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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.

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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; Schicklberger *et al*., 2013) or avert cellular stress by repurposing exsting 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 Δ^1-pyrroline-5-carboxylate reductase. Multiple enzymes for the first (ProB, ProJ) and last (ProI, 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

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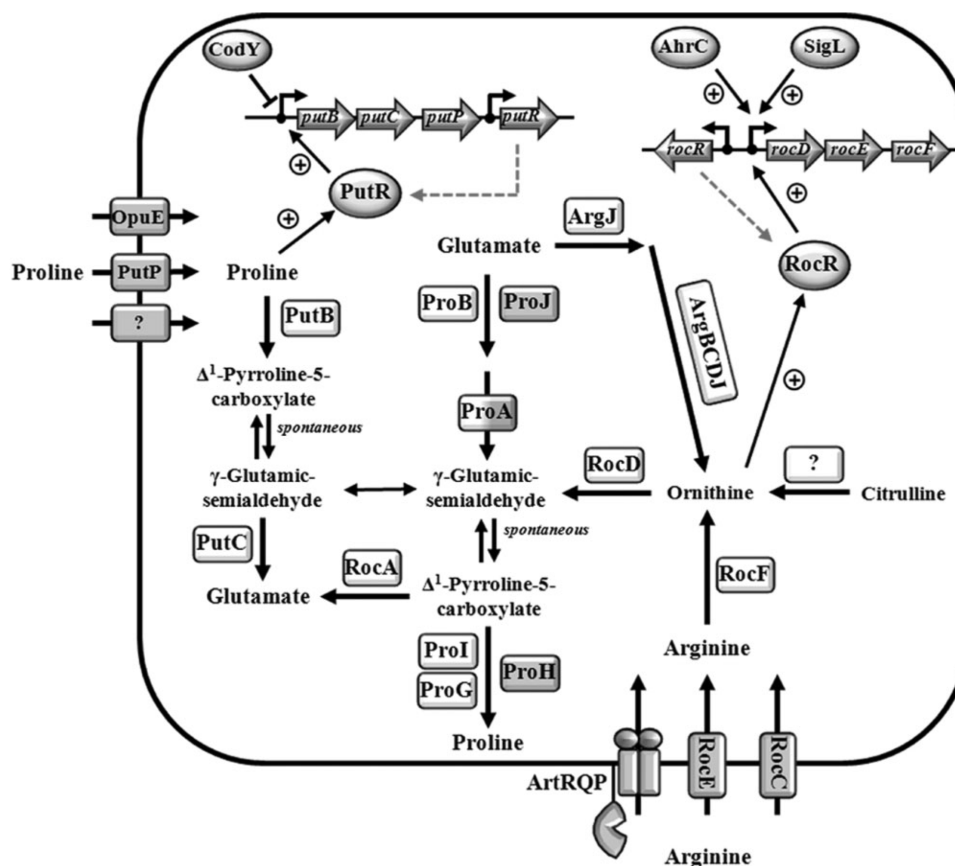

Fig. 1. Biosynthetic routes for anabolic and osmostress-adaptive proline production, and proline catabolism and their connection to the arginine synthesis and degradation systems of *B. subtilis*.

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 mechanism, 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*

