Mutational activation of the RocR activator and of a cryptic rocDEF promoter bypass loss of the initial steps of proline biosynthesis in Bacillus subtilis

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Summary

The gamma-glutamyl-phosphate reductase (ProA) interlinks both the anabolic and osmostress adaptive proline biosynthetic routes of Bacillus subtilis. Because no paralogous protein to ProA exists in this microorganism, proA mutants should exhibit a tight proline auxotrophic growth phenotype. Contrary to expectations, proA mutants formed microcolonies on agar plates lacking proline and faster growing Pro+ suppressor mutants arose. These mutants carried alterations in the rocR-rocDEF region encoding enzymes of the arginine degradation pathway and its transcriptional activator RocR. They were of two types: (i) mutants carrying single amino acid substitutions in RocR resulting in partial inducer-independent variants and (ii) mutants carrying single base-pair changes in the vicinity of the SigL/Sig-54-dependent −12/−24 class rocDEF promoter that activate a cryptic SigA-type promoter. Consequently, enhanced rocDEF transcription should lead to increased cellular amounts of the RocD ornithine aminotransferase, an enzyme that synthesizes the same reaction product as ProA, gamma-glutamic-semialdehyde/delta-1-pyrroline-5-carboxylate. This compound can be enzymatically converted into proline. The Pro+ suppressors also exhibited a new regulatory pattern by allowing enhanced rocDEF transcription in response to proline availability when ammonium is present. Our work provides an example how flexibly bacteria can genetically develop routes to bypass constraints imposed on their biosynthetic networks and evolve new regulatory mechanisms.

Introduction

Bacteria frequently respond to changes in their environment by changing the activity and/or the amount of their enzymes. The mechanisms of these adaptive cellular responses have been the subject of numerous studies (Copley, 2012). However, in recent years, it has become increasingly clear that adaptation to environmental cues and constraints also occurs at the genomic level (Nam et al., 2011; Shou et al., 2011). Whenever bacteria encounter unfavorable conditions, mutants better adapted to the new conditions will be successful in meeting the environmentally and metabolically imposed restrictions (Commichau et al., 2008; Florez et al., 2011; Gunka and Commichau, 2012). Hence, genome plasticity provides an additional level of adaptation that allows bacteria to rapidly conquer novel habitats (Hoffmann et al., 2013a), broaden their metabolic abilities (Solopova et al., 2012; Summers et al., 2012; Schickberger et al., 2013) or avert cellular stress by repurposing existing pathways (Veeravalli et al., 2011). This rapid genomic adaptation is certainly one of the reasons for the evolutionary success of bacteria (Blount et al., 2012).

We are interested in proline synthesis in Bacillus subtilis (Belitsky et al., 2001; Brill et al., 2011a) and use of this amino acid as an osmostress-protectant (Whatmore et al., 1990; von Blohn et al., 1997; Brill et al., 2011b; Hoffmann et al., 2012; 2013b; Zaprasis et al., 2013) and nutrient (Belitsky, 2011; Moses et al., 2012). Proline biosynthesis in B. subtilis, as in many other microbial species (Csonka and Leisinger, 2007), proceeds from the precursor glutamate (Gunka and Commichau, 2012) and involves three enzymes: the γ-glutamate kinase, the γ-glutamyl-phosphate reductase and the Δ1-pyrroline-5-carboxylate reductase. Multiple enzymes for the first (ProB, ProJ) and last (ProL, ProH, ProG) steps in proline biosynthesis are present in B. subtilis, but it possesses only a single γ-glutamyl-phosphate reductase (ProA) (Fig. 1) (Belitsky et al., 2001). Proline biosynthesis serves two physiological...
functions in *B. subtilis*: (i) to fuel protein synthesis (Brill *et al.*, 2011a) and (ii) to provide cellular protection against high osmolarity (Whatmore *et al.*, 1990; Brill *et al.*, 2011b). The sizes of the intracellular proline pools required to accomplish these two tasks differ greatly (Whatmore *et al.*, 1990; Brill *et al.*, 2011a,b; Hoffmann *et al.*, 2012). Both transcriptional and post-transcriptional control mechanisms, and two interconnected proline biosynthetic routes (Fig. 1) are used by *B. subtilis* to set and fine-tune the pool size of this amino acid in order to meet the cell’s physiological needs under different growth conditions.

Proline production for anabolic purposes is mediated by the ProB-ProA-ProI biosynthetic route (Brill *et al.*, 2011a). When the intracellular proline pool is sufficiently high to support the ongoing protein biosynthetic activities of the cell, *B. subtilis* prevents a wasteful overproduction of proline by limiting the expression of the anabolic *proBA* and *proI* genes through a *cis*-acting and proline-responsive RNA-based regulatory device (Brill *et al.*, 2011a), a member of the T-box regulatory system (Gutierrez-Preciado *et al.*, 2009). It further controls the flow of the precursor glutamate into the ProB-ProA-ProI biosynthetic route through a proline-mediated feedback mechanism (Fujita *et al.*, 2003; Perez-Arellano *et al.*, 2010) that acts on the activity of the *B. subtilis* ProB protein (Chen *et al.*, 2006), the first enzyme of this pathway (Fig. 1). As a result of these regulatory measures, *B. subtilis* cells grown in chemically defined media typically maintain a proline pool between 10 and 20 mM (Whatmore *et al.*, 1990; Hoffmann *et al.*, 2012; Moses *et al.*, 2012; Zaprasis *et al.*, 2013).

Proline is a well-recognized member of osmostress-relieving organic compounds, the compatible solutes (Csonka, 1989; Kempf and Bremer, 1998). *Bacillus subtilis* makes use of it to offset the negative effects of high external salinity (or osmolarity) on cellular water content, turgor and growth (Whatmore *et al.*, 1990; Bremer and Krämer, 2000; Hoffmann *et al.*, 2012; Zaprasis *et al.*, 2013). The amounts of proline synthesized by osmotically challenged *B. subtilis* cells is linked in a finely tuned fashion to the degree of the environmentally imposed osmotic stress (Brill *et al.*, 2011b; Hoffmann *et al.*, 2013b), and the size of the proline pool can reach magnitudes of about 500 mM when the osmotic stress is severe (Whatmore *et al.*, 1990; Hoffmann *et al.*, 2012; 2013b; Zaprasis *et al.*, 2013). To meet the increased cellular demand for proline under these circumstances, *B. subtilis*
has developed a dedicated and osmotically controlled proline production pathway that uses isoenzymes of the first (ProJ) and last (ProH) steps of the anabolic ProB-ProA-Prol proline synthesis route but uses from this latter route the ProA enzyme (Brill et al., 2011b). As a consequence, the anabolic and the osmotic stress adaptive proline biosynthetic pathways are interconnected through the enzymatic activity of the γ-glutamyl-phosphate reductase (ProA) (Fig. 1) (Belitsky et al., 2001; Brill et al., 2011b). Because of the redundancy of the enzymes for the first and last steps of proline synthesis (Fig. 1), a disruption of the proB, proJ, prol, proG and proH genes does not cause a proline auxotrophic growth phenotype of B. subtilis (Belitsky et al., 2001; Brill et al., 2011b).

Such a phenotype is expected, however, for proA mutants (Belitsky et al., 2001). Surprisingly, we found that such mutations did not confer a tight proline auxotrophie. Instead, these strains formed tiny colonies on minimal media agar plates lacking proline and faster growing ProA suppressors were observed in which the need for proline as a building block for protein biosynthesis was satisfied, but the large proline pools required to achieve cellular osmoprotection could not be attained.

