An activator for pyruvoyl-dependent \( \alpha \)-aspartate \( \alpha \)-decarboxylase is conserved in a small group of the \( \gamma \)-proteobacteria including \textit{Escherichia coli}

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**Abstract**

In bacteria, \( \beta \)-alanine is formed via the action of \( \alpha \)-aspartate \( \alpha \)-decarboxylase (PanD) which is one of the small class of pyruvoyl-dependent enzymes. The pyruvoyl cofactor in these enzymes is formed via the intramolecular rearrangement of a serine residue in the peptide backbone leading to chain cleavage and formation of the covalently-bound cofactor from the serine residue. This reaction was previously thought to be uncatalysed. Here we show that in \textit{Escherichia coli}, PanD is activated by the putative acetyltransferase YhhK, subsequently termed PanZ. Activation of PanD both in vivo and in vitro is PanZ-dependent. PanZ binds to PanD, and we demonstrate that a PanZ(N45A) site-directed mutant is unable to enhance cleavage of the proenzyme PanD despite retaining affinity for PanD. This suggests that the putative acetyltransferases domain of PanZ may be responsible for activation to enhance the processing of PanD. Although \( \text{panD} \) is conserved among most bacteria, the \( \text{panZ} \) gene is conserved only in \textit{E. coli}-related enterobacterial species including \textit{Shigella}, \textit{Salmonella}, \textit{Klebsiella} and \textit{Yersinia}. These bacteria are found predominantly in the gut flora where pantothenate is abundant and regulation of PanD by PanZ allows these organisms to closely regulate production of \( \beta \)-alanine and hence pantothenate in response to metabolic demand.

**Introduction**

Pantothenate (vitamin B5) is required in all organisms for the synthesis of coenzyme A (CoA) and acyl carrier proteins, however its biosynthesis is limited to prokaryotes, fungi and plants in which it is produced from D-pantoate and \( \beta \)-alanine (Webb et al. 2004; Fig. 1A). In bacteria, \( \beta \)-alanine is synthesized via decarboxylation of \( \alpha \)-aspartate, a reaction catalyzed by the pyruvoyl-dependent enzyme \( \alpha \)-aspartate \( \alpha \)-decarboxylase (ADC). ADC-dependent synthesis of \( \beta \)-alanine is the sole source of \( \beta \)-alanine and deficiency in ADC \( \beta \)-alanine therefore leads to \( \beta \)-alanine auxotrophy which can be relieved with either \( \beta \)-alanine or pantothenate. In \textit{Escherichia coli}, supplementation of culture media with pantothenate or \( \beta \)-alanine markedly increases the cellular level of CoA, whereas supplementation with excess pantoate is much less effective (Cronan et al. 1982). This demonstrates that \( \beta \)-alanine supply is limiting for pantothenate biosynthesis in this organism. Regulation of ADC activity is therefore central to the regulation of intracellular pantothenate and CoA concentrations.