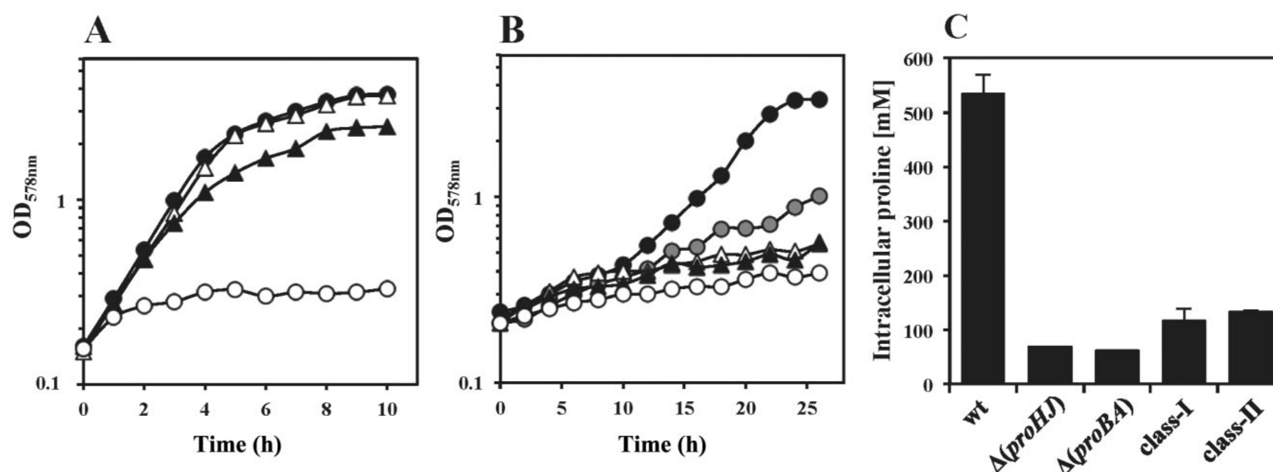

Fig. 2. Influence of Pro+ suppressor mutations on growth and intracellular proline pools. Cultures of *B. subtilis* wild-type strain JH642 (black circles), the *proHJ* mutant strain JSB8 (grey circles), the *proBA* mutant strain GWB101 (white circles) and two representatives of the isolated Pro+ suppressor strains [the class-I RocR* (L250H) mutant strain GWB128 (black triangles) and the class-II SigA-P1 mutant strain GWB120 (white triangles)] were used to inoculate (A) SMM or (B) SMM containing 1.2 M NaCl. Growth of the cultures was monitored over time by measuring the OD_{578}. (C) Cells grown at high salinity (SMM containing 1.2 M NaCl) were harvested by centrifugation once the cultures reached an OD_{578} of about 1.8–2 and were then assessed for their proline content (Bates *et al*., 1973). For each sample analysed, the intracellular proline content was determined twice. The values represent the means of two independently grown cultures, and the error bars indicate standard deviations.

has developed a dedicated and osmotically controlled proline production pathway that uses isoenzmes of the first (ProJ) and last (ProH) steps of the anabolic ProB-ProA-ProI proline synthesis route but uses from this latter route the ProA enzyme (Brill *et al*., 2011b). As a consequence, the anabolic and the osmostress 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*, *proI*, *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 Pro+ 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 Pro+ 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 artifically provides, via the enzymatic reaction of the RocD ornithine aminotransferase, the Pro+ suppressor strains with increased amounts of γ-glutamyl-semialdehyde/Δ^1-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 Pro+ 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-glutamyl-phosphate-reductase of *B. subtilis* (Belitsky *et al*., 2001), and a tight proline auxotrophic growth penotype is therfore 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*)1] or GWB101 [Δ(*proBA*::*cat*)2] on SMM agar plates lacking proline. However, the colonies