Here, we present the genetic analysis of these ProA suppressor strains, and we demonstrate that they harbor mutations leading to increased expression of the rocDEF operon in the absence of its natural inducers. The rocDEF gene cluster is part of the arginine degradation system of B. subtilis (Gardan et al., 1995; Fischer and Debarbouille, 2002; Ali et al., 2003), and its increased expression artificially provides, via the enzymatic reaction of the RocD ornithine aminotransferase, the ProA suppressor strains with increased amounts of γ-glutamyl-semialdehyde/Δ1-pyrroline-5-carboxylate. These are the same metabolites that are also synthesized by the ProA enzyme, and they can be converted into proline by several pyrroline-5-carboxylate reductases operating in B. subtilis (Belitsky et al., 2001) (Fig. 1). As a consequence, the ProA suppressor mutations bypass the first two steps in proline synthesis and synthetically harness part of the B. subtilis arginine catabolic system for proline production.

Results
Isolation of suppressor mutants bypassing a defect in proA

The proA-encoded protein is the only γ-glutamylphosphate-reductase of B. subtilis (Belitsky et al., 2001), and a tight proline auxotrophic growth phenotype is therefore predicted for proBA or proA mutants. It should be noted in this context that the enzyme activity of ProB can be functionally substituted for by the paralogous ProJ enzyme (Fig. 1) (Belitsky et al., 2001; Brill et al., 2011b). Indeed, the disruption of the proBA or proA loci greatly impaired growth of B. subtilis when such strains were cultivated in a minimal medium [Spizizen’s minimal medium (SMM)] lacking proline. This is documented in Fig. 2A for the Δ(proBA::cat)2 mutant strain GWB101; however, some low-level growth of proBA and proA mutant strains was observed. This was also borne out when we plated strain GWB99 [Δ(proA::spc)] or GWB101 [Δ(proBA::cat)] on SMM agar plates lacking proline. However, the colonies
developed by both strains were very small in comparison with those formed by the Pro⁺ wild-type strain JH642 (Fig. 3), but they formed colonies with the same plating efficiency as their proBA⁺ parent strain JH642 (data not shown).

We often observed that cultures of strains carrying proA or proBA gene disruption mutations attained the same optical density (OD₅₇₈) values as the parent strain JH642 after overnight growth in SMM lacking proline. We traced this unexpected phenomenon to the appearance of faster growing colonies on SMM agar plates lacking proline (Fig. 3); such colonies have thus the potential to overgrow their proA parent strains in liquid cultures. When we purified such faster growing colonies, they possessed the antibiotic resistance markers expected from the presence of the [Δ(proA::spc)1 or [Δ(proBA::cat)2] alleles in the parent strains. Consequently, in these faster growing strains, the integrity of the proA or proAB genes were not somewhat restored; instead, they must harbour suppressor mutations that allow a bypass of the ProA defect. The size of the colonies formed by the Pro⁺ suppressor strains on SMM agar plates matched that of the B. subtilis wild-type strain JH642 upon replating (Fig. 3), and they also grew with kinetics and growth yields comparable with strain JH642 in liquid cultures in SMM lacking proline (Fig. 2A).

We chose 11 and 8 independently isolated Pro⁺ suppressor mutants derived from a Δ(proBA::cat)2 and a Δ(proA::spc)1 genetic background, respectively, for further study.

The Pro⁺ suppressors are unable to attain osmstres protective levels of proline via de novo synthesis

*Bacillus subtilis* cells cultivated in minimal media typically possess a proline pool between 10 and 20 mM (Whatmore et al., 1990; Zaprasis et al., 2013; Hoffmann et al., 2013b). We measured the proline content of two representatives (see later) of the Pro⁺ suppressor mutants grown in SMM and found that strains GWB128 (a class-I mutant) and GWB120 (a class-II mutant) had proline pools of 8 ± 0.2 and 7 ± 0.4 mM respectively; the wild-type strain JH642 possessed under these growth conditions a proline pool of 9 ± 0.2 mM. Hence, the tested Pro⁺ suppressor mutants did not overproduce proline.

Because the ProA protein is involved not only in the anabolic proline biosynthetic route of *B. subtilis* (Brill et al., 2011a) but also in the production of proline as a cellular protectant against osmotic stress (Brill et al., 2011b) (Fig. 1), we also assessed the influence of the Pro⁺ suppressor mutant strains on the ability of *B. subtilis* to cope with high salinity through the synthesis of large amounts of proline (Brill et al., 2011b; Hoffmann et al., 2012; 2013b). For this experiment, we grew the wild-type strain JH642, the Δ(proBA::cat)2 strain GWB101, the osmotically sensitive Δ(proHJ::tet)1 mutant strain JSB8 (Brill et al., 2011b), and the Pro⁺ suppressor mutant strains GWB128 and GWB120 in SMM containing 1.2 M NaCl (Fig. 2B). Neither one of the two tested Pro⁺ suppressor mutants was able to grow effectively under high salinity conditions (Fig. 2B) despite the fact that they possess an intact proHJ locus encoding the central enzymes for the osmstres-relieving proline production system (Brill et al., 2011b) (Fig. 1). This growth defect of the Pro⁺ suppressor strains at high salinity is a reflection of their inability to attain large proline pools via de novo synthesis (Fig. 2C). The proline pool in the wild-type strain was increased in response to osmotic stress from about 9 mM to about 550 mM, whereas the corresponding proline pools of strains GWB128 and GWB120 reached only magnitudes of about 87 and 115 mM, respectively, under high-salinity growth conditions (Fig. 2C). Taken together, the isolated Pro⁺ suppressor strains can produce enough proline to support the protein biosynthetic activities of *B. subtilis* cells propagated in a chemically defined medium but synthesize...
Fig. 4. Organization of the rocR-rocDEF region, genetic regulation of rocDEF expression and position of the RocR* suppressor variants within the RocR activator protein.

A. Transcriptional regulation of the rocDEF operon. Transcription of the rocDEF operon occurs from a SigL dependent −12/−24 type promoter (Gardan et al., 1995) and is positively regulated by the RocR and AhrC proteins (Gardan et al., 1997; Miller et al., 1997). The rocR gene is expressed from a SigA-type promoter (10/35); the RocR protein functions only as an activator of rocDEF transcription in the presence of an inducer (e.g. ornithine) (indicated by a black triangle). AhrC is a second activator protein stimulating rocDEF transcription (Miller et al., 1997; Garnett et al., 2008); its activity is negatively regulated by the antisense RNA sr1 (Heidrich et al., 2007). The RocR protein possesses three functional domains: The N-terminal domain (amino acids 1–146, Nw) includes a PAS domain (Gu et al., 2000) and acts an intramolecular repressor; the N-terminal domain interacts with the central domain (amino acids 146–380, CD); the region between amino acids 171 and 242 comprises the AAA+ domain which is involved in ATP binding and hydrolysis required for the transcriptional activator function of RocR (Bush and Dixon, 2012; Joly et al., 2012). The C-terminal domain (amino acids 380–461, Cw) contains the DNA-binding region of the RocR protein (Calogero et al., 1994; Gardan et al., 1997). B. Positions of the amino acid substitutions in RocR (RocR*) present in the strains suppressing the defects in proA (class-I). C. Positions of the amino acid substitutions in RocR found by Gardan et al. (Gardan et al., 1997) in a genetic screen searching for enhanced expression of a rocD-lacZ reporter fusion.

insufficient amounts of proline when it is needed by the cell as an osmotic stress protectant.