The enzyme is a member of the small class of pyruvoyl-dependent enzymes, which contain a covalently-bound pyruvoyl cofactor (Williamson and Brown 1979). This enzyme class includes human \( S \)-adenosyl methionine decarboxylase, human phosphatidylserine decarboxylase and histidine decarboxylase from \textit{Lactobacillus} 30a (van Poelje and Snell 1990). For all of these enzymes, the protein is initially translated as an inactive proenzyme (designated the \( \pi \)-protein) subsequently is activated via post-translational modification. A serine residue in the peptide backbone rearranges to generate an ester intermediate which subsequently cleaved to generate the \( \alpha \)-subunit and \( \beta \)-subunit observed in the active
Figure 1. Supplements for growth defects of the ΔyhhK mutant and in vivo and in vitro assay for cleavage of panD. (A) Biosynthetic pathway of pantothenate and coenzyme A. (B) Growth of the ΔpanZ (ΔyhhK) mutant on agar plates of the M9 glucose medium with or without supplements is indicated above each panel. Cell culture overnight was applied at a series of dilutions of 1:10. These cells were spotted on the plates and incubated at 37°C for 2 days. Wild-type; MG1655, ΔpanZ; SN202, ΔpanZ/vector; SN202 harboring pCA24N(-GFP), ΔpanZ/His-panZ; SN202 harboring pCA24N(-GFP)-his-panZ. (C) Growth of the panD-flag strain on agar plates of the M9 glucose medium with or without β-alanine. The ΔpanD strain was used as negative control. Wild-type; MG1655, ΔpanD; SN208, ΔpanD-flag; SN208. (D) In vivo cleavage of panD-FLAG was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblot analysis using anti FLAG antibody. Wild type; MG1655, panD-flag; SN208, ΔpanZ, panD-flag; SN216, pCA24N(-GFP)-his-panZ expressing His-PanZ are designated his-PanZ. Expression of his-PanZ was induced by IPTG. (E) In vitro cleavage of purified panD-FLAG was analyzed by SDS-PAGE and detected by immunoblot analysis using anti FLAG (Upper) or anti His antibody (Lower). Purified His-PanZ (40 μmol/L) and PanD-FLAG (2.5 μmol/L) are shown in lane 1, 2, respectively. Purified PanD-FLAG (2.5 μmol/L) was incubated at 37°C for 3 h with or without His-PanZ.
enzyme. Cleavage of the peptide chain generates a dehydro-
alanyl residue at the N-terminus of the α-subunit which
hydrolyses to generate the pyruvoyl group (van Poelje and
Snell 1990; Schmitzberger et al. 2003). In all cases, cleavage
of the α-chain is autocatalytic; the π-chain of purified E.
coli PanD is activated to generate the α-subunit and the
β-subunit of ADC by auto-activation in vitro (Ramjee
et al. 1997). Auto-activation of purified E. coli PanD is
however slow; although activation occurs to some extent
after incubation at 37°C for 24 h, stoichiometric activation
requires higher temperatures or longer incubation times.
Considering the doubling time of E. coli cells, which is
about 20 min at 37°C under optimal conditions, this rate
for activation of PanD to generate ADC is relatively slow
compared with in vivo. Naturally, it was therefore
proposed that there might be a specific catalyst for proenzyme
processing in vivo (Ramjee et al. 1997). Activation of pyru-
voyl-dependent S-adenosylmethionine decarboxylase is
stimulated by addition of putrescine and spermidine, these
molecules react with the product of S-AdometDC, and
thereby can regulate its production. More recently it has
been found that cleavage of histidine decarboxylase, is acti-
vated by a protein activator, HdcB, which acts enzymati-
cally in maturation (Trip et al. 2011). However, no such
activator has been identified for ADC.

Reinvestigation of the genetic analysis of mutants
requiring pantothenate suggested the existence of an activ-
ator in Salmonella typhimurium and E. coli. Mutations
generating β-alanine auxotrophy were identified in the
1970s and 1980s. These mutations were mapped to two
different chromosomal positions at 5 min and 89 min in
Salmonella typhimurium (Ortega et al. 1975; Primerano
and Burns 1983). The gene encoding ADC, PanD, was
mapped at 5 min in S. typhimurium and genetically
linked to panB and panC, which encode the two other
essential enzymes for pantothenate biosynthesis (Primer-
ano and Burns 1983). In E. coli, PanD was mapped at
3 min and was closely linked to panC (Cronan 1980) cor-
responding, therefore, to the mutation in S. typhimurium
at 5 min. However, the second mutation at 89 min was
forgotten for decades without further study, during which
time it had been thought that the metabolic pathway of
pantothenate was completely elucidated in bacteria (Webb
et al. 2004). Here we report that this “forgotten muta-
tion” is required for activation of ADC.

Results

Requirement of β-alanine for pantothenate
synthesis in the yhhK gene deletion mutant

