A bacterial checkpoint protein for ribosome assembly moonlights as an essential metabolite-proofreading enzyme

Ankita J. Sachla & John D. Helmann

In eukaryotes, adventitious oxidation of erythrose-4-phosphate, an intermediate of the pentose phosphate pathway (PPP), generates 4-phosphoerythronate (4PE), which inhibits 6-phosphogluconate dehydrogenase. 4PE is detoxified by metabolite-proofreading phosphatases such as yeast Pho13. Here, we report that a similar function is carried out in Bacillus subtilis by CpgA, a checkpoint protein known to be important for ribosome assembly, cell morphology and resistance to cell wall-targeting antibiotics. We find that ΔcpgA cells are intoxicated by glucose or other carbon sources that feed into the PPP, and that CpgA has high phosphatase activity with 4PE. Inhibition of 6-phosphogluconate dehydrogenase (GndA) leads to intoxication by 6-phosphogluconate, a potent inhibitor of phosphoglucose isomerase (PGI). The coordinated shutdown of PPP and glycolysis leads to metabolic gridlock. Over-expression of GndA, PGI, or yeast Pho13 suppresses glucose intoxication of ΔcpgA cells, but not cold sensitivity, a phenotype associated with ribosome assembly defects. Our results suggest that CpgA is a multifunctional protein, with genetically separable roles in ribosome assembly and metabolite proofreading.
The ribosome is an abundant and exceptionally complex structure whose assembly and function dominates bacterial physiology. During rapid growth of bacteria such as *Bacillus subtilis*, the majority of RNA polymerase is engaged in the synthesis of ribosomal RNA (rRNA). Ribosomes comprise up to 50% of cell mass, and translation consumes up to 2/3 of cellular energy. As expected for such an energetically demanding process, ribosome synthesis and assembly is highly regulated, and defects can impose severe fitness costs on cells.

Ribosome assembly requires the efficient processing of the precursor rRNA transcripts followed by the ordered assembly of ribosomal proteins to generate the 30S and 50S subunits. Assembly of the ribosome occurs rapidly in vivo, in a process facilitated by RNA helices, rRNA modification enzymes, and ribosome-assembly GTPases (RA-GTPases). The RA-GTPases are universally conserved proteins that couple GTP hydrolysis to specific checkpoints in assembly. In *B. subtilis*, the six RA-GTPases include RbgA, YphC, and YscC implicated in assembly of the 50S subunit, and Era, Yqeh, and CpgA to facilitate assembly of the 30S subunit. Of these six RA-GTPases, four are essential and mutants in *yqeh* and *cpgA* are growth impaired. CpgA (circularly-permuted GTase) is also important for normal cell morphology, proper deposition of the peptidoglycan sacculus, and intrinsic resistance to antibiotics affecting both the ribosome and cell wall synthesis. Whether or not these various phenotypes are related to the ribosome-assembly role is not resolved.

CpgA is also a target for PrkC, a eukaryotic-like Hanks Ser/Thr kinase with surface-exposed penicillin binding protein and Ser/Thr kinase associated (PASTA) domains implicated in muropeptide sensing. Indeed, *cpgA* is co-transcribed with *prkC* and *prpC*, encoding the cognate phosphatase for PrkC. Using phosphomimetic and phosphoablative variants of CpgA, it has been proposed that phosphorylation of CpgA at Thr166 increases intrinsic GTPase activity, enhances association with 30S ribosomal subunits to aid in ribosome maturation, and is necessary for normal cell morphology. However, whether CpgA phosphorylation affects its activities related to peptidoglycan deposition and antibiotic sensitivity is not clear.

Here, we report studies in which we unexpectedly discovered that CpgA functions as a broad specificity phosphatase that protects cells against the deleterious effects of underground metabolism. A Δ*cpgA* mutant is sensitive to β-lactam antibiotics and is unable to grow in the presence of glucose or gluconate. These metabolic restrictions arise from the inhibition of 6-phosphogluconolactone dehydrogenase (*GndA*) by 4-phosphoerythronate (4PE), an adventitious oxidation product arising from the pentose phosphate pathway (PPP) intermediate erythrose-4-phosphate. 4PE initiates an inhibition cascade in which 6-phosphogluconolactone accumulates and competitively inhibits phosphoglucone isomerase (PGI), leading to metabolic gridlock (Fig. 1), bacteriostasis, and ultimately cell death. CpgA prevents metabolic intoxication by cleansing the metabolite pool of potentially toxic molecules, including 4PE. These results document an unexpected link between CpgA and carbon catabolism that is important in preventing deleterious effects arising from enzyme promiscuity on cell physiology.

![Fig. 1](image-url)
Results

A ΔcpgA mutant is intoxicated by glucose or gluconate. Several substrates of the PrkC kinase/PrpC phosphatase system have been linked to cell wall assembly and intrinsic antibiotic resistance, including WallR, GlmR/YvcK, GpsB, and CpgA. The prpC-prkC-cpgA operon is conserved in the Firmicutes (Fig. 2a), and CpgA is a target for PrkC phosphorylation, suggesting that these proteins may function in the same pathway. We therefore assessed the role of these genes in intrinsic resistance to cefuroxime (CEF), a β-lactam antibiotic that serves as a sensitive indicator of cell wall assembly defects in B. subtilis. We used the BKE/BKK collection of gene disruptants to generate in-frame, clean deletions in prkC and prpC. The resulting ΔprkC strain was more sensitive to CEF than WT, whereas the ΔprpC strain was slightly more resistant (Fig. 2a). The ΔcpgA mutant was slightly more sensitive to CEF than WT, whereas the ΔcpgA ΔprkC strain was slightly more resistant (Fig. 2a). The ΔcpgA mutant was much more sensitive than ΔprkC, indicating that CpgA has functions relevant to cell wall homeostasis independent of PrkC. In liquid LB medium, we noted a 10-fold increase in the sub-lethal concentration of CEF for ΔcpgA strains (~0.01 μg ml⁻¹) as compared with WT (~0.1 μg ml⁻¹) (Supplementary Fig. 1a and b). Moreover, in the ΔcpgA background, mutation of either prkC or prpC no longer had an observable effect on CEF sensitivity (Fig. 2a).