The Pro* suppressor mutants are genetically connected to the arginine degradation pathway

It has been reported in previous studies that the growth defect of B. subtilis strains carrying genetically not fully characterized lesions in proline biosynthetic genes (probably at the proBA operon) can be corrected not only by proline but also by an external supply of arginine, citrulline or ornithine (Baumberg and Harwood, 1979; Buxton, 1980; Belitsky et al., 2001). This was also the case for the genetically well-defined chromosomal Δ(proBA::catf2 and Δ(proA::spc)1 alleles that we have constructed by targeted gene disruptions (Fig. 3). The cross-feeding of proBA or proA mutant strains by arginine, or other intermediates in the arginine degradation pathway of B. subtilis (Gardan et al., 1995; 1997; Fischer and Debarbouille, 2002) can be understood when one considers that the rocD-encoded ornithine aminotransferase (RocD) produces the same reaction products as the γ-glutamyl-phosphate-reductase (ProA), namely γ-glutamic-semialdehyde/Δ1-pyrroline-5-carboxylate (Belitsky, 2002; Fischer and Debarbouille, 2002) (Fig. 1). The activity of the RocD enzyme should therefore be able to bypass the ProB- and ProA-catalysed initial two steps in proline biosynthesis when B. subtilis is cultured in a minimal medium proline. Indeed, when we combined the Δ(proBA::catf2) mutation present in strain GWB101 with a gene disruption in rocD, the microcolonies were no longer observed, and the growth defect of a proBA rocD double-mutant could not be rescued by an external supply of arginine (Fig. 3). We therefore conclude that the microcolonies formed by proA or proB mutant strains of B. subtilis on agar plates lacking proline result from a low-level conversion of ornithine to γ-glutamic-semialdehyde/Δ1-pyrroline-5-carboxylate by the RocD ornithine aminotransferase and the subsequent biotransformation of this intermediate into proline by the three paralogous Δ1-pyrroline-5-carboxylate reductases (ProI, ProG, ProH) (Fig. 1) operating in B. subtilis (Belitsky et al., 2001).

In view of the previously outlined physiological considerations (Baumberg and Klingel, 1993; Fisher, 1993; Belitsky et al., 2001; Belitsky, 2002), the consultation of the SPABBATS pathway discovery tool (Florez et al., 2011), and previous studies linking the suppression of proA mutations in Salmonella typhimurium and Escherichia coli to arginine metabolism (Kuo and Stocker, 1969; Berg and Rossi, 1974), we surmised that an upregulation in the cellular amount of the RocD protein or an enhancement of its enzymatic activity might underlie genetically the formation of the Pro* suppressor mutants (Fig. 1). To explore this possibility, we determined the DNA sequence of the rocR gene encoding the central regulator of the arginine degradation pathway (Calogero et al., 1994; Gardan et al., 1997; Fischer and Debarbouille, 2002), the regulatory region between the divergently oriented rocR gene and the rocDEF operon (Gardan et al., 1995) and part of the 5′-region of the rocD gene (Fig. 4A). In this way, we were able to pinpoint the mutation present in all of the 19 independently isolated Pro* suppressor strains.

RocR is a member of the family of NtrC/NifA prokaryotic enhancer-binding proteins that act together with Sig-54 transcription factors to activate transcription (Bush and...
The Pro* suppressor strains differ in their dependence on SigL

The expression of the rocDEF operon is strictly dependent on the alternative transcription factor SigL (Debarbouille \textit{et al}., 1991; Gardan \textit{et al}., 1995; 1997). The two classes of Pro* suppressor strains that we have isolated differ in their dependence on SigL to promote growth in a proline-free minimal medium. The class-I Pro* suppressor mutants (located in rocR) were all dependent on a functional SigL protein for their growth in SMM lacking proline, whereas class-II mutants (located near the rocR-rocDEF promoter) were able to grow in the absence of proline even when SigL was defective (Fig. 5).

Single amino acid substitutions result in partial inducer-independent variants of the RocR activator protein

Expression of the rocABC and rocDEF operons is induced by the presence of arginine, ornithine or citruline in the growth medium, with ornithine probably functioning as the true inducer of the RocR activator protein (Calogero \textit{et al}., 1994; Gardan \textit{et al}., 1995; 1997; Ali \textit{et al}., 2003). RocR possesses a helix-turn-helix DNA binding motif near its carboxy terminus and a domain positioned near its amino terminus interacts with the central domain and functions as an intramolecular repressor module (Gardan \textit{et al}., 1997). The inhibiting activity of the intramolecular repressor domain is relieved upon inducer binding to RocR, and this then fosters a productive interaction of the RocR protein with RNA-polymerase molecules complexed with SigL to promote transcription of the rocABC and rocDEF gene clusters and of the rocG gene (Debarbouille \textit{et al}., 1991; Calogero \textit{et al}., 1994; Gardan \textit{et al}., 1995; Belitsky and Sonenshein, 1999; Ali \textit{et al}., 2003). Missense mutations that disturb the intramolecular repressor function of the N-terminal domain of RocR with its central domain lead to partially inducer-independent RocR variants (Gardan \textit{et al}., 1997).

The 11 Pro* class-I suppressor mutants all carry single amino acid substitutions in the RocR activator protein (Fig. 4B); we refer in the following to these mutants as the RocR* variants. None of these missense mutations are located in the carboxy-terminal domain of the RocR protein that contains the DNA-binding module, and none are located in the PAS domain (amino acid 10–75) positioned near the N-terminus of the polypeptide chain; the predicted PAS domain (Gu \textit{et al}., 2000) is of unknown relevance for the functioning of the RocR activator. The RocR* mutants cluster in two regions: the substitutions K83N, K93E, D118N, V119M and E123D are present in the N-terminal intramolecular repressor domain of the RocR protein (Fig. 4B) with which the intramolecular repressor domain has been proposed to interact (Gardan \textit{et al}., 1997). Hence, none of the amino acid substitutions present in the RocR* variants were located in the AAA* domain (located between position 171 and 242) that is involved in ATP binding and hydrolysis of Sig-54 type activator proteins (Bush and Dixon, 2012; Gourse and Landick, 2012; Joly
The positions of the amino acid substitutions recovered in our Pro\(^{-}\) suppressor screen (Fig. 3) resemble those of a set of partially inducer independent RocR variants that were previously isolated by Gardan and colleagues (1997) using a genetic screen that relied on the expression of an rocD-lacZ reporter fusion in the absence of an inducer for the RocR protein. Despite the fact that very different genetic screens were used for the isolation of the corresponding rocR mutant strains, the D118N missense mutation was independently isolated both by Gardan and colleagues (1997) and in this study (Fig. 4B and C). This suggested to us that the genetic explanations for the Pro\(^{-}\) suppressor phenotype of our RocR\(^{*}\) mutants and that of the partially inducer-independent RocR mutants isolated previously were one and the same: enhanced expression of the rocDEF operon in the absence of an inducer.

To test this hypothesis, we combined all of the Pro\(^{-}\) RocR\(^{*}\) variants with a rocD-lacZ transcriptional reporter gene fusion and studied the response of the reporter construct to the presence of the RocR activating molecules arginine and ornithine (Table 1). Consistent with previous studies (Gardan \textit{et al.}, 1995; 1997), we found that the expression of the rocD-lacZ reporter fusion was strongly enhanced in a strain expressing the wild-type RocR protein when the inducers arginine or ornithine was present in the growth medium (Table 1). This was also the case when we assessed the influence of the various RocR\(^{*}\) variants on the transcriptional profile of the rocD-lacZ reporter fusion (Table 1). However, there was an important difference between the rocD-lacZ reporter fusion strain carrying the wild-type rocR gene and the strains harbouring the various rocR\(^{*}\) alleles. In this latter group, rocD-lacZ expression was enhanced (between 6- and 57-fold) relative to the wild-type fusion construct in SMM Orn Arg Pro Pro \([-\text{NH}_4^+]\) (Table 1). Consequently, the RocR\(^{*}\) variants recovered by us as Pro\(^{-}\) suppressor strains all function as partially inducer-independent activators of the rocDEF operon. Their Pro\(^{-}\) suppressor phenotype can thus be readily understood in terms of the above outlined working hypothesis that invokes an enhanced level of the RocD ornithine aminotransferase to produce increased cellular levels of \(\gamma\)-glutamyl-semialdehyde/\(\Delta^1\)-pyrroline-5-carboxylate that then can be enzymatically converted by \(\Delta^1\)-pyrroline-5-carboxylate reductases into proline (Belitsky \textit{et al.}, 2001) (Fig. 1). Like the authentic RocR protein, each of the RocR\(^{*}\) variants was fully dependent on SigL for its influence on the uninduced and induced levels of rocD-lacZ expression (Table 1). This fits nicely with our finding that the growth of the class-I Pro\(^{-}\) suppressor mutant strains in SMM lacking proline is critically dependent on the functioning of the alternative transcription factor SigL (Fig. 5).