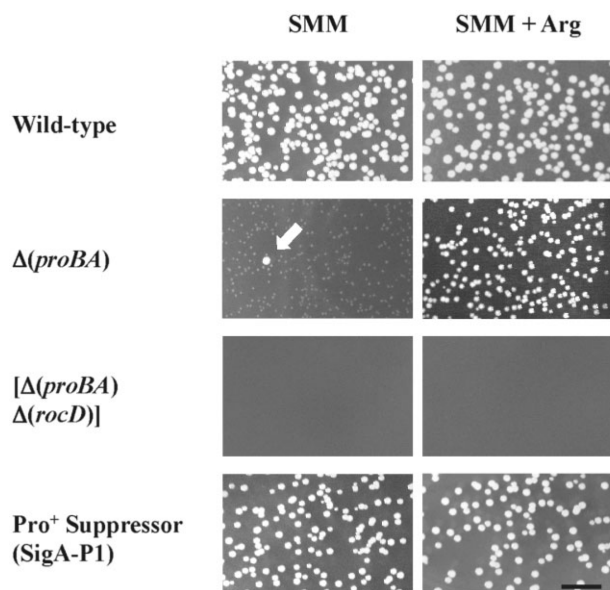

Fig. 3. Appearance and properties of Pro+ suppressor strains in a Δ(*proBA*) genetic background. Cells of the *B. subtilis* wild-type strain JH642, its Δ(*proBA::cat*) mutant derivative strain GWB101, the [Δ(*proBA*) Δ(*rocD*)] deletion strain GWB90 and a Pro+ suppressor strain (GWB120) containing the SigA-P1-*rocDEF* promoter mutation were grown overnight in SMM containing 10 mM proline. The cultures were carefully washed with SMM, and 100 μl of appropriate dilutions (10^{-5}; undiluted in case of the [Δ(*proBA*) Δ(*rocD*)] deletion strain GWB90) were plated on SMM agar plates lacking or containing 10 mM arginine. The agar plates were incubated at 37°C for 24 h (cells grown on SMM plates containing arginine) or 48 h (cells grown on SMM plates lacking arginine). The arrow indicates the appearance of a Pro+ suppressor mutant strain that appeared in the Δ(*proBA::cat*) deletion strain GWB101. The bar represents 5 mm in the photographic picture of the various agar plates.

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 (OD_{578}) 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 somewhow 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 osmostress protective levels of proline via de novo synthesis

Bacillus subtilis cells cultivated in minimal media typically posess 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 osmostress-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

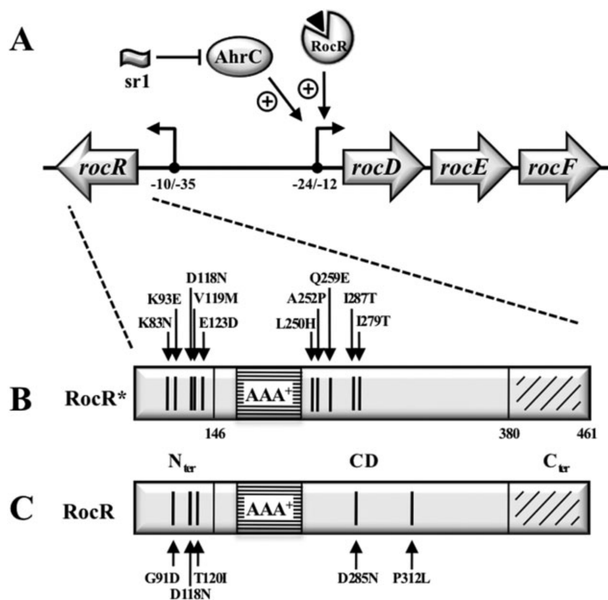

Fig. 4. Organization of the *rocR-rocDEF* region, genetic regulation of *rocDEF* expression and position of the RocR* suppressor variants within the RocR activator protein.
A. Transcriptional regulation of the *rocDEF* operon. Transcription of the *rocDEF* operon occurs from a SigL dependent −12/−24-type 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, N_{ter}) 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, C_{ter}) 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 osmostress 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*::*cat*)2 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/Δ^1-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*::*cat*)2 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 *proBA* mutant strains of *B. subtilis* on agar plates lacking proline result from a low-level conversion of ornithine to γ-glutamic-semialdehyde/Δ^1-pyrroline-5-carboxylate by the RocD ornithine aminotransferase and the subsequent biotransformation of this intermediate into proline by the three paralogous Δ^1-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

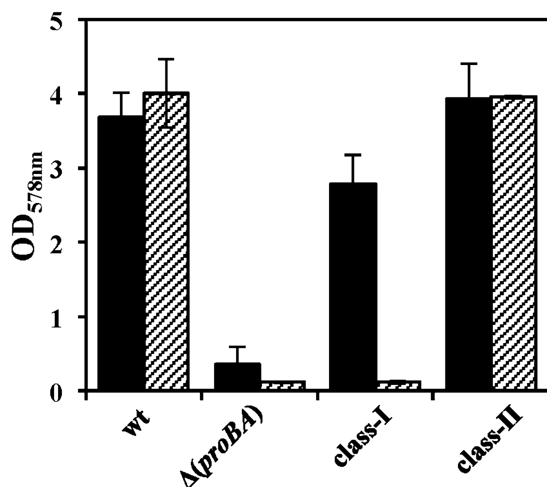

Fig. 5. Influence of SigL on the growth of different types of Pro+ suppressor strains. Growth of various *B. subtilis* strains with an intact *sigL* gene (black bars) and its *sigL* (striped bars) mutant derivatives were assessed in SMM lacking proline. The following strains were used: the wild-type strain JH642 (wt), the *proBA* mutant (GWB101), a class-I Pro+ suppressor mutant (GWB128) carrying the RocR* (L250H) allele and a class-II Pro+ suppressor mutant (GWB120) carrying the SigA-P1-*rocDEF* promoter mutation. The cells were pregrown in SMM containing 10 mM proline, carefully washed two times to remove the proline present in the growth medium and were then used to inoculate prewarmed SMM lacking proline (to an OD_{578} of 0.08). The growth yield of the cultures was measured after 16 h of incubation at 37°C. The values represent the means of two independently grown cultures, and the error bars indicate standard deviations.