Based on these findings, we conclude that CpgA plays a dominant role in intrinsic CEF resistance, and the more modest effects noted for the PrkC/PrpC system may be due to modification of CpgA.

We used mariner transposon (mTn) mutagenesis to select for colonies with increased CEF resistance in the ΔcpgA parent strain on LB medium. The mTn insertion sites were mapped by sequencing to within or near genes encoding an alternate lipotechoic acid synthase LtaSa (YfnI), the transcription termination factor Rho, endonuclease YazA, and the glucose permease PtsG (Fig. 2b). Transformation experiments confirmed that the mTn insertions were linked to the increased CEF resistance phenotype. The ΔcpgA mutant displayed a three-fold increase in the diameter of the zone-of-growth inhibition with CEF (42 mm vs. 14 mm for WT; Fig. 2b), which correlates well with the 10-fold difference in MIC considering that the diffusion zone (area) increases as the square of the diameter. This phenotype was complemented by reintroduction of CpgA from the parent strain (Supplementary Fig. 1a and b). Moreover, in the ΔcpgA background, mutation of either prkC or prpC no longer had an observable effect on CEF sensitivity (Fig. 2a).

Based on these findings, we conclude that CpgA plays a dominant role in intrinsic CEF resistance, and the more modest effects noted for the PrkC/PrpC system may be due to modification of CpgA.

We used mariner transposon (mTn) mutagenesis to select for colonies with increased CEF resistance in the ΔcpgA parent strain on LB medium. The mTn insertion sites were mapped by sequencing to within or near genes encoding an alternate lipotechoic acid synthase LtaSa (YfnI), the transcription termination factor Rho, endonuclease YazA, and the glucose permease PtsG (Fig. 2b). Transformation experiments confirmed that the mTn insertions were linked to the increased CEF resistance phenotype. The ΔcpgA mutant displayed a three-fold increase in the diameter of the zone-of-growth inhibition with CEF (42 mm vs. 14 mm for WT; Fig. 2b), which correlates well with the 10-fold difference in MIC considering that the diffusion zone (area) increases as the square of the diameter. This phenotype was complemented by reintroduction of CpgA from the parent strain (Supplementary Fig. 1a and b). Moreover, in the ΔcpgA background, mutation of either prkC or prpC no longer had an observable effect on CEF sensitivity (Fig. 2a).

Based on these findings, we conclude that CpgA plays a dominant role in intrinsic CEF resistance, and the more modest effects noted for the PrkC/PrpC system may be due to modification of CpgA.

Next, we tested the ability of a ΔcpgA Δzwf double mutant expressing various zwf alleles to grow on glucose. When the only copy of zwf was present as an IPTG-inducible copy at amyE,

Reduced Zwf activity suppresses ΔcpgA glucose sensitivity. To gain insight into the molecular basis for the observed glucose intoxication, we isolated spontaneous suppressors from within the zone of clearance when ΔcpgA was plated on MH medium with glucose (Fig. 3a). Suppressors were not likely to be null mutations. Suppression was also not observed when a second, IPTG-inducible copy of zwf was induced in the ΔcpgA background (Fig. 3c). We thus hypothesized that these suppressor alleles might encode altered or reduced function G6PDH.

Next, we tested the ability of a ΔcpgA zwf double mutant expressing various zwf alleles to grow on glucose. When the only copy of zwf was present as an IPTG-inducible copy at amyE,
Fig. 2 A ΔcpgA mutant is intoxicated by glucose or gluconate. a Top panel: prpC-prkC-cpgA genomic locus organization and bottom panel: CEF sensitivity of the strains shown as measured for 6 μg of CEF using a disk diffusion assay (8 mm diameter) performed on LB media (n = 10). b The CEF sensitivity (shown as zone of inhibition, ZOI, mm) of the ΔcpgA strain can be partially suppressed by deletions in genes identified by mTn insertions (yfln, rho, yazA, and ptsG) (n = 3). The complemented strain (ΔcpgA pMUTIN4-cpgA) was at locus. Results shown are representative of three-independent biological replicates. c Disk diffusion assays to measure glucose and gluconate sensitivity were determined on MH medium. Under our conditions, 1% glucose (10 mg) is 55 μmols and 1% gluconate is 46 μmols on the filter. d Growth (OD₆₀₀ vs time) for various strains in defined MSMM supplemented with 0.6%(w/v) glucose plus 0.6%(w/v) malate. Growth measurements were recorded at every 15 min. for WT (green line), ΔcpgA (red line) and complement (blue line) cultures growing aerobically at 37 °C for 48 h. e Spot dilution experiments (10 μl) were used to measure cell viability as a function of time after cells growing in MSMM at 37 °C were subjected to 4%(w/v) glucose. f Spot dilution experiments (10 μl) were used to measure cell viability after exposure to 4% gluconate (as for panel e). These experiments are representative of three-independent biological replicates; all images were captured following 18 h of incubation at 37 °C. Significant observations related to CFU fitness are indicated with red boxes in e and f.
(amyE::Pspac(hy)-zwf), the ΔcpgA strain grew on glucose when the construct was not induced, consistent with a small amount of leaky expression from this promoter, but not when 1 mM IPTG was present (Fig. 3c). This suggests that a low level of G6PDH leaky expression from this promoter, but not when 1 mM IPTG was induced, consistent with a small amount of glucose-intoxication. In contrast, when the Δzwf1 Δzwf7 ΔcpgA Δzwf14 strain was needed to support growth, but that higher levels led to a ~63% reduction in Zwf activity (Fig. 3d). Moreover, induction of wild-type Zwf cannot rescue the poor growth of ΔcpgA strain on glucose, whereas induction of several hypomorphic alleles does (Supplementary Fig. 4). Since G6PDH is a dimer, we hypothesized that heterodimer formation might reduce Zwf activity in the merodiploid cells. Mutations in the human Zwf ortholog (G6PDH) give rise to G6PDH deficiency, an X chromosome-linked disease that primarily affects males. These mutations are also often found at the dimer interface and lead to a reduction of enzyme activity. It is not clear whether any of these mutant alleles are dominant since there is stochastic X-chromosome inactivation in females, and therefore the phenotype of cells expressing both WT and mutant alleles is not obvious.