### Table 1. Effect of single amino acid substitutions in RocR on rocD-lacZ expression.

<table>
<thead>
<tr>
<th>Strain(^{a})</th>
<th>Mutation</th>
<th>SigL(^{-})</th>
<th>Beta-galactosidase Activity ([\text{U mg protein}^{-1}])</th>
<th>SigL(^{b})</th>
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<tr>
<td></td>
<td></td>
<td>SMM</td>
<td>Orn</td>
<td>Arg</td>
</tr>
<tr>
<td>ACB136</td>
<td>–</td>
<td>1 ± 1</td>
<td>389 ± 40</td>
<td>388 ± 18</td>
</tr>
<tr>
<td>ACB145</td>
<td>RocR(^{-})-D118N</td>
<td>57 ± 1</td>
<td>338 ± 40</td>
<td>322 ± 10</td>
</tr>
<tr>
<td>ACB146</td>
<td>RocR(^{-})-I287T</td>
<td>6 ± 1</td>
<td>341 ± 13</td>
<td>340 ± 20</td>
</tr>
<tr>
<td>ACB147</td>
<td>RocR(^{-})-L250H</td>
<td>13 ± 1</td>
<td>324 ± 13</td>
<td>325 ± 27</td>
</tr>
<tr>
<td>ACB148</td>
<td>RocR(^{-})-I279T</td>
<td>22 ± 1</td>
<td>389 ± 19</td>
<td>382 ± 17</td>
</tr>
<tr>
<td>ACB149</td>
<td>RocR-K83N</td>
<td>33 ± 1</td>
<td>355 ± 21</td>
<td>364 ± 24</td>
</tr>
<tr>
<td>ACB150</td>
<td>RocR-A252P</td>
<td>56 ± 8</td>
<td>341 ± 26</td>
<td>308 ± 15</td>
</tr>
<tr>
<td>ACB151</td>
<td>RocR-K93E</td>
<td>26 ± 4</td>
<td>344 ± 14</td>
<td>341 ± 17</td>
</tr>
<tr>
<td>ACB152</td>
<td>RocR-Q258E</td>
<td>30 ± 1</td>
<td>340 ± 24</td>
<td>322 ± 13</td>
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<tr>
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<td>RocR-E123D</td>
<td>18 ± 1</td>
<td>323 ± 13</td>
<td>313 ± 19</td>
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<tr>
<td>ACB282</td>
<td>RocR-V119M</td>
<td>16 ± 1</td>
<td>326 ± 27</td>
<td>370 ± 45</td>
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\(a\). All \textit{B. subtilis} strains are derived from the \textit{B. subtilis} wild-type laboratory strain JH642 and harbour the \(\psi(rocD-lacZ)\) reporter gene fusion stably integrated as a single copy in the chromosomal \textit{amyE} gene. The \(rocD-lacZ\) reporter gene fusion construct is expressed from the authentic \(-12/-24\) \(rocDEF\) promoter (Gardan \textit{et al.}, 1995; 1997). Cells were grown in SMM (15 mM ammonium sulfate) or SMM without ammonium \([-\text{NH}_4^+]\). Ornithine (Orn), arginine (Arg) and proline (Pro) were added to the growth medium at final concentrations of 20 mM. Cultures were grown to early exponential phase (\(OD_{600}\) of about 0.6–0.8) and then harvested for beta-galactosidase (LacZ) enzyme activity assays. The values for the LacZ activity given for each strain represent at least two independently grown cultures, and for each sample analysed, the LacZ activity was determined twice.

\(b\). In addition to the \(\psi(rocD-lacZ)\) reporter gene fusion, all strains carry the \(\lambda(sigL:aphA3)\) gene disruption mutation. Disruption of the \(sigL\) genes lead to proline auxotrophic strains in a \(proA\) mutant genetic background; therefore, the cells were grown in the presence of 10 mM proline.

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\(\gamma\)-glutamyl-semialdehyde/\(\Delta^1\)-pyrroline-5-carboxylate that then can be enzymatically converted by \(\Delta^1\)-pyrroline-5-carboxylate reductases into proline (Belitsky \textit{et al.}, 2001) (Fig. 1). Like the authentic RocR protein, each of the RocR\(^{*}\) variants was fully dependent on SigL for its influence on the uninduced and induced levels of rocD-lacZ expression (Table 1). This fits nicely with our finding that the growth of the class-I Pro\(^{-}\) suppressor mutant strains in SMM lacking proline is critically dependent on the functioning of the alternative transcription factor SigL (Fig. 5).

Single base-pair changes result in the activation of a cryptic SigA-type promoter in the rocDEF regulatory region

The \(rocDEF\) operon is transcribed from a sigma-54 (SigL)-dependent \(-12/-24\) type promoter, and the transcriptional initiation site for this gene cluster has been identified through primer extension analysis in cells grown in the presence of arginine (Gardan \textit{et al.}, 1995) (Fig. 6). The eight class-II Pro\(^{-}\) suppressor mutations that were located...
in the rocR-rocDEF intergenic regions (Fig. 4A) were all single point mutations and these were located between the −12 region of the SigL-dependent rocDEF promoter and the rocDEF transcriptional initiation site (Fig. 6). In six of the mutants, an A•T bp was inserted 6 bp upstream of the mRNA initiation site (referred to in the following as the SigA-P1 mutation) and in the remaining two Pro+ suppressor mutants (SigA-P1; SigA-P2) are marked in red. The position of the transcription initiation site of the wild-type and SigA-P1 mutant are indicted by red circles.

B. Cells of the wild-type strain JH642 [pZAZA21] (wild-type) and its sigL mutant derivative TMB152 [pZAZA22] (SigA-P1) were grown in SMM, in SMM with 20 mM proline (+ Pro) or 20 mM arginine (+ Arg) to midexponential growth phase (OD578nm of approximately 1). Total RNA was isolated, hybridized to a rocD mRNA-specific antisense DNA primer, and a reverse transcription reaction was carried out. The sequence reactions of the corresponding plasmids (pZAZA21 and pZAZA22) were loaded on a polyacrylamide gel, along the primer extension reaction products, to allow the identification of the 5′ end of the rocD-mRNA (indicated by arrows).
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Bypassing the ProA-catalysed enzyme reaction

Table 2. Roles of the decryptified SigA-type promoters and the presence of proline on rocD-lacZ expression.