A gene-knockout library of E. coli strains revealed a num-
ber of gene knock-out mutants which, while essential for
growth of E. coli in minimal synthetic media (Baba et al.
2006; Joyce et al. 2006), are dispensable in nutrient-rich
media. This media-dependent growth inhibition suggests
that these knock-out mutations cause nutritional deficien-
cies. However, in most cases, the biological functions of
these genes remain unknown. One such gene knock-out
mutant is ΔyhhK. Supplementation of growth media with
pantothenate restored the growth of ΔyhhK cells even in
synthetic minimal media (Adams et al. 1990); it seemed
likely therefore that ΔyhhK cells are deficient in the bio-
synthetic pathway for pantothenate. We examined which
intermediate during pantothenate synthesis was required
for normal growth (Fig. 1B). Pantothenate is generated
via the condensation of β-alanine and pantoate. Supple-
mentation of the growth medium with β-alanine, but not
with pantoate, restored the growth of ΔyhhK cells,
whereas supplementation with l-aspartate, the precursor
of β-alanine, did not. Complementation of ΔyhhK cells,
using a plasmid encoding a his-tagged YhhK, efficiently
restored the normal growth of ΔyhhK cells. We therefore
concluded that biosynthesis of β-alanine is prevented by
the knock-out mutation of yhhK. In bacteria, β-alanine is
formed by decarboxylation of l-aspartate. This enzymatic
reaction is catalyzed by l-aspartate-α-decarboxylase
(ADC), encoded by PanD (Webb et al. 2004). In E. coli,
the locus of yhhK does not correspond to the locus of
PanD. In S. typhimurium, two loci for β-alanine auxotro-
phy have been identified, one corresponding to PanD and
the other located at 89 min. The genetic configuration of
E. coli and S. typhimurium chromosomes resemble each
other, and so we theorized that the map position of the
latter mutant in S. typhimurium might correspond to that
of yhhK in E. coli, which was indeed the case. We will
therefore henceforth refer to yhhK as PanZ.

Activation of cleavage of PanD by PanZ
(YhhK) for the maturation of ADC

How does the PanZ gene product influence the decarbox-
ylation of l-aspartate to generate β-alanine? We hypothe-
sized that the conversion of PanD to an active enzyme
could be accelerated by PanZ. To assess whether PanZ is
an activator for the maturation of ADC, we examined the
cleavage of PanD in ΔPanZ cells. To detect cleavage
of PanD in vivo, FLAG peptides were added to the C-termi-
minus of PanD (PanD-FLAG). The growth rate of the cells
with PanD-FLAG was slower on minimal synthetic
medium as compared to wild type cells, but they did not
require β-alanine as a supplementary nutrient (Fig. 1C)
demonstrating that PanD-FLAG is a functional enzyme. In
PanZ+ cells, two bands at 18 kDa and 15 kDa correspond-
ing to the unactivated PanD gene product and the α-chain
of the activated ADC were detected by Western blotting.
In vitro, it appears that this process is not catalytic. PanD is cleaved in a PanZ-dependent manner in vivo and insufficient to fully activate PanD-FLAG. Thus, while (3 h) however, addition of 16 equivalents of His-PanZ is complete activation of PanD-FLAG. On the timescale shown (Fig. 1E). However, addition of His-panz leads to complete activation of PanD-FLAG. On the timescale shown (3 h) at 37°C, PanD-FLAG on its own is unactivated (Fig. 1E). However, addition of His-panz leads to complete activation of PanD-FLAG. On the timescale shown (3 h) however, addition of 16 equivalents of His-PanZ is insufficient to fully activate PanD-FLAG. Thus, while PanD is cleaved in a PanZ-dependent manner in vivo and in vitro, it appears that this process is not catalytic.

**Involvement of the GNAT domain in PanD cleavage**

Bioinformatics analysis showed that PanZ is a putative N-acetyltransferase and a member of the GNAT (Gcn5-related N-acetyltransferase) superfamily of acetyltransferases (Fig 2 A). Indeed, the structure of PanZ (YhhK) bound to CoA has been solved using nuclear magnetic resonance (NMR, PDB ID: 2k5t). We used ITC to confirm the interaction between PanZ and CoA, and also acetyl CoA (data not shown). Several residues in the acetyltransferase domain are well conserved in the GNAT superfamily between bacteria and humans. Residue Asn45 is one such residue, and it is completely conserved in those bacterial species which have the PanZ gene. We constructed a single mutation in PanZ at one of these positions to generate a N45A site-directed mutant, this mutant PanZ(N45A) could not compensate for the requirement for β-alanine in ΔPanZ cells (Fig 2B).