### 6-phosphogluconate intoxicates ΔcpgA cells

Spontaneous suppressors were also recovered from ΔcpgA cells challenged with gluconate (Fig. 4a). In this case, whole genome re-sequencing mapped a suppressor mutation to gntP, a permease involved in gluconate uptake (Fig. 4a; Supplementary Table 1). Similarly, a ΔcpgA gntK double mutant (lacking the gluconokinase responsible for converting D-gluconate to 6-phosphogluconate; Fig. 1) was also tolerant of gluconate (Fig. 4a). These results indicate that gluconate toxicity requires import and phosphorylation. As predicted, mutations in zwf that suppress glucose toxicity did not suppress gluconate toxicity, which enters the PPP downstream of Zwf (Fig. 1).

To test whether growth inhibition was due to accumulation of 6-phosphogluconate or a downstream metabolite, we over-expressed GndA within ΔcpgA cells. Induction of either GndA (the major, NADP-dependent GndA) or the glucose-inducible paralog, GntZ, restored growth of the ΔcpgA mutant strain on modified Spizizen’s minimal medium (MSMM) containing glucose (Fig. 4b, c). We conclude that 6-phosphogluconate, rather than a downstream metabolite, is toxic to cells. Over-expression of GndA also partially suppressed the CEF sensitivity of ΔcpgA, suggesting that CEF sensitivity results, at least in part, from dysregulation of central carbon metabolism (Supplementary Fig. 5).
6-phosphogluconate inhibits PGI. 6-phosphogluconate has been widely studied as a competitive inhibitor of phosphoglucoisomerase (PGI), the initiating enzyme that directs glucose-6-phosphate into glycolysis. PGI is constitutively expressed and is amongst the most abundant proteins during growth on glucose27,28 (Fig. 1). Previously, 6-phosphogluconate was found to be a potent inhibitor of PGI from Bacillus caldotenax with a $K_i$ of 30 μM29. Consistent with the hypothesis that 6-phosphogluconate can inhibit PGI, we found that overproduction of PGI restored the ability of ΔcpgA cells to grow on MSMM with glucose as a sole carbon source (Fig. 4d), with an even greater reduction in lag time than noted for overexpression of GndA or GntZ (Fig. 4b, c). In contrast, overproduction of several other enzymes of central carbon metabolism (including Zwf, YkgB, Rpe, FbaA, GapA, Pgm, Pgk, and Eno) did not restore growth on glucose-6-phosphate, thereby bypassing the need for PGI (Fig. 1).

4PE inhibits GndA. Next, we searched for known inhibitors of GndA activity. In both yeast and mammals, it was recently noted that 4PE is a potent inhibitor of GndA that is degraded by a broad specificity phosphatase30,31. This phosphatase, known as Pho13 (4-nitrophenyl phosphatase) in yeast and PGP (phosphoglycolate phosphatase) in mammals, dephosphorylates 4PE. 4PE is enriched when glyceraldehyde-3-phosphate dehydrogenase (GAPDH) uses the PPP intermediate erythrose-4-phosphate as an accidental substrate30. Although CpgA is not homologous to Pho13/PGP, this led us to hypothesize that CpgA may function as a phosphatase to cleanse the metabolite pool of potentially toxic inhibitors.

To determine if the growth defects of the ΔcpgA strain might be related to 4PE accumulation and consequent inhibition of GndA, we expressed the yeast Pho13 phosphatase in ΔcpgA cells. Induction of Pho13 (with 50 μM IPTG) protected ΔcpgA cells against gluconate-mediated killing (Fig. 5a) and allowed growth in MSMM supplemented with glucose (Fig. 5b). In general, ΔcpgA cells are slow growing even in LB medium, and Pho13 expression restored growth comparable to that of WT. Since Pho13 is specific for the hydrolysis of 4PE30, these results strongly suggest that 4PE is responsible for GndA inhibition in ΔcpgA cells.

CpgA functions as a metabolite-proofreading phosphatase. To determine if CpgA functions as a metabolite-proofreading phosphatase, we used LC-MS to compare intracellular metabolite levels in WT and ΔcpgA cells 30 min. after shift from LB to MSMM containing 0.8% glucose. Strikingly, loss of cpG led to at least a 100-fold increase in intracellular 4PE, which was below the detection limit in WT cells (Fig. 5c, d). In addition, we noted a more modest increase in intracellular accumulation of 6-phosphogluconate (2.2-fold), as well as GTP (1.7-fold) and erythrose-4-P (1.4-fold). This compares with an 8-fold increase in 6-phosphogluconate in yeast lacking Pho1330. In contrast, other phosphorylated intermediates were largely unchanged including hexose-1-phosphates (the assay used did not distinguish glucose-1-phosphate and fructose-1-phosphate). Thus, CpgA may function analogously to Pho13 to dephosphorylate 4PE, and perhaps other metabolites, and thereby prevent inhibition of GndA.
To explore the substrate range of CpgA, we purified the protein and tested a variety of candidate substrates (GTP, G6P, F6P, 6-phosphogluconate, erythrose-4-phosphate, and 4PE) (Fig. 5d). The highest levels of phosphatase activity were observed for erythrose derivatives: erythrose-4-phosphate and 4PE (~25–28 μmole min⁻¹ mg⁻¹ of CpgA). This is nearly identical to the rate measured with Pho13 using 4PE (~30 μmole min⁻¹ mg⁻¹), although Pho13 had very low activity with erythrose-4-phosphate. GTP and 6-phosphogluconate were also moderately good substrates for CpgA (~10–12 μmole min⁻¹ mg⁻¹). Much lower rates were observed for G6P and F6P (~2–3 μmole min⁻¹ mg⁻¹). Previously, a K177A mutation the Walker A motif of CpgA was shown to abolish GTPase activity. Consistent with a central role for the CpgA active site in metabolite proofreading, expression of a CpgA(K177A) mutant did not allow growth on glucose (Supplementary Fig. 3a). These data strongly suggest that CpgA is a broad specificity phosphatase with a preference for erythrose-derived phosphosugars.

