as in strain 8R. The *bmrA* transcript is indicated by an arrow.

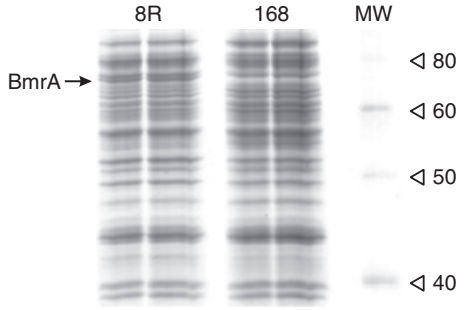

Fig. 5. Protein identification. *Bacillus subtilis* 168 and *B. subtilis* mutant 8R strains were grown in LB medium, sonicated in ice, and membrane vesicles were collected by centrifugation at 100 000 ***g*** for 60 min, washed and suspended in 20 mM Tris-HCl pH 8.0, 300 mM sucrose, 1 mM EDTA and lysed in 1% *n*-dodecyl β-D-maltoside. Separation was in a 12% polyacrylamide-gel using MagicMark Protein standard (Invitrogen). Size markers are indicated in kDa; BmrA indicates the position of the additional band in the mutant strain identified by LCQ Classic Ion-trap-ESI.

The −35 and the +6 mutations cooperate to yield 5 μM CmC resistance

A mutant strain of *B. subtilis* 168 containing only the single mutation in the −35 box of the *bmrA* promoter designated *B. subtilis* YH2M grew only in the presence of 3 μM CmC, but not at 4 μM CmC, in contrast to the fragment containing both mutations that transformed *B. subtilis* to resistance against 5 μM CmC. A fragment comprising just the +6 mutation was used to transform *B. subtilis* 168 and *B. subtilis* YH2M. The resulting double transformant containing the −35 and the +6 mutation grew in the presence of 5 μM CmC. Transformation of *B. subtilis* 168 with this fragment did not yield any transformants growing in the presence of >1 μM CmC. *In vitro* studies using EMSA and transcription experiments showed no influence of this +6 mutation on the promoter activity. These data show that the stepwise increase in CmC resistance during mutant selection is due to the cumulative effect of two mutations in the promoter region. Apparently, both mutations cooperate to yield the 5 μM CmC resistance found in the double mutant 8R. All constructs were proven by sequencing PCR fragments obtained from their genomic DNA.

The downstream mutation stabilizes *bmrA* mRNA

Because the results of the *lacZ* reporter gene fusions, EMSAs and *in vitro* transcription indicated that only the upstream mutation in the −35 box affected RNAP binding, and hence, the total amount of *bmrA* mRNA, we can now draw the conclusion that the downstream mutation in the noncoding region of *bmrA* is responsible for the stabilization of *bmrA* mRNA. To substantiate these data, half-life

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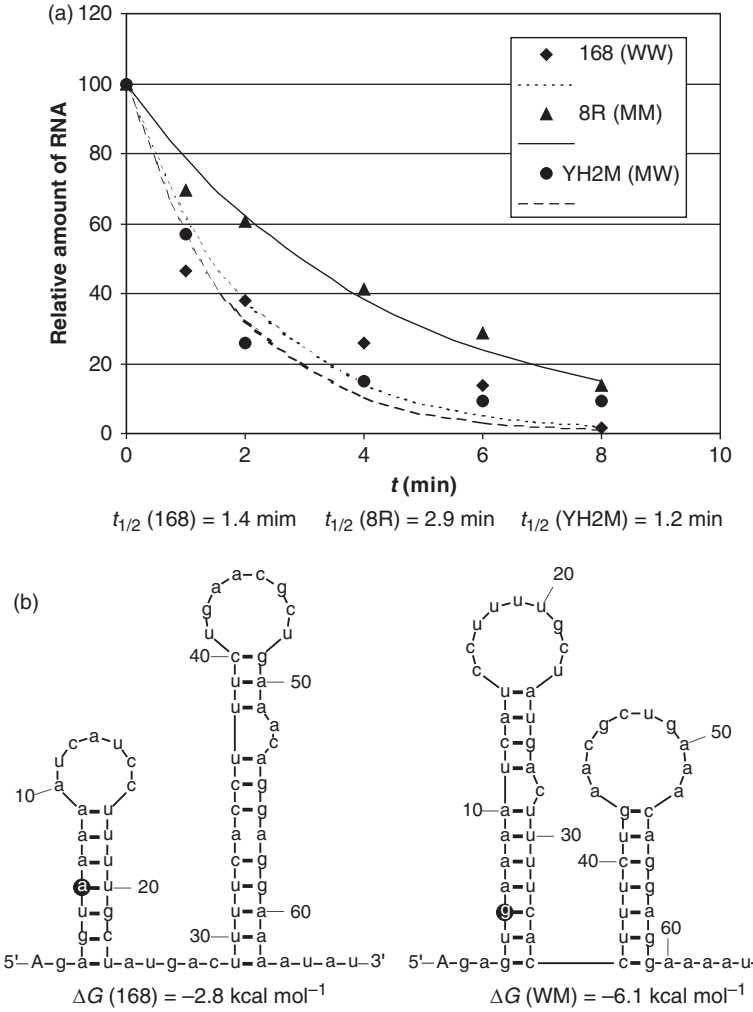