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<th>Straina</th>
<th>Mutationb</th>
<th>Relevant genotypec</th>
<th>Beta-galactosidase Activity [U mg protein−1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SigL RocR AhrC</td>
<td>Om Arg Pro Pro [-NH4+]</td>
</tr>
<tr>
<td>ACB136</td>
<td>WT</td>
<td>+ + +</td>
<td>403±3 432±9 4±1 134±12</td>
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<tr>
<td>ACB137</td>
<td>SigA-P1</td>
<td>+ + +</td>
<td>424±26 446±7 148±9 274±26</td>
</tr>
<tr>
<td>ACB138</td>
<td>SigA-P2</td>
<td>+ + +</td>
<td>438±19 453±16 225±9 282±19</td>
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<tr>
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<td>WT</td>
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<td>53±11 42±9 148±9 274±26</td>
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<tr>
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<td>+ + +</td>
<td>78±4 97±16 50±9 170±11 285±15</td>
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</table>

a. All B. subtilis strains are derived from the B. subtilis wild-type laboratory strain JH642 and harbour the ψ(rocD-lacZ)1 reporter gene fusion stably integrated as a single copy in the chromosomal amylE gene. The designation WT (wild-type) indicates that the authentic –12/-24 rocDEF promoter (Gardan et al., 1995; 1997) is present in the ψ(rocD-lacZ) fusion construct. The designations SigA-P1 and SigA-P2 indicate that in addition to the authentic –12/-24 rocDEF promoter, decryptified SigA-type promoters direct the transcription in the ψ(rocD-lacZ) fusion construct. Cultures were grown to early exponential growth phase (OD578 of about 0.6–0.8), and ornithine (Orn), arginine (Arg) and proline (Pro) were added to the cultures.

b. + denotes the presence of the wild-type gene; − denotes its absence. The mutant alleles present in the used strains are: Δ(rocR::aphA3), Δ(sigL::aphA3) and Δ(ahrC::ery1).

c. Primer extension analysis of the decryptified SigA-P1 promoter

The genetic data presented in Table 2 are consistent with our notion that the two types of point mutations in the vicinity of the the AhrC-, RocR- and SigL-dependent –12/–24-type rocDEF promoter create new SigA-type promoters that operate independently of these transcription factors. To prove this directly, we carried out a primer extension experiment with the SigA-P1 promoter variant to map the 5′ end of the rocDEF mRNA and compared this with that produced by the wild-type rocDEF promoter. Our primer extension analysis of the wild-type rocDEF promoter showed the same 5′ end of the rocDEF mRNA that has been mapped previously (Gardan et al., 1995). Fully consistent with the rocD-lacZ reporter gene fusion data (Table 2), the amount of this transcript was enhanced in

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cells grown in the presence of arginine but not when proline was present (Fig. 6B). We mapped the 5′ end of the rocDEF mRNA of the SigA-P1 variant in a strain that was defective for SigL because the rocD-lacZ reporter fusion data (Table 2) predict that a constitutively synthesized transcript should originate from the decryptified SigA-type promoter with a 5′ end different from that produced from the SigL-dependent −12/−24 rocDEF promoter. As expected, the wild-type arginine-inducible rocDEF transcript was absent, and instead, a new and constitutively produced mRNA species was detected (Fig. 6B). Its 5′ end is located 2 bp upstream of the mRNA initiation site used by the wild-type −12/−24 rocDEF promoter (Fig. 6A). Hence, the A•T bp insertion present in the class II SigA-P1 Pro+ suppressor strains has indeed created a new promoter and in view of our genetic data (Table 2), we surmise that this is also the case for the T•A bp to A•T bp substitution mutation present in the SigA-P-2 variant.

The rocR* and the SigA-P1 and SigA-P2 promoter mutations create a new regulatory pattern for rocDEF expression

In addition to arginine, ornithine and citrulline, proline is also an inducer of the rocABC and rocDEF operons (Calogero et al., 1994; Gardan et al., 1995; 1997). However, induction of roc gene expression by proline differs in two important aspects from that mediated by the other compounds. Induction by proline is relatively weak in comparison with that mediated by arginine in a minimal medium containing ammonium as the nitrogen source, but proline functions as a relatively good inducer for rocDEF gene expression in a modified minimal medium lacking ammonium (Baumberg and Harwood, 1979; Gardan et al., 1995; 1997). The transcriptional response of our rocD-lacZ reporter gene fusion carrying the wild-type −12/−24 rocDEF promoter faithfully reflects this previously reported pattern of rocDEF expression (Tables 1 and 2). The molecular mechanism(s) underlying the different effects of proline on rocDEF transcription in media that contain or that lack ammonium, one of the most preferred nitrogen sources of B. subtilis (Fisher, 1993), is not understood (Baumberg and Harwood, 1979; Gardan et al., 1995; 1997).

In contrast with the transcriptional profile of the rocD-lacZ reporter gene fusion expressed in a rocR wild-type genetic background and transcribed from the authentic −12/−24 type rocDEF promoter (Tables 1 and 2), we found that both the RocR* mutants (Table 1) and the two newly created SigA-type promoters (Table 2) generated a novel regulatory pattern of rocDEF transcription. Both types of genetic changes allowed a strong induction of rocD-lacZ expression by proline despite the fact that the growth medium contained 15 mM ammonium as the primary nitrogen source.

One possible scenario to explain the induction of the rocDEF operon by proline in ammonium-containing minimal medium would be that externally provided proline is imported via PutP, catabolized to Δ1-pyrroline-5-carboxylate via the PutB enzyme (Moses et al., 2012), and this metabolite could then be converted into ornithine by the backward enzyme reaction of RocD (Gardan et al., 1995; 1997). Consequently, ornithine formed by this sequence of events could then trigger the RocR-dependent induction of transcription of the arginine catabolic operons. Such a model implies that the induction of the rocDEF operon by proline in ammonium-containing medium is abolished in strains carrying either deletions of the putBCP operon or of the rocD gene. The data summarized in Table S1 demonstrate conclusively that the previously outlined model is incorrect because both class I and class II Pro+ suppressor mutations allowed induction of rocDEF transcription by proline in ammonium-containing medium in strains harbouring disruptions of either the putBCP operon or of rocD (Table S1).

Discussion

In this work, we demonstrate that B. subtilis rapidly responds to a perturbation in proline biosynthesis (Belitsky et al., 2001; Brill et al., 2011a,b) by the accumulation of suppressor mutants that harness and integrate enzymes from the arginine degradation system (Baumberg and Klingel, 1993) into proline production to circumvent a mutationally imposed bottleneck at the ProA-catalysed step (Fig. 1). Two classes of Pro+ suppressors of proA mutations were characterized, and we found that the expression of the structural gene for the RocD aminotransferase was uncoupled in both groups of mutants from its normal pattern of regulation, i.e. the requirement of arginine, ornithine or citrulline to genetically induce rocDEF expression (Gardan et al., 1995; 1997). The expression of the rocDEF operon is partially constitutive in all Pro+ suppressor mutants, and it became clear in our analysis that an increase in the cellular level of the RocD ornithine aminotransferase is responsible for the observed Pro+ suppressor phenotype. Indeed, this enzyme produces the same reaction products, namely γ-glutamic-semialdehyde/Δ1-pyrroline-5-carboxylate, as the ProA enzyme and thus allows the circumvention of the ProB and ProA-catalysed steps in proline biosynthesis (Fig. 1). Our finding that a deletion of the rocD gene abolishes the bypass of the ProA-catalysed reaction (Fig. 3) fully supports our proposal that an increased amount of RocD is the biochemical underpinning of the growth phenotype of the Pro+ suppressor strains in a proA mutant background.