These results support a model in which the CpgA protein functions as a metabolite repair phosphatase with activity against the accidental metabolite 4PE. By analogy with the findings in yeast and mammals, 4PE is likely generated by the essential enzyme GapA. In the absence of CpgA, 4PE triggers an inhibition cascade in which 4PE binding to GndA leads to elevated levels of 6-phosphogluconate, a potent inhibitor of PGI. The coordinated inhibition of both glycolysis (PGI) and the PPP (GndA) creates metabolic gridlock, thereby precluding growth in the presence of glycolytic and PPP-dependent carbon sources.
CpgA metabolite proofreading is independent of PrkC. CpgA is encoded as part of a complex operon including the prpC-prkC-cpgA triad of genes (Fig. 2a), an organization conserved in the Firmicutes. Moreover, CpgA has been shown to be phosphorylated by PrkC on Thr166, and this modification increases GTPase activity and association with 70S ribosomes. These findings suggest that PrkC may regulate one or more activities of CpgA. To determine whether or not PrkC-mediated phosphorylation affects the metabolite-proofreading role of CpgA, we replaced cpgA with alleles encoding phosphoablative (CpgA T166A) or phosphomimetic (CpgA T166E) CpgA variants. Strikingly, cells expressing any of the three CpgA variants grew similarly in either LB medium (Supplementary Fig. 6a) or MSMM plus glucose (Supplementary Fig. 6b). In addition, they were equally resistant to CEF as monitored by either disk diffusion (Supplementary Fig. 6c) or liquid culture MIC determinations (Supplementary Fig. 7). Our microscopic observations are consistent with those previously reported, with a subset of cells displaying obvious morphological and cell division defects (Supplementary Fig. 6d). These morphology defects were not affected by phosphorylation status of Thr166 in our investigation.

CpgA functions in two distinct pathways. Cells lacking CpgA are slow growing even in rich medium (LB, MH, MH + 0.4% fructose), and this defect is exacerbated at lower temperatures. Cold-sensitivity is characteristic of ribosome-assembly defects, consistent with the assigned role of CpgA as a RA-GTPase. However, our results suggest that CpgA additionally functions as a metabolite proofreading phosphatase. To determine if these are two distinct functions, or if the reported defects in ribosome assembly might also be related to metabolite intoxication, we tested whether ΔcpgA strains overexpressing either PGI or Pho13 would overcome the cold-sensitivity of parental ΔcpgA strain on LB agar. Although induction of PGI or Pho13 significantly increased the fitness of ΔcpgA cells at 37 °C (Fig. 6a), their expression was unable to rescue cell growth at 30 °C (Fig. 6b). In contrast, complementation with CpgA, which provides both the ribosome assembly and metabolite proofreading functions, was beneficial.

**Fig. 6** CpgA has separable functions in ribosome assembly and metabolite proofreading. Box plot of colony area (n ≥ 45) determined with ImageJ software for various strains (WT in blue, ΔcpgA in red, complement in gray, ΔcpgA expressing Pgi with (orange) and without IPTG (yellow), and ΔcpgA expressing Pho13 with (pink) and without IPTG (green) plated at 37 °C (a) and at 30 °C (b) for 40 h. c All strains were grown to OD₆₀₀ of ~0.4, serially diluted, and spotted onto LB agar plates with incubation at 22 °C, 30 °C, or 37 °C for 40 h. Boxplot depicting centerline represents the second quartile (median) and the bottom and top of the box are the first and third quartiles, respectively and the X inside the box is the mean. Whiskers extend 1.0 quartile range. Outliers are shown as single dots.
functions, fully rescues growth at both temperatures. CpgA, as well as its phosphomimetic and phosphoablative variants, were equivalent in their ability to support growth at a variety of temperatures (Fig. 6c). Although PrkC-dependent phosphorylation might influence the activity of CpgA in ribosome assembly and metabolite proofreading, we have not found conditions where this impact is apparent.

Our analyses support the notion that CpgA is a bifunctional protein, with activities in both ribosome assembly and metabolite proofreading, where the relative importance of these two activities depends on growth conditions. Our genetic suppression studies suggest that both functions may contribute to fitness in the presence of β-lactam antibiotics under the conditions tested. In our original selection for CEF resistance on LB medium, the strongest effect was found for ptsG::mTrn, which likely reduced metabolic stress elicited by import of glucose. However, we also recovered an insertion just upstream of yazzA, encoding a predicted endonuclease. The yazzA gene is co-transcribed with yabB (encoding a tRNA methyltransferase) and yabC (encoding a likely 16 S rRNA methyltransferase). We speculate that alterations in the expression of one or more of these genes may partially compensate for the lack of the ribosome assembly function of CpgA.

In previous work, strong genetic interactions were observed between rsgA (the E. coli cpgA ortholog) and a variety of genes implicated in ribosome assembly34. We postulated that mutations that compensate for the ribosome assembly defect of ΔcpgA cells might be selected if the metabolite proofreading function was provided by the heterologous metabolite-proofreading enzyme Pho13. To test this idea, we selected for suppressor mutations that increased growth of ΔcpgA on medium containing glucose plus gluconate, but with induction of Pho13. In this condition, several of the recovered mutations affected ribosome related functions (e.g. ribosome recycling factor, rRNA methyltransferase) (Supplementary Table 1). Thus, under conditions conducive to 4PE formation (glucose plus gluconate and loss of CpgA), Pho13 can compensate for metabolite intoxication, and we propose that the resulting suppressor mutations in this strain are instead compensating for defects in ribosome assembly.