To confirm that this was due to a deficiency in the catalytic activity of PanZ(N45A), we tested cleavage of PanD using purified proteins. Native PanD was purified from cell lysates that overproduced PanD (Fig 2C). In contrast, PanZ and PanZ(N45A) proteins were purified using his-tags and affinity purification (Fig 2D). After incubation of a mixture including purified PanD and PanZ-His, cleaved fragments of PanD were detected by SDS gel electrophoresis (Fig. 2E). This cleavage reaction was independent of CoA or acetyl-CoA, and no change in the cleavage reaction could be detected by the addition of either (data not shown). However, the cleavage reaction could not be detected when PanZ(N45A)-His was used in place of PanZ-His; the single amino acid substitution in the GNAT domain prevents PanZ from activating PanD. Hence, the GNAT domain is responsible for maturation of PanD to ADC.

**yhhK is conserved only among γ-proteobacteria**

Orthologous genes encoding ADC are conserved among almost all bacterial species, whereas homologous genes of PanZ are found only in some members of the γ-proteobacteria including E. coli (Fig. 3A).

The observation of PanD in bacterial species lacking PanZ homologues suggests that other activating factors might be involved in the activation of PanD or that, in these organisms, auto-activation of PanD alone might produce active ADC. We tested whether PanD derived from *Bacillus subtilis*, which lacks a PanZ homologue, could suppress the β-alanine auxotrophy of *E. coli* ΔPanZ cells. The cloned PanD gene of *B. subtilis* (PanDBS) was substituted for PanD on the *E. coli* chromosome. *E. coli* PanZ⁺ cells in which PanD is replaced with PanDBS grew as well as wild type *E. coli* in M9 glucose medium (Fig. 3B). The same substitution in *E. coli* ΔPanZ generates cells that can also grow without pantothenate supplementation. These results suggest that activation of PanD⁰⁰ does not require PanZ. We confirmed the cleavage of PanD⁰⁰ in *E. coli*. A C-terminal FLAG tag was added to the chromosomal PanD⁰⁰ and cell extracts were monitored by western blotting. Two bands at ~17 kDa (corresponding to the PanD⁰⁰ proenzyme) and ~14 kDa (the activated z-subunit) were observed in both panZ⁺ and ΔPanZ cells (Fig. 3C). Although the proenzyme form of PanD⁰⁰-FLAG was observed in the cell extract, almost all of the proenzyme form was completely changed to the cleaved form during purification (data not shown) preventing study of the activation of PanD⁰⁰ in vitro.

**Physical interaction of PanD with PanZ**

Acceleration of PanD activation by PanZ suggests that non-covalent interaction of the proteins may be required. We examined the protein-protein interaction between PanZ and PanD in vivo using a bacterial two-hybrid assay (Karimova et al. 1998). Each of PanZ and PanD were fused to fragments of the catalytic domain of adenylate cyclase (T25 or T180). Association between PanZ and PanD leads to association of the fragments of adenylate cyclase, further leading to cAMP production which subsequently induces expression of lacZ. Homotetramerization of PanD was used as a positive control (Ramjee et al. 1997; Albert et al. 1998); cells that co-expressed...
T25-PanD and T18-PanD fusions appeared dark blue when grown on X-gal media (Fig. 4). This intense color reflects the strong interactions within the homotetramer – boiling in the presence of SDS is required to fully dissociate the PanD tetramer (Ramjee et al. 1997). Co-expression of either the T25-PanD and the T18-PanZ fusion, or the T18-PanD and the T25-PanZ fusion led to generation of a pale blue color (Fig. 4A) indicating an interaction between PanZ and PanD (though weaker than the very strong PanD tetramerization). Finally, co-expression of T25-PanZ and T18-PanZ leads to a very pale blue color suggesting multimerisation of PanZ is occurring, although however this could be a PanD tetramer mediated interaction.