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The deregulation of rocDEF expression in the Pro⁺ suppressor mutants is caused by either partially constitutive activity of the activator protein RocR (Table 1) or by the generation of constitutively active (Table 2) SigA-type rocDEF promoter (Fig. 6A). As a consequence of these changes, the former class of Pro⁺ suppressors are fully dependent for their growth on SigL in a medium-lacking proline, whereas in the latter class of Pro⁺ suppressors, this requirement has been lost (Fig. 5). In those mutants that acquired a new promoter (Fig. 6A), both the SigL-dependent –12/–24-type and the decoupled SigA-type promoter are simultaneously active (Table 2). However, loss of the SigL protein caused an enhancing effect on the activity of the newly generated SigA promoter (Table 2). How can this be understood? RNA-polymerase complexed with a Sig-54 type sigma factor (e.g. SigL) can bind tightly to 12/24 promoters to form a closed but transcriptionally inactive complex until a productive interaction of the Sig-54/RNA polymerase complex with a transcriptional enhancer factor (e.g. RocR) is established (Bush and Dixon, 2012; Gourse and Landick, 2012; Joly et al., 2012). Because the Sig-54/RNA-polymerase complex is prebound at –12/–24-type promoters (Reichenbach et al., 2009; Friedman and Gelles, 2012; Gourse and Landick, 2012), the SigL/RNA polymerase complex will inhibit access of the SigA/RNA-polymerase complexed to the newly created SigA-P1 or SigA-P2 promoters (Fig. 6A). Loss of SigL relieves this inhibition (Table 2).

The differential effects of proline on roc gene expression in the presence or absence of ammonium (Tables 1 and 2), an excellent nitrogen source for B. subtilis (Fisher, 1993; Fischer and Debarbouille, 2002), is a long-known phenomenon (Gardan et al., 1997), but the underlying regulatory events have not yet been elucidated. The two classes of suppressor mutations that we have studied modify the regulatory pattern of the rocDEF gene cluster. Although the molecular events underlying enhanced rocDEF expression in the two classes of the Pro⁺ suppressor strains are different, both allow enhanced rocDEF expression in response to proline availability in ammonium-rich medium (Tables 1 and 2). This finding needs to be taken into account when an explanation for the different transcriptional response of the rocDEF operon to proline availability in the presence and absence of ammonium is sought. We genetically tested a model that invokes the uptake of externally provided proline by the PutP transporter, its catabolism to Δ¹-pyrroline-5-carboxylate via the PutB enzyme (Moses et al., 2012) and its subsequent conversion to the inducer ornithine by a reversal of the RocD-catalysed enzyme reaction (Gardan et al., 1997) (Fig. 1). However, the data summarized in Table S1 demonstrate that this model does not withstand scrutiny.

The pathways for the synthesis of proline and the catabolism of arginine are closely related in many microorganisms, and this is also true for B. subtilis (Baumberg and Klingel, 1993; Belitsky, 2002) (Fig. 1). Phenotypically, the Pro⁺ suppressor mutants of ProA defects isolated by us in B. subtilis resemble those reported previously for S. typhimurium and E. coli (Kuo and Stocker, 1969; Berg and Rossi, 1974), but the underlying molecular events are completely different. The ProA bypass suppressors in S. typhimurium and E. coli rely on the inactivation of the argD-encoded N-acetylornithine aminotransferase, an enzyme that is involved in the synthesis of arginine. In these suppressor mutants, enhanced levels of γ-glutamim semialdehyde/Δ¹-pyrroline-5-carboxylate are produced via the ArgABCE arginine biosynthetic enzymes (Kuo and Stocker, 1969; Berg and Rossi, 1974). In contrast, our Pro⁺ suppressors rely on regulatory events that increase the level of the catabolic RocD ornithine aminotransferase, an enzyme that forms γ-glutamic-semialdehyde/Δ¹-pyrroline-5-carboxylate directly, metabolites that then can be used as a substrate by the three Δ¹-pyrroline-5-carboxylate reductases (Prol, ProH, ProG) operating in B. subtilis to form proline (Belitsky et al., 2001) (Fig. 1).

As manifested by the size of the colonies formed by proBA and proA mutants on SM agar plates (Fig. 3), and by the very limited growth of such strains in a minimal medium lacking proline (Fig. 2A), the RocD-catalysed step for the formation of γ-glutamic-semialdehyde/Δ¹-pyrroline-5-carboxylate from ornithine is not an effective way to produce proline by B. subtilis wild-type strains. However, when inducers (e.g. arginine) of the arginine catabolic system are present in the growth medium, B. subtilis can synthesize enough proline via the RocD-catalysed shunt to fuel the cells ongoing protein biosynthetic activities (Fig. 3) (Belitsky et al., 2001). An external supply of arginine can also provide osmotic protection with an efficiency similar to that of proline (Zaprasis et al., 2013), a process that relies on its catabolism to γ-glutamic-semialdehyde/Δ¹-pyrroline-5-carboxylate and the subsequent ProH-mediated conversion of these intermediates into the compatible solute proline (M. Bleisteiner and E. Bremer, unpubl. data).

Where does the ornithine come from that the Pro⁺ suppressor strains use to produce γ-glutamic-semialdehyde/Δ¹-pyrroline-5-carboxylate in the RocD-catalysed reaction? One possibility is the de novo synthesis of arginine, its catabolism to ornithine and subsequent conversion to γ-glutamic-semialdehyde/Δ¹-pyrroline-5-carboxylate via the RocD enzyme (Fig. 1). However, such a process entails an energy and substrate wasting futile cycle of synthesis and degradation of arginine. A more likely route of ornithine formation in the Pro⁺ suppressor strains would be its production from glutamate via the B. subtilis arginine biosynthetic enzymes.
Bacillus Genetic Stock Center (BGSC), OH, USA.

c. genetically linked to a chloramphenicol resistance gene (JSB8 gene as a single copy, thereby rendering the fusion strains defective in the extracellular

GWB98
Δ(rocD::aphA3)1
This study
GWB99
Δ(proA::spc)1
This study
GWB101
Δ(proB::cat)2
This study
GWB120
Δ(proB::cat)2 SigA-P1 – rocDEF promoter
This study
GWB127
Δ(proB::cat)2 SigA-P2 – rocDEF promoter
This study
GWB128
Δ(proB::cat)2 RocR'–L250H
This study
ACB136
[amyE::(rocD::lacZ)1 cat]
This study
ACB137
[amyE::(rocD::lacZ)1 cat]
This study
ACB138
[amyE::(rocD::lacZ)1 cat]
This study
ACB166
Δ(ahrC::ery)1
This study
TM151
Δ(sigL::spc)1
This study
TM152
Δ(proB::cat)2 Δ(sigL::spc)1 (SigA-P1 – rocDEF)
This study
JSB8
Δ(proH::tet)
(Blount et al., 1994)
SMB44
Δ(puBCP::spc)1
S. Moses
QB5505
Δ(sigL::aphA3) trpC2
(Debarbouille et al., 1991)
QB5533
Δ(rocR::aphA3) trpC2
(Calogero et al., 1994)

a. All strains, except QB5505 and QB5533, are derivatives of the B. subtilis wild-type laboratory strain JH642 (Srivatsan et al., 2008) and therefore carry the trpC2 pheA1 mutations in addition to the genetic markers indicated. Strains QB5505 and QB5533 are derivatives of the B. subtilis laboratory strain 168.

b. The designation [amyE::(rocD::lacZ)1 cat] indicates that the rocD-lacZ operon reporter fusion is stably integrated into the chromosomal amyE gene as a single copy, thereby rendering the fusion strains defective in the extracellular α-amylase AmyE. The ϕ(rocD-lacZ)1 reporter fusion is genetically linked to a chloramphenicol resistance gene (cat).

c. Bacillus Genetic Stock Center (BGSC), OH, USA.

(Mountain et al., 1984; Baumberg and Klingel, 1993) (Fig. 1).

Collectively, our study on genetic suppressors circumvent the ProA-catalysed step in proline biosynthesis of B. subtilis provides an example how flexibly microorganisms can bypass constraints imposed on their biosynthetic routes and how readily they can create novelty in their regulatory networks (Nam et al., 2011; Shou et al., 2011; Veeravalli et al., 2011; Blount et al., 2012).