Discussion

CpgA is one of 17 P-Loop type GTPases encoded in B. subtilis, 12 of which are thought to have functions related to translation (translation factor-related, or TRAFAC, GTPases35). A subset of these, including CpgA, are specifically involved in ribosome assembly and have been designated as RA-GTPases2. Previous genetic studies have revealed that ΔcpgA mutants have a range of phenotypes, many of which are not obviously explained by a defect in ribosome assembly. For example, ΔcpgA mutants are growth compromised, and the severity of the growth defect is dependent on media conditions. Indeed, cpgA was even assigned as an essential gene in early studies8. In addition to the observed growth defects, ΔcpgA mutants are sensitive to a range of antibiotics including both those targeting the ribosome and some cell wall synthesis inhibitors5. While sensitivity to ribosome-targeting antibiotics might be expected for cells with a defect in ribosome assembly, the basis for the sensitivity to cell wall inhibitors is less obvious. Cells depleted for (or lacking) CpgA also display altered cell morphology6.

Here, using forward genetics, we ascribe an unexpected function to CpgA as a broad specificity, metabolite proofreading phosphatase. This function is essential during growth on carbon sources that feed directly into upper glycolysis or the PPP. Indeed, cells lacking CpgA are intoxicated by growth on glucose or gluconate, even when other preferred carbon sources are present (Fig. 2). We ascribe this intoxication to an inhibition cascade in which accidentally generated 4PE inhibits the primary GndA and this leads to an elevation in cellular levels of 6-phosphogluconate, which inhibits the glycolytic enzyme PGI. The simultaneous inhibition of both glycolysis (PGI) and the PPP (GndA) leads to metabolic gridlock and eventually cell death. Evidence in support of this model includes (i) a drastic (>100×) increase in 4PE in cpgA mutant cells after shift to glycolytic carbon sources (Fig. 5c), (ii) suppression of toxicity by overproduction of either GndA, the GndA paralog GntZ, or PGI (Fig. 4b–d), (iii) suppression of toxicity by yeast Pho13, an enzyme that specifically catalyzes 4PE (Fig. 5a, b), and (iv) biochemical evidence that CpgA is a broad specificity phosphatase with 4PE as a preferred substrate (Fig. 5d).

Although B. subtilis uses a wide range of organic compounds as sources of carbon and energy, glucose and malate are preferred carbon sources. During growth on glucose, a significant fraction (~43%) of G6P is diverted to the PPP, while during growth on malate as little as ~8% of flux is allocated to the PPP loop24. In cells lacking CpgA, high flux through the PPP loop during growth on glucose or gluconate results in growth inhibition, even when other preferred carbon sources are present. Our results suggest that the proximal cause of growth inhibition is the loss of PGI activity, since overproduction of PGI effectively suppresses glucose or gluconate toxicity (Fig. 4). Consistently, cells grow comparatively well on sucrose (Supplementary Fig. 3), which provides both glucose-6-phosphate and fructose-6-phosphate, thereby compensating for the inhibition of PGI in the ΔcpgA mutant (Supplementary Fig. 3f; Fig. 1). Interference with PGI activity may also be related to the observed CEF sensitivity in ΔcpgA cells. Indeed, we have previously shown that availability of F6P, a branch point intermediate critical for the production of aminosugars used in peptidoglycan synthesis, can affect CEF sensitivity20.

The generation of 4PE as a toxic metabolite is an example of what is often termed underground metabolism. The enzymes of glycolysis and the PPP are among the most abundant in the cytosol36, and it is increasingly appreciated that these and other metabolic enzymes may sometimes function with alternative substrates. In some cases, these secondary activities may be physiologically beneficial37,38. However, in other cases the products of these adventitious reactions may be toxic or may represent metabolic dead-ends, in which case they may be considered as accidents of metabolism30,39. Recently, the PPP intermediate erythrose-4-P was found to adventitiously react with the promiscuous enzyme GAPDH to generate 4PE, an inhibitor of GndA. Mammals contain a phosphoglycolate phosphatase capable of hydrolyzing 4PE, and in yeast this activity was associated with the ortholog Pho1330,31. These phosphatases thus function as metabolite proofreading enzymes that destroy toxic compounds generated during glycolytic growth. Here, we provide evidence that CpgA has an analogous function.

In E. coli 4PE is not an accidental metabolite, but a precursor used to support the synthesis of vitamin B6 by the deoxyxylulose-5-phosphate (DXP) pathway40. In this pathway, 4PE is synthesized by erythrose-4-P dehydrogenase (Epd), a GAPDH homolog. In contrast, B. subtilis synthesizes vitamin B6 by a short pathway involving two enzymes PdxST, and without formation of 4PE40. Rosenberg et al.41 engineered a B. subtilis strain lacking pdxST to express the last two enzymes of the DXP pathway (PdxH and PdxI), and were then able to evolve strains that could grow in the absence of PLP. Their findings imply that, at least in their evolved strains, B. subtilis produces sufficient 4PE to support the function of the engineered DXP pathway41.

In conclusion, we propose that CpgA is a bifunctional protein with roles in both ribosome assembly and metabolite
proofreading. It is increasingly appreciated that proteins may have two unrelated functions, a phenomenon referred to as protein moonlighting. For example, aconitase functions as an enzyme in the tricarboxylic acid cycle, and also as an RNA-binding protein to regulate gene expression. For CpgA, the relative importance of its two activities depends on both the growth medium and temperature. The metabolite-proofreading role of CpgA is essential for growth of B. subtilis in the presence of glucose or glucanate. In contrast, the role in ribosome assembly seems to be most critical for growth at low temperatures where ribosome assembly is potentially compromised. These findings highlight the perils of assigning protein functions solely based on homology, and serve as a reminder that proteins may participate in multiple, independent pathways.