Fig. 6. The +6 mutant stabilizes the 5′ untranslated region (UTR) of *bmrA* mRNA. (a) Half-life measurements. *Bacillus subtilis* 168 wild-type, 8R (double-mutant) and YH2M (− 35 mutation) were grown in LB medium containing 5 μmol CmC till OD 1; 200 μg mL^{-1} rifampicin were added and time samples taken as indicated. Total RNA was prepared as described in Materials and methods. Reverse transcription with random hexamer primers was performed according to the recommendations of the suppliers. Subsequently, the RNA was hydrolyzed with 10 μL 1 M NaOH at 70 °C for 10 min, neutralized with 25 μL 1 M HEPES pH 7.0. Alternatively, RNase H was used to remove the mRNA. PCR was performed according to the recommendations of the manufacturers. Primers located in the mRNA of the *gyrB* gene served as a reference. Two primers in the mRNA of the *bmrA* gene were used as targets (a list of all primers can be found in Table S1). Strains were harvested at time points t_n indicated after addition of rifampicin (t_0), total RNA was isolated and cDNA synthesized, used in qRT-PCR. Using equation $\Delta\Delta C_{Ttn} = (C_{T\ bmrA} - C_{T\ gyr})^{168} - (C_{T\ bmrA} - C_{T\ gyr})^{8R}$, we determined. $2^{-\Delta\Delta C_{Ttn}}$ for each time point (Pfaffl, 2002). These values were transformed to the respective % ratios. (b) Computer prediction of the influence of the +6 mutation on the mRNA secondary structure for the 5′ UTR of *bmrA*. A transition at position +6 leads to a change of the predicted secondary structure and a decrease in free energy Δ*G*, according to http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi. Black indicates the transition from A in *B. subtilis* 168 to G in mutant 8R.

measurements of *bmrA*-mRNA were performed for *B. subtilis* 168, YH2M (MW) and the double mutant 8R. As shown in Fig. 6a, the half-life of 168 and single-mutant MW was ≈ 1.5 min, whereas the half-life of 8R was calculated to be ≈ 3 min. This twofold increase of the half-life of the double-mutant must be due to a contribution of single-mutant WM at position +6, demonstrating that this mutation leads to the stabilization of the *bmrA* mRNA.

Figure 6b shows the mRNA secondary structures predicted for the *bmrA* 5′ untranslated region. The transition at position +6 leads to a change of the predicted structure and a decrease in Gibbs free energy Δ*G*. According to http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi, the first stem–loop is stabilized. This is in accordance with previous observations on the mRNA-stabilizing function of 5′-terminal stem–loops (Hansen *et al.*, 1994; Hambraeus *et al.*, 2000).

Because antibiotic resistance is most often only transiently advantageous to bacteria, an efficient way to escape the lethal action of drugs is the regulation of resistance gene expression at the transcriptional or the translational level following mutations or the movement of mobile genetic elements (Depardieu *et al.*, 2007). Piddock (2006) reported that chromosomally encoded efflux pumps may be overexpressed due to mutations in the local repressor, mutations in global regulatory genes, promoter mutations or insertion sequences. In an induction experiment, we confirmed the finding of Steinfels *et al.* (2004) that *bmrA* is not inducible by any specific substrate. Furthermore, using EMSA and a radioactively labelled fragment of the *bmrA* upstream region, no specific binding protein acting as an activator or a repressor could be identified in crude protein extracts of the mutant or the wild-type strain (data not shown).

Instead, we identified a mechanism of adaptation without fine-tuning, resulting in antibiotic resistance by constitutively upregulated expression of a specific protein. Such proteins may encompass ABC transporters, permeases, transcription factors or sigma factors. For instance, Stirrett *et al.* (2008) reported the upregulated expression of several

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efflux pumps in *Y. pestis* by overexpression of the transcriptional regulator RobAYp from a multicopy plasmid.

So far, spontaneous constitutively resistant mutants in Gram-positive bacteria revealing overexpression due to promoter mutations have only been detected in a few cases (Piddock, 2006). For instance, the triclosan efflux pump of *Pseudomonas aeroginosa* was upregulated by a mutation in the −35 region of the promoter (Mima *et al.*, 2007), while in *M. smegmatis* a G to T transversion in the −10 region of the promoter increased the copy number of the D-alanine racemase conferring resistance to D-cycloserine (Cáceres *et al.*, 1997). Similar data were obtained by Ohki & Tateno (2004), who reported the increased expression of the *bmr3* efflux transporter due to a +4 mutation that also resulted in the stabilization of the corresponding *bmr3* mRNA. However, in this case, no mutation that improved RNAP binding was needed for the observed effects.

Our data contribute to these few cases, as we describe the adaptation of gene expression of a constitutively expressed ABC exporter due to the cumulative effect of a promoter upmutation and a mutation stabilizing the corresponding mRNA.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligodeoxyribonucleotides used in this study.
Table S2. Antibiotic resistance profile of *Bacillus subtilis* 8R compared with 168.

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