Dixon, 2012; Joly *et al.*, 2012). SigL is the equivalent of Sig-54 in *B. subtilis*, and it mediates transcription from –12/–24 class promoters (Debarbouille *et al.*, 1991; Calogero *et al.*, 1994; Gardan *et al.*, 1995). Such a promoter is present in front of both the *rocDEF* and *rocABC* operons, and of the *rocG* gene encoding the components for the arginine degradation system of *B. subtilis* (Gardan *et al.*, 1995; Belitsky and Sonenshein, 1999; Fischer and Debarbouille, 2002; Ali *et al.*, 2003). Eleven of the Pro+ suppressor mutations were located in *rocR* (class-I), and eight mutations were located in the regulatory region in front of the *rocDEF* operon (class-II). Both types of mutations were obtained regardless whether a Δ(*proBA*::*cat*)2 or a Δ(*proA*::*spc*)1 genetic background had been initially used for the isolation of the Pro+ suppressor strains.

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 *et al.*, 1991; Gardan *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 *et al.*, 1994; Gardan *et al.*, 1995; 1997; Ali *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 *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 *et al.*, 1991; Calogero *et al.*, 1994; Gardan *et al.*, 1995; Belitsky and Sonenshein, 1999; Ali *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 *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 *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 RocR; the V119M RocR* variant was isolated twice. The substitutions L250H, A252P, Q259E, I279T and I287T are found closely spaced in the central domain of the RocR protein (Fig. 4B) with which the intramolecular repressor domain has been proposed to interact (Gardan *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

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

		Beta-galactosidase Activity [U mg protein^{-1}]						
		SigL^{+}					SigL^{-b}	
Strain[a]	Mutation	SMM	Orn	Arg	Pro	Pro [-NH_4^+]	SMM	Arg
ACB136	–	1 ± < 1	389 ± 40	388 ± 18	3 ± 1	117 ± 10	2 ± 10	2 ± < 1
ACB145	RocR*-D118N	57 ± < 1	338 ± 40	322 ± 10	346 ± 23	239 ± 48	1 ± 0	1 ± < 1
ACB146	RocR*-I287T	6 ± < 1	341 ± 13	340 ± 20	45 ± 4	128 ± 43	1 ± 0	1 ± < 1
ACB147	RocR*-L250H	13 ± < 1	324 ± 13	325 ± 27	99 ± 19	223 ± 26	2 ± 0	1 ± < 1
ACB148	RocR*-I279T	22 ± < 1	389 ± 19	382 ± 17	157 ± 18	246 ± 10	1 ± 0	2 ± < 1
ACB149	RocR*-K83N	33 ± < 1	355 ± 21	364 ± 24	239 ± 6	255 ± 32	1 ± 1	1 ± < 1
ACB150	RocR*-A252P	56 ± 8	341 ± 26	308 ± 15	248 ± 27	198 ± 18	2 ± 0	2 ± < 1
ACB151	RocR*-K93E	26 ± 4	344 ± 14	341 ± 17	191 ± 10	247 ± 20	2 ± 0	2 ± < 1
ACB152	RocR*-Q259E	30 ± 1	340 ± 24	322 ± 13	265 ± 13	210 ± 7	2 ± 1	2 ± < 1
ACB281	RocR*-E123D	18 ± < 1	323 ± 13	313 ± 19	98 ± 1	129 ± 7	1 ± 2	1 ± < 1
ACB282	RocR*-V119M	16 ± 1	326 ± 27	370 ± 45	80 ± 5	142 ± 33	1 ± 1	1 ± < 1

a. All *B. subtilis* strains are derived from the *B. subtilis* wild-type laboratory strain JH642 and harbour the φ(*rocD-lacZ*)1 reporter gene fusion stably integrated as a single copy in the chromosomal *amyE* gene. The *rocD-lacZ* reporter gene fusion construct is expressed from the authentic –12/–24 *rocDEF* promoter (Gardan *et al*., 1995; 1997). Cells were grown in SMM (15 mM ammonium sulfate) or SMM without ammonium [-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_{578} 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 φ(*rocD-lacZ*)1 reporter gene fusion, all strains carry the Δ(*sigL*::*aphA3*)1 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.