Methods

Bacterial strains, growth conditions, and media. The strains used in this study are listed in Supplementary Table 2. The primary strains for gene deletions were obtained from the BKE/BKK collection of disruptants containing erythromycin/kanamycin cassettes available from the Bacillus Genetic Stock Center (BGSC). Mutations were moved into B. subtilis 168 and then the antibiotic marker removed using pDR244 plasmid to generate clean, in-frame deletions as described. For ectopic expression, genes were amplified with primers listed in Supplementary Table 3 from B. subtilis subspecies 168 chromosomal DNA using Phusion high-fidelity polymerase (NEB) and subjected to restriction enzyme digestion, purification, and ligation into pre-digested pPL82 vector for propagation in E. coli DH5α on LB medium (Ampicillin) supplemented with 100 μg ml⁻¹ of ampicillin.

Table 3 from location of mTn insertion. The ultra-sonication of cells in organic solvent was performed at 40 °C. Cells were gently scraped and subjected to rinse using 0.9% ammonium acetate in water (pH 4.3) at 37 °C for 30 min. followed by overnight ligation of cohesive ends to generate a circular transposon-gDNA plasmid. The PCR reaction from ends of the mTn performed on chimeric library was further subjected to sequencing analysis to identify the orientation and location of mTn insertion.

SpOT dilutions and survival curves. Overnight cultures grown on LB agar plates were used to inoculate 5 ml liquid LB with aerobic growth at 37 °C until OD₆₀₀ ~ 0.4, then cultures were centrifuged, washed multiple times with phosphate buffered saline, (pH 7.4). The washed cells were then re-suspended into 5 ml of MSM containing 4% glucose, (pH 7.4) or 5 ml of MSM containing 4% of sodium glutamate, (pH 7.4). 100 μl of samples was collected at various time intervals (t = 0, 1, 2, 3, 4, 5, 6, and 7 h) during incubation at 37 °C under 300 rpm shaking conditions. All of the samples were then washed and serial dilutions (10⁻⁰ to 10⁻⁴) using PBS, and 10 μl of diluent along with undiluted samples were spotted onto freshly prepared, square-grid LB agar plates (25 ml). Spotted cultures were air-dried in a laminar-hood for 15 min. and plates were initially grown at 30 °C for 6 h and then were shifted to 37 °C for 72 h. Growth for 6 h and observations were made by counting colony forming units at the end of the incubation period. For phospho-replacement study strains were grown to OD₆₀₀ of 0.4 and were serially diluted and 10 μl of cultures were spotted and air-dried for 15 min. in laminar air flow and plates were incubated at 22 °C, 30 °C, and 37 °C for 40 h and images were capture using standard camera.

Whole genome re-sequencing. Spontaneous suppressor mutants were picked and their phenotype confirmed. Chromosomal DNA was extracted and purified using Qiagen genomic DNA extraction kit. Genomic DNA was quantified by Qubit dsDNA HS assay kit (ThermoFisher scientific) and 50 ng of DNA samples were submitted to Biotechnology Resources at Cornell University core facility (Ithaca, NY). Whole genome sequencing data were performed using Illumina HiSeq 2500 (100 cycles) with paired end reads were analyzed using CLC genomic workbench (Qiagen) to trim, map, and align against the B. subtilis 168 reference genome (Ref Seq: NC_000964.3) to detect SNPs. Results were confirmed using Sanger DNA sequencing.

Zwf enzyme assay. G6P dehydrogenase (Zwf) enzyme activity was measured from crude extracts. Extracts were prepared from 5 ml of cell culture at OD₆₀₀ of ~0.8 harvested in 15 ml of Mid Tissu buffer (7.4) containing 10 μM of G6P and 10 μM of EDTA and sonicated for 60 sec. Briefly, 0.25 mM NAD⁺, 2.5 mM of MgCl₂, and 50 μl of crude bacterial extract in 20 mM dicylgene buffer, pH 9.4 was mixed. The reaction was initiated with 12.5 mM G6P (Sigma) and absorbance at 340 nm was monitored before and after addition using a Synergy H1 plate reader (BioTek Instruments, Inc. VT) as a function of time.

Phosphate (P) release assay. N-terminal histidine-tagged CpgA was purified as described by Absalon and coworkers. Then, His-tagged CpgA was dialyzed in 20 mM HEPES (pH 8) buffer, and protein concentration was determined using Bradford colorimetric assay kit. In a 96 well plate, P, release was measured using 10 μM CpgA with 1 mM of substrates: gluonosine-5'-triphosphate (Roche), D-glucose-6-phosphate (Sigma), D-fructose-6-phosphate (Sigma), D-glucanate-6-phosphate (Sigma), D-erythrose-4-phosphate (Sigma), and D-erythronate-4-phosphate (Santa Cruz Biotechnology), in a 100 mM MOPS buffer (pH 7.5) containing 1 mM MgCl₂. The reactions were incubated at 37 °C for 20 min. and released phosphates were measured spectroscopically at 620 nm by adding 25 μl of molybdate green staining reagent (Sigma-Aldrich, MAK308) incubated for 30 min in room temperature.

Metabolite extraction. For metabolite measurements, cell cultures of cpgA mutant and its isogenic B. subtilis 168 WT strains were grown aerobically in LB media to OD of 0.6 at 37 °C. In total, 40 ml was centrifuged and subjected to several washing steps using PBS and cells were resuspended in MSM containing 0.8% glucose, and cells were further incubated at 37 °C for 30 min. Cells were vacuum filtered, and the filter was dropped into quenching solution of 60% methanol containing 0.9% ammonium bicarbonate held at ~4 °C. Cells were gently scraped and washed-off of membrane filters and subjected to rinse using 0.9% ammonium bicarbonate (4°C). Recovered cells were then transferred to 70% methanol solution pre-cooled to ~40 °C. The ultra-sonication of cells in organic solvent was performed on ice (at power 30 W) for 5 min and cell debris was subjected to centrifugation at 10,000 x g for 15 min. at 4°C. The supernatant was collected and vacuum dried and was stored at ~80 °C until further use.