Physical interaction between PanZ and PanD in vitro was then confirmed by gel filtration with purified PanZ and PanD. Although PanZ-His and PanD have similar molecular weights, 15.6 kDa and 13.8 kDa respectively, retention time of the purified protein, PanZ-His and PanD are distinctive (Fig. 5A and B). Rather, the retention time of PanD corresponds to a tetramer. After co-incubation of PanZ-His and PanD, both proteins co-eluted together with an earlier retention time (Fig. 5D). Analysis by gel electrophoresis indicated that PanD had been cleaved and the PanZ-His was co-purified with the cleaved PanD. This suggests that PanZ tightly binds to the PanD tetramer even after maturation of the inactive proenzyme. In order to confirm the affinity of PanZ(N45A)-His for the unactivated proenzyme form of PanD, the purified PanZ(N45A)-His was also eluted using gel filtration chromatography (Fig. 5C and E). PanZ (N45A)-His co-purified with PanD, suggesting that the mutant PanZ still retains the ability to bind to PanD. However, the processing of the unactivated proenzyme form of PanD was completely inhibited. Thus, the mutated residue is critical for cleavage of the proenzyme

Figure 2. Effect of a single mutation in the GNAT domain on cell growth and in vitro cleavage of panD. (A) Amino acid sequence and a schematic structure of PanZ with the position of the point mutation in GNAT family domain. (B) Complementation of phenotype of ΔpanZ by panZ(N45A)-his. Growth of ΔpanZ derivative strains on agar plates of M9 glucose medium with or without β-Alanine. Wild-type; MG1655/pBAD24, ΔpanZ; SN202/pBAD24, ΔpanZ/Vector; SN202/pBAD24-panZ-his, ΔpanZ/panZ-his; SN202/pBAD24-panZ-his, ΔpanZ/panZ(N45A)-his; SN202/pBAD24-panZ(N45A)-his. (C) Purification of PanD. Lane 1; total cell lysate, lane2; total cell lysate after addition of IPTG, lane3; soluble fraction of cell lysate, lane4; fraction of 40–50% ammonium sulphate precipitation, lane5; fraction of anion-exchange column purification. (D) Purification of PanZ-His and PanZ(N45A)-His. Lane1; total cell lysate after addition of IPTG, lane2; soluble fraction, lane3; fraction of 50–80% ammonium sulphate precipitation, lane4; fraction of Ni-sepharose column purification. (E) In vitro cleavage of purified PanD. Purified proteins were incubated at 37°C for 60 min in 50 mmol/L HEPES/KOH, 100 mmol/L potassium acetate, 1 mmol/L EDTA, pH 7.6 and subjected to SDS-PAGE and CBB staining.
rather than perturbing its affinity for the substrate. Addition of CoA or acetyl-CoA did not affect the elution of PanD, PanZ and the mixture as before (data not shown).

Discussion

The putative acetyltransferase PanZ is crucial for the activation of the proenzyme PanD in vivo. This function of PanZ appears to not be catalytic, but rather to provide a cofactor to stimulate self-activation of PanD. Although PanZ can bind to CoA and acetyl-CoA, we could not detect any influence of the addition of CoA and acetyl-CoA to the in vitro reaction. PanZ with the single mutation of N45A demonstrates that the well conserved feature of this domain between the GNAT family domain, which can bind CoA, is involved in enhancing the self-processing of PanD. This suggests that binding of a CoA derivative to PanZ might regulate activation of the processing of proenzyme PanD in vivo. It is possible that a CoA derivative has been co-purified with PanZ and is therefore present in our experiments to investigate the interaction between PanZ and PanD. More precise biochemical measurements are therefore needed to observe the influence of CoA and acetyl-CoA on the in vitro reaction.

Is it possible and physiologically significant that somehow CoA or acetyl-CoA binding regulates the activation of PanD in the generation of ADC? As CoA is derived from pantothenate, we would therefore expect CoA to negatively regulate the synthesis of pantothenate. Such a regulatory pathway would provide a second negative feedback in the pantothenate synthetic pathway. In most organisms except archaea, pantothenate kinase, CoaA, phosphorylates pantothenate to produce phosphopantothenate (Brown 1959) (Fig. 6A). CoaA activity is regulated by CoA in a negative feedback manner (Vallari and Rock 1987; Vallari and Jackowski 1988). Inhibition of CoaA leads to accumulation of pantothenate and the excess pantothenate is secreted from the cells via the sodium-dependent transporter PanF (Jackowski and Rock 1981; Vallari and Rock 1985). This

Figure 3. Evolutionary relationships in the panD and panZ families. (A) Phylogenetic tree of panD in representative bacterial species. A gray box indicates that homologues of the panZ (yhKK) gene are conserved (B) Growth of the E. coli cells with panDBS was tested on agar plates of the M9 glucose medium with or without β-alanine. Overnight cell culture was spotted at a series of dilutions of 1:10. wild type; MG1655, ΔpanZ, SN202, panDBS; SN219, panDBS, ΔpanZ, SN223. (C) In vivo cleavage of PanDBS-FLAG was analyzed by SDS-PAGE and detected by western blotting using anti FLAG antibody. Wild type; MG1655, panD-flag; SN208, ΔpanZ, panD-flag; SN216, panDBS-flag; SN220, ΔpanZ, panDBS-flag; SN224.