et al., 2012). 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). Fully consistent with previous studies (Gardan *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 habouring 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 the absence of an inducer (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 γ-glutamic-semialdehyde/Δ^1-pyrroline-5-carboxylate that then can be enzymatically converted by Δ^1-pyrroline-5-carboxylate reductases into proline (Belitsky *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 *et al*., 1995) (Fig. 6). The eight class-II Pro$^+$ suppressor mutations that were located

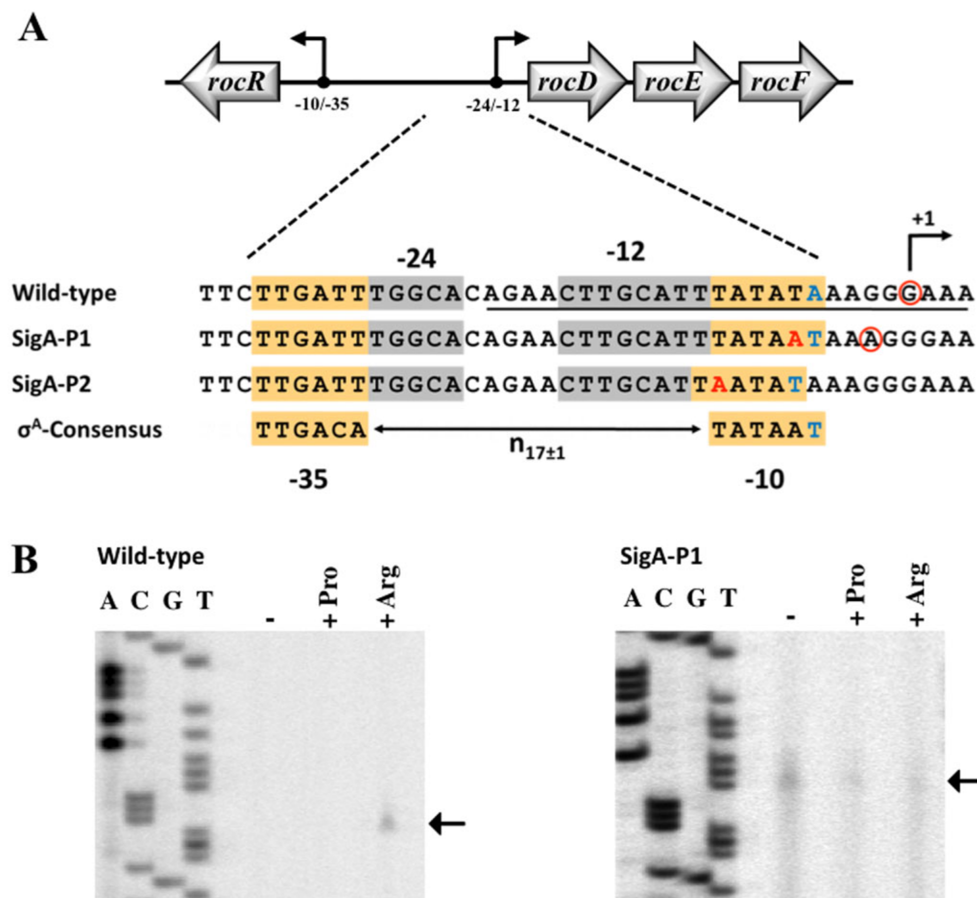

Fig. 6. Primer extension analysis of the *rocDEF* transcript and mapping of the transcriptional start sites of the SigA-P1-*rocDEF* promoter mutant.
A. DNA sequence of the *rocDEF* promoter region and nucleotide changes in the SigA-P1 and SigA-P2 suppressor mutants. The –24/–12 regions of the SigL-dependent *rocDEF* promoter are indicated by grey boxes, and the AhrC binding site is underlined (Miller *et al.*, 1997). The RocR-binding site is located upstream of the *rocDEF* promoter region (Gardan *et al.*, 1995; 1997). The potential –10 and –35 regions of the cryptic SigA-dependent promoter (Helmann, 1995) are indicated by orange boxes; the position of the 'invariant' T in the –10 region is highlighted in blue, and the two promoter mutations (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 (OD_{578nm} 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).