Metabolite detection. Samples were re-suspended with 150 μl 60% ACN prior to LC/MS analysis. Chromatographic separation was performed on a Vanquish UPLC system with a SeQuant ZIC-PHILIC column (5 μm, 2.1 x 150 mm) coupled to a Q Exactive Hybrid Quadrupole-Orbitrap High Resolution Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of (A) 10 mM ammonium acetate in water (pH = 9.8), 0.1% formic acid and (B) acetonitrile. The gradient was as follows: 0–15 min, 90–30% solvent B; 15–18 min, 90–10% solvent B; 18–18.5 min, 30–90% solvent B; 18.5–20 min, 90% solvent B; followed by 3 min of re-equilibration of the column before the next run. The flow rate was 250 μl min⁻¹ and the Injection volumes were set to 2 μl. All of the samples were analyzed by negative electrospray ionization (ESI) in full scan MS mode. Nitrogen as sheath, auxiliary, and sweep gas was set at 50, 8, and 1, respectively. Over the following resolution, 120,000 full width at half maximum; automatic gain control target, 3 x 10⁶ ions; maximum injection time, 100 ms; scan range, 67–1000 m/z (mass to charge ratio); spray...
References

1. Neidhardt, F. C., Schaechter, M. Physiology of the Bacterial Cell A Molecular Approach. 506 (Sinauer, Sunderland, MA, 1990).

2. Britton, R. A. Role of GTPases in bacterial ribosome assembly. Annu. Rev. Microbiol. 63, 155–176 (2009).

3. Gulati, M., Jain, N., Anand, B., Prakash, B. & Britton, R. A. Mutational analysis of the ribosome assembly GTPase RhoB provides insight into ribosome interaction and ribosome-stimulated GTPase activation. Nucleic Acids Res. 41, 3217–3227 (2013).

4. Ni, X. et al. YphC and YscX GTPases assist the maturation of the central protuberance, GTPase associated region and functional core of the 50S ribosomal subunit. Nucleic Acids Res. 44, 8442–8455 (2016).

5. Campbell, T. L., Daigne, D. M. & Brown, E. D. Characterization of the Bacillus subtilis GTPase YloQ and its role in ribosome function. Biochem. J. 389, 843–852 (2005).

6. Pompeo, F. et al. Phosphorylation of CpgA protein enhances both its GTPase activity and its affinity for ribosome and is crucial for Bacillus subtilis growth and morphology. J. Biol. Chem. 287, 20830–20838 (2012).

7. Morimoto, T. et al. Six GTP-binding proteins of the Era/Obg family are essential for cell growth in Bacillus subtilis. Microbiology 148, 3539–3552 (2002).

8. Uicker, W. C., Schafer, L. K., Koenigsknecht, M. & Britton, R. A. The essential GTPase Yqeh is required for proper ribosome assembly in Bacillus subtilis. J. Bacteriol. 189, 2926–2929 (2007).

9. Hunt, A., Rawlins, J. P., Thomaides, H. B. & Errington, J. Functional analysis of 11 putative essential genes in Bacillus subtilis. Microbiology 152, 2905–2907 (2006).

10. Absalon, C. et al. The GTPase CpgA is implicated in the deposition of the peptidoglycan sacculus in Bacillus subtilis. J. Bacteriol. 190, 3786–3790 (2008).

11. Shah, I. M., Laaberki, M. H., Popham, D. L. & Dworkin, J. A eukaryotic-like Ser/Thr kinase signals bacterial exit dormancy in response to peptidoglycan fragments. Cell 135, 486–496 (2008).

12. Squeglia, F. et al. Chemical basis of peptidoglycan discrimination by PrkC, a key kinase involved in bacterial resuscitation from dormancy. J. Am. Chem. Soc. 133, 20676–20679 (2011).

13. Pompeo, F., Foulquier, E. & Galinier, A. Impact of Serine/Threonine Protein Kinases on the Regulation of Sporulation in Bacillus subtilis. Front. Microbiol. 7, 588 (2016).

14. Iwaniwki, A., Hinc, K., Seror, S., Wegrzyn, G. & Obuchowski, M. Transcription in the prpC-yloQ region in Bacillus subtilis. Arch. Microbiol. 183, 421–430 (2005).

15. Libby, E. A., Goss, L. A. & Dworkin, J. The Eukaryotic-Like Ser/Thr Kinase PrkC Regulates the Essential WalRK Two-Component System in Bacillus subtilis. PLoS Genet. 11, e1005275 (2015).

16. Foulquier, E. et al. PrkC-mediated phosphorylation of overexpressed YvcK protein regulates PRP1 protein localization in Bacillus subtilis mreB mutant cells. J. Biol. Chem. 289, 23662–23669 (2014).

17. Pompeo, F., Foulquier, E., Serrano, B., Grangeasse, C. & Galinier, A. Phosphorylation of the cell division protein GpsS regulates PrkC kinase activity through a negative feedback loop in Bacillus subtilis. Mol. Microbiol. 97, 139–150 (2015).

18. Absalon, C. et al. CpgA, EF-Tu and the stressosome protein YezB are substrates of the Ser/Thr kinase/phosphatase couple, PrkC/PrpC, in Bacillus subtilis. Microbiology 155, 932–943 (2009).

19. Manuse, S., Fleuere, A., Zucchini, L., Lesterlin, C. & Grangeasse, C. Role of eukaryotic-like serine/threonine kinases in bacterial cell division and morphogenesis. FEBS Microbiol. Rev. 40, 41–56 (2016).

20. Patel, V., Wu, Q. & Chandrasangsu, P. A metabolic checkpoint protein GlmR is important for diverting carbon into peptidoglycan biosynthesis in Bacillus subtilis. PLoS Genet. 14, e1007689 (2018).

21. Koo, B. M. et al. Construction and analysis of two genome-scale deletion libraries for Bacillus subtilis. Cell Syst. 4, 291–305 e7 (2017).