Figure 4. Bacterial two hybrid assay. The combinations of the prey and the bait are indicated in the right panel.
Figure 5. Gel filtration chromatography for analysis of affinity between PanD and PanZ. Purified proteins were chromatographed by gel filtration and elution profiles were monitored by absorbance at 280 nm. (A) PanD. (B) PanZ-His. (C) PanZ(N45A)-His. (D) PanD and PanZ-His. (E) PanD and PanZ(N45A)-His. (F) Molecular weight standards. Molecular weights are indicated in the elution curve (kDa). Fractions between the broken line (from fraction number 34 to 60) were subjected to SDS-PAGE and CBB staining as shown in the right panels. Left lane in each panel indicates molecular weight (kDa).
means that ΔPanZ cells can grow by using pantothenate secreted by wild type cells (Fig. 6B). Indeed, slight growth of ΔPanZ cells was often seen in proximity to growing PanZ+ cells. When the ΔPanZ cells were inoculated near pantothenate transport-deficient cells (ΔpanF) however, no growth was observed (Fig. 6B). Thus, secreted pantothenate can be used by those bacteria that cannot produce or otherwise do not produce pantothenate.

In the gut flora, vast numbers of microorganisms live and produce pantothenate. However feedback control of CoA on CoA activity does not effectively regulate this and produce pantothenate. However feedback control of otherwise do not produce pantothenate.

In contrast to PanD from E. coli, PanDbs does not require PanZ for its activation. Overexpression of PanD from Corynebacterium glutamicum in E. coli has previously been reported as a high yielding strategy for production of β-alanine and hence pantothenate (Dusch et al. 1999). This is, perhaps, consistent with our findings: an analogue of the PanZ gene is not present in C. glutamicum and so, like PanDbs, PanD from C. glutamicum might be fully activated by auto-processing. Previous attempts to engineer the pathway in plants by Fouad et al. (Fouad and Rathinasabapathi 2006) and Chakauya et al. (Chakauya et al. 2008) have used PanDfc. As PanDfc requires PanZ for its activation, PanDbs would therefore be more effective in these applications. It remains unclear which structural differences between these orthologs lead to rapid activation of PanDbs without the addition of PanZ. Alignment of orthologs of PanD (Fig. S1) reveals that the N-terminal (1-111) portions of the proteins are highly conserved among all bacterial species. However, the last 20 aa are poorly conserved among most bacterial species, but are well conserved in those bacterial species in which orthologs of PanZ are found. This region is distant from the site of cleavage (Gly24-Ser25) so it is not clear how this region could prevent activation of PanD in these species. Recently, it has been reported that this portion of the protein can bind intermolecularly to the active site of adjacent proteins as a result of crystal packing (Webb et al. 2012); it is therefore possible that such intramolecular interactions might act to inhibit activation in solution.

In summary, we have identified an essential new component in the pantothenate biosynthetic pathway. The pathway of pantothenate biosynthesis has been previously proposed as a target for antimicrobial chemotherapy (Spry et al. 2008). But as the pathway is common to all bacteria, the opportunity for selective chemotherapy is limited. PanZ homologues are however only found in the genomes of E. coli and related bacteria, such as Shigella, Salmonella, Klebsiella and Yersinia, all of which are resident in the animal gut. PanZ therefore provides a potential target by which to inhibit only pantothenate biosynthesis in these pathogenic bacteria, while sparing the gut flora where pantothenate is abundant.