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 initation 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 a T•A bp located 10 bp upstream of the transcriptional start site was substituted by an A•T base pair (referred to in the following as the SigA-P2 mutation) (Fig. 6A).

A visual inspection of the DNA sequences around the –12/–24 *rocDEF* promoter (Gardan *et al.*, 1995) revealed the presence of –35 and –10 regions typical for SigA-type *B. subtilis* promoters (Helmann, 1995). These putative –35 and –10 regions are separated by 17 bp, the most prefered spacer length of SigA-dependent promoters (Fig. 6A). While the putative –35 and –10 regions in the vicinity of the 12/24 *rocDEF* promoter possess overall rather good matches to the consensus sequence of SigA-type promoters, the potential –10 region lacks the T•A bp that is most critical for the functioning of SigA-dependent promoters, the so-called 'invariant T' (Helmann, 1995). Both the A•T bp insertion and the T•A bp to A•T bp substitution mutation present in the SigA-P1 and SigA-P2 Pro+ suppressor mutants, respectively, reconfigure the putative –10 region in such a way that new –10 boxes are created that possess an T•A bp at the critical position of

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

		Relevant genotype[c]			Beta-galactosidase Activity [U mg protein^{-1}]				
Strain[a]	Mutation[b]	SigL	RocR	AhrC	SMM	Orn	Arg	Pro	Pro [-NH_4^+]
ACB136	WT	+	+	+	1 ± < 1	403 ± 35	432 ± 9	4 ± 1	134 ± 12
ACB137	SigA-P1	+	+	+	53 ± 11	424 ± 26	446 ± 7	148 ± 9	274 ± 26
ACB138	SigA-P2	+	+	+	56 ± 8	438 ± 19	453 ± 16	225 ± 9	282 ± 19
ACB139	WT	+	–	+	1 ± < 1	1 ± < 1	1 ± 1	2 ± < 1	5 ± 1
ACB140	SigA-P1	+	–	+	51 ± 2	58 ± 5	53 ± 3	189 ± 4	226 ± 29
ACB141	SigA-P2	+	–	+	56 ± 4	62 ± 10	61 ± 10	240 ± 6	254 ± 24
ACB142	WT	–	+	+	1 ± <1	2 ± < 1	1 ± < 1	2 ± < 1	2 ± 1
ACB143	SigA-P1	–	+	+	173 ± 9	182 ± 16	134 ± 23	269 ± 42	224 ± 35
ACB144	SigA-P2	–	+	+	148 ± 21	139 ± 17	134 ± 11	271 ± 29	249 ± 30
ACB167	WT	+	+	–	2 ± < 1	2 ± < 1	2 ± < 1	3 ± < 1	70 ± 2
ACB168	SigA-P1	+	+	–	68 ± 5	86 ± 1	48 ± 3	174 ± 24	257 ± 8
ACB169	SigA-P2	+	+	–	78 ± 4	97 ± 16	50 ± 9	170 ± 11	285 ± 15

a. All *B. subtilis* strains are derived from the *B. subtilis* wild-type laboratory strain JH642 and harbour the φ(*rocD-lacZ*)1 reporter gene fusion stably integrated as a single copy in the chromosomal *amyE* gene. The designation WT (wild-type) indicates that the authentic –12/–24 *rocDEF* promoter (Gardan *et al.*, 1995; 1997) is present in the φ(*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 φ(*rocD-lacZ*) fusion construct. Cultures were grown to early exponential growth phase (OD_{578} of about 0.6–0.8), and ornithine (Orn), arginine (Arg) and proline (Pro) were added to the cultures at a final concentration of 20 mM. Cells were typically grown in SMM with ammonium sulfate (15 mM) as the nitrogen source; the designation [-NH_4^+] indicates that the ammonium sulfate was left out of the SMM medium, and instead, 20 mM proline was used at the nitrogen source and as an inducer for φ(*rocD-lacZ*) expression. 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. SigA-P1: single base-pair A•T insertion; SigA-P2: single base-pair T•A to A•T mutation in the promoter region of the *rocDEF* operon to activate SigA-type promoters.
c. + 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*::*ery*)1.