Experimental procedures

Chemicals

L-proline, L-arginine and L-ornithine, the ninhydrine reagent for the quantification of proline, and the antibiotics chloramphenicol, kanamycin, erythromycin, tetracycline, lincomycin and spectinomycin were all purchased from Sigma-Aldrich (Steinheim, Germany). The chromogenic substrate for the measurement of β-galactosidase (LacZ) enzyme activity, ortho-nitrophenyl-β-D-galactopyranoside (ONPG), was purchased from Serva (Heidelberg, Germany).

Bacterial strains

The E. coli K-12 strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for routine cloning purposes, maintenance of cloning vectors and recombinant plasmids. Bacteria were grown and maintained on Luria-Bertani (LB) liquid medium and agar plates (Miller, 1972). Solid and liquid media contained, when necessary, antibiotics to select for the presence of plasmids or chromosomal gene disruption mutations marked with an antibiotic resistance cassette. The B. subtilis strain JH642 and its various mutant derivatives were used throughout this study (Tables 1–3). Strain JH642 is a member of the domesticated lineage of B. subtilis laboratory strains (Srivatsan et al., 2008) and was kindly provided to us by Dr. James A. Hoch (The Scripps Research Institute, La Jolla, CA, USA).

Media and growth conditions for B. subtilis strains

Bacillus subtilis strains were cultivated in SMM (Harwood and Archibald, 1990), with 0.5% (w/v) glucose as the carbon source and L-tryptophan (20 mg l⁻¹) and L-phenylalanine (18 mg l⁻¹) to satisfy the auxotrophic growth requirements of strain JH642 (trpC2 pheA1) and its derivatives (Table 3). A solution of trace elements (Harwood and Archibald, 1990) was added to SMM to improve the growth of B. subtilis strains. When ammonium-free medium was required for growth studies with B. subtilis strains, the ammonium sulfate normally present in SMM (Harwood and Archibald, 1990) was left out, and 20 mM of proline was used instead as sole nitrogen source (Moses et al., 2012). The osmolarity of SMM was increased by adding NaCl to it from a 5 M stock solution. Amino acid solutions were sterilized by filtration and added to
the growth medium at concentrations indicated in the individual experiments. For growth experiments that involved proline auxotrophic strains, cultures were pregrown in SMM with an excess of proline (10 mM), the cells were carefully washed two times with SMM without added proline, and these were then used to inoculate SMM medium. All *B. subtilis* cultures were inoculated from exponentially growing precultures in prewarmed (37°C) minimal media to OD\textsubscript{600} of 0.1. The cultures (20 ml culture volume in a 100 ml Erlenmeyer flask) were then propagated at 37°C in a shaking water bath set to 220 rpm. The growth of bacterial cultures was monitored by measuring their OD\textsubscript{600} at a wavelength of 578 nm (OD\textsubscript{578}). The antibiotics chloramphenicol (5 μg ml\(^{-1}\)), tetracycline (10 μg ml\(^{-1}\)), erythromycin-lincomycin (0.4 μg ml\(^{-1}\) and 15 μg ml\(^{-1}\) respectively), and spectinomycin (100 μg ml\(^{-1}\)) were used for the selection of *B. subtilis* strains carrying chromosomal copies of gene disruption mutations with insertions of an antibiotic resistance cassette or of \([\varphi|\text{rocD-lacZ}\text{cat}|\text{spc}]\) reporter fusion constructs inserted into the non-essential chromosomal *amyE* locus (Table 3). Ampicillin was used at a final concentration of 100 μg ml\(^{-1}\) for *E. coli* strains carrying plasmids encoding a beta-lactamase resistance gene.

Recombinant DNA techniques, constructions of plasmids and of *B. subtilis* strains

The routine manipulation of plasmid DNA, the construction of recombinant plasmids, the amplification of selected regions of the *B. subtilis* genome via PCR, the isolation of chromosomal DNA from *B. subtilis*, and the transformation of *B. subtilis* strains with plasmid or chromosomal DNA were all carried out using standard procedures (Cutting and Vander Horn, 1990; Harwood and Archibald, 1990). The mutant strains GWB99 \([\Delta(proA|\text{spc})]\), GWB101 \([\Delta(proBA|\text{cat})]\), GWB102 \([\Delta(proBA|\text{tet})]\), ACB166 \([\Delta(ahrC|\text{ery})]\) and TMB151 \([\Delta(sigL|\text{spc})]\) (Table 3) were constructed by using a two-step PCR-based method (Kuwayama et al., 2002). Regions flanking the gene of interest were first amplified by PCR and then connected in a second step with a PCR-generated DNA fragment encoding an antibiotic resistance gene. The antibiotic resistance genes used for this purpose were derived from plasmids pDG1726 (spc), pDG1515 (tet), pDG646 (ery) (Guerout-Fleury et al., 1995) and pJMB1 (cat) (M. Jebbar and E. Bremer, unpubl. data). The generated hybrid DNA fragments were then used for the transformation of the *B. subtilis* wild-type strain JH642; the integration of the gene disruptions into the chromosome was selected for by plating the transformation mixture on LB agar plates containing the appropriate antibiotic. Subsequently, PCR reactions using DNA primers flanking the deleted gene regions were used to verify the correct insertion of the various gene disruption constructs into the *B. subtilis* chromosome. Chromosomal DNA of the *B. subtilis* strains GWB99 \([\Delta(proA|\text{spc})]\), GWB101 \([\Delta(proBA|\text{cat})]\), GWB102 \([\Delta(proBA|\text{tet})]\), ACB166 \([\Delta(ahrC|\text{ery})]\), QB5533 \([\Delta(rocR|\text{aphA3})]\), QB5505 \([\Delta(sigL|\text{aphA3})]\) and TMB151 \([\Delta(sigL|\text{spc})]\) (Table 3) were used to introduce selected gene disruption mutations via DNA transformation and homologous recombination events into the chromosome of different *B. subtilis* recipient strains by selecting for colonies on LB agar plates containing the appropriate antibiotic. The relevant genetic markers of all constructed strains are listed in Tables 1–3 and Table S1.

Isolation of Pro\(^r\) suppressor mutants from *B. subtilis* strains carrying mutations in proA

Single colonies of strains GWB99 \([\Delta(proA|\text{spc})]\) and GWB101 \([\Delta(proBA|\text{cat})]\) were used to inoculate SMM medium that was supplemented with 10 mM of proline and contained glucose as the carbon source. The cultures were incubated overnight at 37°C and washed two times with SMM lacking proline; serial dilutions were then plated on SMM agar plates with 0.5% (w/v) glucose as the carbon source that did not contain proline. After 3 days of incubation at 37°C, faster growing colonies (Pro\(^r\) suppressor strains) were picked from the original agar plates and purified by streaking on the same medium. Subsequently, the resistance of these colonies to the expected antibiotics was tested on LB agar plates to ensure that the \([\Delta(proA|\text{spc})]\) and \([\Delta(proBA|\text{cat})]\) mutations present in strains GWB99 and GWB101, respectively, were still retained by the Pro\(^r\) suppressor strains. This was the case in each of the originally picked 19 independent isolates. Molecular analysis showed that these 19 Pro\(^r\) suppressor mutations were located to the rocR-rocDEF region (Gardan et al., 1995) of the *B. subtilis* genome. To this end, the corresponding regions were amplified by PCR from chromosomal DNA of Pro\(^r\) suppressor strains using DNA primers flanking the rocR-rocDEF segment of the *B. subtilis* genome. The DNA sequences of these regions were determined by Eurofins MWG Operon (Ebersberg, Germany) and compared with the known DNA sequence of the corresponding regions from the wild-type strain JH642 (Srivatsan et al., 2008).