**Experimental Procedures**

**Bacterial strains and plasmids**

Table 1 shows bacterial strains and plasmids used in this report. To construct the PanD-flag gene of SN208, we used a DNA fragment encoding the FLAG octapeptide repeated three times in tandem (3×flag) with the cat gene inserted between frt sites (frt-cat-frt). The frt sites are recognition sequences of the sequence specific recombinase FLP. The DNA fragment (3×flag-frt-cat-frt) was amplified by polymerase chain reaction (PCR) using primers PanDflagU and PanDflagL. We used high-fidelity KOD-plus DNA polymerase (TOYOBO, Osaka) for PCR. The resulting fragment was used for transformation into MG1655 according to the Datsenko and Wanner’s procedure (Datsenko and Wanner 2000). Then the 3×flag tag was thereby fused with the carboxyl terminus of the PanD gene. If necessary, the cat genetic marker was removed using the FLP-FRP recombination (Datsenko and Wanner 2000).

To construct the PanDbs strain (SN219), the PanD gene of B. subtilis 168 was amplified using BSPD1 and BSPD21 as PCR primers. The frt-cat-frt fragment was also amplified from template DNA of PanD-flag-frt-cat-frt using primers of BSPD31 and PanDflagL. As the sequence of BSPD21 is complementary to the sequence BSPD31,
The amplified DNA fragment encoding PanD of *B. subtilis* could anneal with the PanD-flag-frt-cat-frt DNA fragment to, yielding the PanD\textsubscript{BS}-frt-cat-frt DNA fragment. This DNA fragment was used to substitute *E. coli* PanD with PanD\textsubscript{BS} by recombination according to Datsenko and Wanner’s procedure (Datsenko and Wanner 2000). Similarly a strain with PanD\textsubscript{BS}-flag (SN220) was constructed using primers BSPD1 and BSPD4 for amplification of PanD\textsubscript{BS}, and primers of BSPD5 and PanDflagL for that of flag-frt-cat-frt.

To construct ΔyhhK\textsubscript{(PanZ)} derivatives, ΔyhhK::\textit{kan}, which is one of the deletion mutants in the Keio collection (Baba et al. 2006), was used to transduce each parental strain by P1 transduction, resulting in SN202, SN216, SN223 and SN224. The \textit{kan} gene marker was eliminated according to Datsenko and Wanner’s procedure (Datsenko and Wanner 2000). To construct the ΔpanF strain (SN225), ΔpanF::\textit{kan} of the KEIO collection was introduced to MG1655 by P1 transduction.

pCA24N(-GFP)-\textit{his}-PanZ is one of the expression plasmids of the ASKA clone (-) library, in which each cloned gene can be expressed by addition of IPTG (isopropyl-\beta-D-thiogalactopyranoside) with the gene product having an N-terminal His tag (Kitagawa et al. 2005).
To construct pBAD24-PanD-flag, the PanD-flag fragment was amplified by PCR using the primers ECPanD-FlagU and PanD-FlagL. The PCR product was digested with EcoRI and HindIII and inserted into the pBAD24 vector plasmid (Guzman et al. 1995). Expression of the PanD-flag gene is induced by addition of arabinose. However, leaky, uninduced expression could complement the phenotype of ΔPanD. The vector pBAD24-PanZ-his was constructed similarly except that the PanZ gene was amplified using the primers PanZBADU and PanZBADL.

To construct pBAD24-PanZ(N45A)-his, a single nucleotide mutation was introduced into the PCR primers PanZN45AL and PanZN45AU. The 5’ portion of the PanZ-his gene was amplified using the primers of PanZBADU and PanZBADL, and the 3’ portion using primers of PanZN45AU and PanZBADL. As the primer sequence of PanZN45AL is complementary to that of PanZN45AU, the PCR products could be annealed and thus the mutated PanZ gene was obtained. This PanZ (N45A)-his fragment was cloned into the pBAD24 vector as described above.

To construct an expression plasmid for PanZ, the PanZ gene was amplified using primers THPanZU and THPanZL. The PCR product was digested with EcoRI and BamHI and inserted into the EcoRI-BamHI site of pUT18C and pKT25 (Karimova et al. 1998), respectively, and BamHI and inserted into the EcoRI-BamHI site of pUT18C and pKT25 (Karimova et al. 1998), respectively, and BamHI and inserted into the EcoRI-BamHI site of pUT18C and pKT25 (Karimova et al. 1998), respectively.