the 'invariant T' (Fig. 6A). These observations suggested to us that the authentic –12/–24 type *rocDEF* promoter (Gardan *et al.*, 1995) overlaps with DNA sequences of a cryptic SigA-type promoter and that the SigA-P1 and SigA-P2 point mutations activate this promoter. Hence, in class II Pro[+] suppressor mutants, the *rocDEF* operon should be expressed even in the absence of SigL and thereby allow growth of the suppressor strains in the absence of proline and without a functional SigL protein; this is exactly what we observed (Fig. 5).

We assessed the influence of the SigA-P1 and SigA-P2 mutations on the transcriptional profile of appropriately modified *rocD-lacZ* reporter gene fusions. One would predict that: (i) a considerable level of *rocDEF* expression should occur both in the absence of inducers for the RocR activator and in a *sigL* mutant (transcription driven by the newly created SigA-type promoter) and (ii) *rocDEF* expression should be inducible by arginine and ornithine and be dependent on the RocR regulatory protein (transcription driven by the –12/–24 *rocDEF* promoter) (Fig. 4A). The AhrC protein is a second activator for *rocDEF* expression (Miller *et al.*, 1997; Heidrich *et al.*, 2007; Garnett *et al.*, 2008), and the SigA-P1 and SigA-P2 mutations are present in its previously mapped binding site (Fig. 4A). The data summarized in Table 2 demonstrate that the above outlined predictions were fully borne out when we separately introduced the SigA-P1 and SigA-P2 mutations into a *rocD-lacZ* fusion and combined these reporter gene constructs with appropriately *rocR*, *ahrC* and *sigL* gene disruption mutations. Notably, the SigA-P1 and SigA-P2 mutations did not abrogate the activating activitity of the AhrC regulatory protein on the expression of the *rocD-lacZ* fusion (Table 2). With respect to the genetic explanation underlying the Pro[+] suppressor phenotype of strains carrying SigA-P1 and SigA-P2 mutations in a *proA* mutant background, the enhanced expression of the *rocDEF* operon in the absence of an inducer for RocR (Table 2) is a key event because it permits increased production of γ-glutamic-semialdehyde/Δ^1-pyrroline-5-carboxylate via the RocD ornithine aminotransferase (Fig. 1).

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 analyis 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

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 initation 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 Δ^1-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/Δ^1-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.

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-dependent promoters that overlap the authentic –12/–24-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 decryptified 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 transcription 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 Δ^1-pyrroline-5-carboxylate via the PutB enzyme (Moses *et al.*, 2012) and its subsequent conversion to the inducer orinithine 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 scruteny.

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-glutamic-semialdehyde/Δ^1-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/Δ^1-pyrroline-5-carboxylate directly, metabolites that then can be used as a substrate by the three Δ^1-pyrroline-5-carboxylate reductases (ProI, 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 SMM 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/Δ^1-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 acitivities (Fig. 3) (Belitsky *et al.*, 2001). An external supply of arginine can also provide osmostress protection with an efficiency similar to that of proline (Zaprasis *et al.*, 2013), a process that relies on its catabolism to γ-glutamic-semialdehyde/Δ^1-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/Δ^1-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/Δ^1-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

Table 3. *Bacillus subtilis* strains used in this study.