22. Vagner, V., Deryvn, E. & Ehrlich, S. D. A vector for systematic gene inactivation in Bacillus subtilis. Microbiology 144( Pt 11), 3097–3104 (1998).

23. Sezovon, G., Joseleau-Petit, D. & D’Ari, R. Escherichia coli physiology in Luria-Bertani broth. J. Bacteriol. 189, 8746–8749 (2007).

24. Klein, R. J. et al. Metabolic fluxes during strong carbon catabolite repression by malate in Bacillus subtilis. J. Biol. Chem. 285, 1587–1596 (2010).

25. Scopes, D. A., Bautista, J. M., Naylor, C. E., Adams, J. M. & Mason, P. J. Amino acid substitutions at the dimer interface of human glucose-6-phosphate dehydrogenase that increase thermostability and reduce the stabilising effect of NADP. Eur. J. Biochem. 251, 382–398 (1998).

26. Cappellini, M. D. & Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. Lancet 371, 64–74 (2008).

27. Ludwig, H. et al. Transcription of glycolytic genes and operons in Bacillus subtilis: evidence for the presence of multiple levels of control of the gapA operon. Mol. Microbiol. 41, 409–422 (2001).

28. Eymann, C. et al. A comprehensive proteome map of growing Bacillus subtilis cells. Proteomics 4, 2849–2876 (2004).

29. Takama, M. & Nosoh, Y. Effect of ATP on glucose-6-phosphate isomerase from Bacillus caldothermus. Biochim. Biophys. Acta 705, 127–130 (1982).

30. Collard, F. et al. A conserved phosphatase destroys toxic glycolytic side ribosome products in mammals and yeast. Nat. Chem. Biol. 12, 801–807 (2016).

31. Beaudoin, G. A. & Hanson, A. D. A Guardian Angel Phosphatase for Mainline Carbon Metabolism. Trends Biochem. Sci. 41, 893–894 (2016).

32. Cladire, L. et al. The GTPase, CpgA(YloQ), a putative translation factor, is implicated in morphogenesis in Bacillus subtilis. Mol. Genom. Genet. 275, 420 (2006).

33. Dammel, C. S. & Noller, H. F. Suppression of a cold-sensitive mutation in 16s rRNA by overexpression of a novel ribosome-binding factor, RfbA. Genes Dev. 9, 626–637 (1995).

34. Campbell, T. L. & Brown, E. D. Genetic interaction screens with ordered overexpression and deletion clone sets implicate the Escherichia coli GTPase YieQ in late ribosome biogenesis. J. Bacteriol. 190, 2537–2545 (2008).

35. Levdikov, V. M. et al. Comprehensive absolute quantification of the cytosolic proteome of Bacillus subtilis by data independent, parallel fragmentation in liquid chromatography/mass spectrometry (LC/MS(E)). Mol. Cell. Proteom. 13, 1008–1019 (2014).

36. Munzel, J. et al. Comprehensive absolute quantification of the cytosolic proteome of Bacillus subtilis by data independent, parallel fragmentation in liquid chromatography/mass spectrometry (LC/MS(E)). Mol. Cell. Proteom. 13, 1008–1019 (2014).

37. Rosenberg, J. & Cimmich, F. M. Harnessing Underground Metabolism for Pathway Development. Trends Biotechnol. 37, 29–37 (2018).

38. Notebaert, R. A., Kintses, B., Feist, A. M. & Papp, B. Underground metabolism: network-level perspective and biotechnological potential. Curr. Opin. Biotechnol. 49, 108–114 (2016).

39. Danchin, A. Coping with inevitable accidents in metabolism. Microb. Biotechnol. 10, 57–72 (2017).

40. Mukherjee, T., Hanes, J., Tews, I., Ealick, S. E. & Begley, T. P. Pyridoxal phosphate: biosynthesis and catabolism. Biochim. Biophys. Acta 1814, 1585–1596 (2011).
41. Rosenberg, J., Yeak, K. C. & Commichau, F. M. A two-step evolutionary process establishes a non-native vitamin B6 pathway in *Bacillus subtilis*. *Environ. Microbiol.* **20**, 156–168 (2018).
42. Jeffery, C. J. An introduction to protein moonlighting. *Biochem. Soc. Trans.* **42**, 1679–1683 (2014).
43. Mascher, T., Hachmann, A. B. & Helmann, J. D. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. *J. Bacteriol.* **189**, 6919–6927 (2007).
44. Le Breton, Y., Mohapatra, N. P. & Haldenwang, W. G. In vivo random mutagenesis of *Bacillus subtilis* by use of TnYLB-1, a mariner-based transposon. *Appl. Environ. Microbiol.* **72**, 327–333 (2006).
45. Ujita, S. & Kimura, K. Glucose-6-phosphate dehydrogenase, vegetative and spore *Bacillus subtilis*. *Methods Enzymol.* **89**(Pt D), 258–261 (1982).
46. Zhu, B. & Stülke, J. SubtiWiki in 2018: from genes and proteins to functional network annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Res.* **46**, D743–D748 (2018).

**Acknowledgements**

This work was supported by National Institutes of Health R35GM122461 awarded to JDH. We are thankful to Diana Herrera, Gumpanat Mahipant, Bacillus Genetic Stock Center, and SubtiWiki database for support. We acknowledge Biotechnology Resources at Cornell (BRC) metabolomics facility for help with metabolite measurements, and Dr. Frédérique Pompeo (CNRS-Laboratoire de Chimie Bactérienne) for the gift of strains harboring pOMG360 construct, which was originally constructed by the Séror lab.

**Author contributions**

Conception, A.J.S. and J.D.H.; Designed and performed experiments, A.J.S.; Manuscript drafted and edited, A.J.S. and J.D.H.

**Additional information**

**Supplementary Information** accompanies this paper at https://doi.org/10.1038/s41467-019-09508-z.

**Competing interests:** The authors declare no competing interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Journal peer review information:** Nature Communications thanks Jörg Stülke and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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

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

© The Author(s) 2019