Construction of reporter strains to analyse rocDEF promoter activity

To determine the transcriptional activity of the rocDEF wild-type promoter and its SigA-P1 and SigA-P2 mutant derivatives, we amplified by PCR a 314-bp DNA fragment encompassing the intergenic region between rocR and the rocDEF operon and part of the rocD coding region from strains JH642 (wild-type rocDEF promoter), GWB120 (SigA-P1-rocDEF promoter region) and GWB127 (SigA-P2 – rocDEF promoter region) using primers AC61 (5’-AAAGGATCCCGAGCGGTTGATAATTGTGGC-3’) and AC62 (5’-AAACC GGTTGATGACCTCGCTACTATTATTTCT-3’). These PCR products were digested with BamHI and Xmal, and cloned into the BamHI-Xmal sites of the lacZ operon fusion vector pAC6 (Stülke et al., 1997). Plasmid pAC6 contains a promoterless lacZ reporter gene, an antibiotic resistance marker (cat), and the 5’- and 3’- regions of the *amyE* gene that allows the integration of the reporter gene constructs into the *B. subtilis* chromosome at the non-essential *amyE* gene via a double-homologous recombination event. DNA of the reporter gene plasmids pZAZA16 \([\varphi|\text{rocD\textsubscript{1-2}}|\text{lacZ}|\text{cat}]\), pZAZA17 \([\varphi|\text{rocD\textsubscript{1-2}}|\text{lacZ}|\text{cat}]\) and pZAZA18 \([\varphi|\text{rocD\textsubscript{1-2}}|\text{lacZ}|\text{cat}]\) was linearized with PsI, and this DNA fragment was then used to transform *B. subtilis* strains. The integration of the rocD-lacZ fusions into the *amyE* locus was selected for by plating the transformation mixture onto LB agar plates containing...
chloramphenicol (5 μg ml−1). Loss of AmyE function caused by the integration of [p(rocD-lacZ) cat] constructs into the amyE gene was assessed by flooding B. subtilis colonies that were grown on agar plates containing 1% starch with Gram's iodine stain and scoring the size of the zone around individual colonies where starch hydrolysis had occurred (Cutting and Vander Horn, 1990). Because plasmid pAC6 contains a chloramphenicol resistance (cat) marker, in some of originally isolated Pro+ suppressor strain, the original present Δ(proBA::cat)2 allele was replaced by a Δ(proBA::tet)3 (from strain GWB102; Table 3) allele.

To increase the level of the 5′-region of the rocD mRNA in B. subtilis cells for the purpose of primer extension analysis, plasmids carrying the rocR-rocDEF intergenic region and part of the rocD coding sequence were constructed by amplifying 800-bp DNA fragments from chromosomal DNA of the wild-type strain JH642 and its mutant derivatives GWB120 [SigA-P1 – rocDEF promoter region] (Gardan et al., 1997) with PCR using DNA primers AC49 (5′-AAAGGATCCCTTCCAGAAGAAAGAG-3′) and AC50 (5′-AAAAAGCTGATGCTGCCATTACAC-3′). The resulting PCR products were cut with BamHI and HindIII, and inserted into the BamHI-HindIII sites of the E. coli–B. subtilis shuttle vector pBR373 (Brückner, 1992), thereby yielding plasmids pZAZA21 (wild-type – rocDEF promoter region) and pZAZA22 (SigA-P1 – rocDEF promoter region). The expected DNA sequence and the absence of undesired mutations were ascertained by DNA sequence analysis.

**Primer extension analysis of the rocDEF regulatory region**

To map the transcriptional start sites of the rocDEF wild-type promoter and its SigA-P1 mutant derivative, we isolated total RNA from cultures of the B. subtilis strain JH642 (pZAZA21) and TMB152 (pZAZA22). These strains were either grown in SMM or in SMM with 20 mM arginine or 20 mM proline to induce rocDEF transcription (Gardan et al., 1997). Total RNA was isolated with the acid-phenol extraction method from log-phase cells (OD578 of about 1) (Majumdar et al., 1991). A reverse transcription reaction of total B. subtilis RNA and 2 pmol of the rocD-specific primer PE-rocD-3 (5′-GGTGCTCCGTAATGAGACGTCTGATGAT-3′) labelled at its 5′-end with the fluorescent dye DY-781 (Biomers, Ulm, Germany) was carried out using the Primer extension method–avian myeloblastosis virus (AMV) reverse transcriptase system (Promega, Mannheim, Germany), as detailed by the manufacturer. The same primer was used for DNA sequence reactions using RNA of plasmids pZAZA21 and pZAZA22 as templates to allow the identification of the 5′-end of the rocDEF mRNA produced from the wild-type and its SigA-P1 mutant derivative. DNA sequencing was performed using the di-deoxy chain termination method with a ‘DNA Cycle Sequencing Kit’ (Jena Biosciences GmbH, Jena, Germany). The products of the primer extension and DNA sequencing reactions were analysed using a DNA sequencer (Model 4000; Li-COR Biosciences, Bad Homburg, Germany).

**β-galactosidase enzyme activity assays**

Bacillus subtilis cells carrying chromosomal amyE::p(rocD-lacZ)1 reporter gene fusions were either grown in SMM or in SMM containing various inducers (arginine, ornithine, proline) of the B. subtilis roc genes (Calogero et al., 1994; Gardan et al., 1995; Ali et al., 2003) at a final concentration of 20 mM. An aliquot (1.6 ml) of the cells was harvested by centrifugation when the culture reached an OD578 of 0.6–0.8, and the cells were then resuspended in 0.5 ml Z buffer (Miller, 1972) (adjusted to pH 7.0) that contained 1 mg ml−1 lysozyme to disrupt the B. subtilis cell wall. After incubation for 10 min at 37°C in an Eppendorf thermomixer with vigorous shaking, cellular debris was removed by centrifugation, and the supernatant was then employed for LacZ activity assays using the chromogenic substrate ONPG. β-galactosidase specific enzyme activity is expressed in units per mg of protein; protein concentrations were estimated from the OD280 of the cell culture (Miller, 1972).

**Determination of cellular proline pools**

The intracellular content of free proline in various B. subtilis strains was determined by a colorimetric assay that detects proline as a coloured proline-ninhydrine complex that can be quantified by measuring the absorption of the solution at 480 nm (Bates et al., 1973). For this assay, the B. subtilis cells were grown in SMM containing 1.2 M of NaCl until midexponential growth phase (the cells had divided at least four times); strains with a growth defect at high salinity were cultured until growth did not increase any further. Aliquots (8 ml) of the cultures were harvested by centrifugation and processed as described previously (Bates et al., 1973; Hoffmann et al., 2012; 2013b). To correlate the coloured proline-ninhydrine complex with cellular proline concentration, a calibration curve was established by treating standard solutions with a known L-proline concentration (0–10 mM) in the same way as the whole B. subtilis cell extracts. Intracellular proline concentrations were calculated using a volume of a B. subtilis cell of 0.67 μl per 1 OD578 unit of cell culture (S. Moses, E.P. Bakker, and E. Bremer, unpubl. data) (Hoffmann et al., 2012). The B. subtilis cell volume was estimated from the internal and total water spaces by measuring the distribution of membrane-permeable 3H2O and membrane-impermeable inulin-[14C]carboxylic acid (Bakker and Mangerich, 1981; Hoffmann et al., 2013b).

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References


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1.** Role of the Proline degradation system (PutBCP) and of the ornithine aminotransferase RocD on proline mediated induction of rocD-lacZ expression.