Strain[a]	Relevant genotype[b]	Source
JH642	*trpC*2 *pheA*1	J. Hoch; BGSC 1A96[c]
GWB88	Δ(*proBA*::*cat*)2 Δ(*sigL*::*aphA3*)	This study
GWB90	Δ(*proBA*::*cat*)2 Δ(*rocD*::*aphA3*)1	This study
GWB92	Δ(*sigL*::*aphA3*)1	This study
GWB97	Δ(*proA*::*spc*)1 Δ(*rocD*::*aphA3*)1	This study
GWB98	Δ(*rocD*::*aphA3*)1	This study
GWB99	Δ(*proA*::*spc*)1	This study
GWB101	Δ(*proBA*::*cat*)2	This study
GWB102	Δ(*proBA*::*tet*)3	This study
GWB120	Δ(*proBA*::*cat*)2 SigA-P1 – *rocDEF* promoter	This study
GWB127	Δ(*proBA*::*cat*)2 SigA-P2 – *rocDEF* promoter	This study
GWB128	Δ(*proBA*::*cat*)2 RocR*-L250H	This study
ACB136	[*amyE*::φ(*rocD*$_{WT}$-*lacZ*)1 *cat*]	This study
ACB137	[*amyE*::φ(*rocD*$_{SigA\text{-}P1}$-*lacZ*)1 *cat*]	This study
ACB138	[*amyE*::φ(*rocD*$_{SigA\text{-}P2}$-*lacZ*)1 *cat*]	This study
ACB166	Δ(*ahrC*::*ery*)1	This study
TMB151	Δ(*sigL*::*spc*)1	This study
TMB152	Δ(*proBA*::*cat*)2 Δ(*sigL*::*spc*)1 (SigA-P1 – *rocDEF*)	This study
JSB8	Δ(*proHJ*::*tet*)1	(Brill *et al*., 2011a)
SMB44	Δ(*putBCP*::*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 *trpC*2 *pheA*1 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*::φ(*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 circumventing 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_{578} 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_{578} at a wavelength of 578 nm (OD_{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 [φ(*rocD-lacZ*) *cat*] 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 [Δ(*proA*::*spc*)1], GWB101 [Δ(*proBA::cat*)2], GWB102 [Δ(*proBA*::*tet*)3], ACB166 [Δ(*ahrC*::*ery*)1] and TMB151 [Δ(*sigL*::*spc*)1] (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 were 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 [Δ(*proA*::*spc*)1], GWB101 [Δ(*proBA*::*cat*)2], GWB102 [Δ(*proBA*::*tet*)3], ACB166 [Δ(*ahrC*::*ery*)1], QB5533 [Δ(*rocR*::*aphA3*)], QB5505 [Δ(*sigL*::*aphA3*)] and TMB151 [Δ(*sigL*::*spc*)1] (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$^+$ suppressor mutants from B. subtilis *strains carrying mutations in proA*

Single colonies of strains GWB99 [Δ(*proA*::*spc*)1] and GWB101 [Δ(*proBA*::*cat*)2] 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$^+$ 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 Δ(*proA*::*spc*)1 and Δ(*proBA*::*cat*)2 mutations orginally present in strains GWB99 and GWB101, respectively, were still retained by the Pro$^+$ suppressor strains. This was the case in each of the originally picked 19 independent isolates. Molecular analysis showed that these 19 Pro$^+$ 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$^+$ 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′-AAAGGATCCC AGCGGGTGATAATTGTTGGC-3′) and AC62 (5′-AAACCCG GGTGTATGAACCTCCCTCAATTATTTTC-3′). These PCR products were digested with BamHI and XmaI, and cloned into the BamHI-XmaI 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 [φ(*$rocD_{WT}$'-lacZ*) *cat*], pZAZA17 [φ(*$rocD_{SigA-P1}$-lacZ*) *cat*] and pZAZA18 [φ(*$rocD_{sigA-P2}$-lacZ*) *cat*] was linearized with PstI, 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 the [φ(*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 originally 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] with PCR using DNA primers AC49 (5′-AAAGGATCCCTCTTCAGAAGAAAGAG-3′) and AC50 (5′-AAAAAGCTTCATGCTCGTCTACCAC-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 pRB373 (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.*, 1995; 1997). Total RNA was isolated with the acid-phenol extraction method from log-phase cells (OD_{578} 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′-GTTGGCTCCGTAATGAGACGTCTGAT-3′) labelled at its 5′-end with the fluorescent dye DY-781 (Biomers, Ulm, Germany) was carried out using the Primer extension system –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 DNA 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*::φ(*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; 1997; 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 OD_{578} 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 OD_{578} 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 OD_{578} 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 ^3H_2O and membrane-impermeable inulin-[^{14}C]carboxylic acid (Bakker and Mangerich, 1981; Hoffmann *et al.*, 2013b).

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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.

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