We converted the gene selection marker of pET28 (Novagen), which is an IPTG-inducible expression vector, from a kanamycin resistance gene to an ampicillin resistance gene. To construct pET28Ap, pET28 plasmid DNA was digested with AlwNI and ClaI to eliminate the kanamycin resistance gene. pBR322 plasmid DNA was digested with AlwNI and ClaI to obtain the ampicillin resistance gene. pBR322 plasmid DNA was digested with AlwNI and ClaI to obtain the ampicillin resistance gene, and the DNA fragment was ligated into the above pET28 plasmid DNA. To construct pET28Ap-PanD, the PanD gene was amplified from MG1655 genomic DNA using the primers PanDPETU and PanDPETL. The PCR product was digested with NcoI and HindIII and introduced into the NcoI-HindII site of pET28Ap.

Strains of the KEIO collection were obtained from the National BioResource Project of the National Institute of Genetics, Mishima, Japan.

**Purification of His-PanZ**

ΔPanZ cells harboring pBAD24-PanD-flag were incubated in 100 ml of L broth containing 50 µg/ml of ampicillin at 30°C until the OD_{600} reached 0.5, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mmol/L in order to express His-PanZ protein. The culture was incubated for an additional 4 h. Cells were harvested by centrifugation and resuspended in 10 ml of Lysis Buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 10 mmol/L imidazole 1 mg/ml lysozyme, pH 8.0) and incubated on ice for 30 min. Cells were disrupted by sonication and cell debris was removed by centrifugation to obtain a cleared lysate. Two ml of 50% slurry of Ni-NTA agarose (QIAGEN) was added to the cleared lysate and mixed by shaking at 4°C for 1 h. The lysate-Ni-NTA mixture was loaded into a column and washed twice with 10 ml of Wash Buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 20 mmol/L imidazole, pH 8.0). His-PanZ protein was eluted with 4× 1 ml of Elution Buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 8.0). Eluted His-PanZ protein was dialyzed against TBSE Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.5) and used for subsequent assays.

**Purification of PanD-FLAG**

ΔPanZ cells harboring pBAD24-PanD-flag were incubated in 100 ml of L broth containing 50 µg/ml of ampicillin at 30°C until the OD_{600} reached 0.5, and then arabinose was added to a final concentration of 0.2% in order to express PanD-FLAG. The culture was incubated for an additional 3 h. Cells were harvested by centrifugation and resuspended in 2 ml of Lysis Buffer and incubated on ice for 30 min. Cells were disrupted by sonication and cell debris was removed by centrifugation to obtain a cleared lysate. The cleared lysate was loaded onto a column with 200 ml of Anti-FLAG M2 affinity gel (SIGMA) and washed twice with 5 ml of TBS (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5). PanD-FLAG protein was eluted with 5× 250 µl of TBS containing 100 µg/ml 3×FLAG peptide. Eluted PanD-FLAG was dialyzed against TBSE Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.5) and used for subsequent assays.

**Purification of PanD**

SN227 harboring pET28Ap-PanD was cultured in L medium containing 50 µg/ml ampicillin until the OD_{600} reached 0.6, and then IPTG was added to a final concentration of 1 mmol/L. The culture was incubated for an additional 3 h. Cells were harvested by centrifugation and resuspended in Lysis buffer (50 mmol/L HEPES/KOH, 100 mmol/L potassium acetate, 1 mmol/L EDTA, 1 mg/ml lysozyme, pH 7.6). Cells were lysed by freeze-thawing three times with liquid nitrogen and a single cycle of sonication and cell debris removed by ultracentrifugation.
Ammonium sulfate was added to the supernatant to 40% saturation and the precipitate was removed by ultracentrifugation. Further ammonium sulfate was added to the supernatant to 50% saturation. After ultracentrifugation, the precipitate was resuspended in K0 buffer (50 mmol/L HEPES/KOH, 1 mmol/L EDTA, pH 7.6) and dialyzed against K0 buffer for more than 3 h to remove ammonium sulfate. The dialyzed solution was loaded on a Resource Q column using an AKTA prime plus liquid chromatography system (GE Healthcare). Gradient elution was carried out from K0 buffer to K100 buffer (50 mmol/L HEPES/KOH, 1 mmol/L EDTA, 1 mol/L potassium acetate, pH 7.6). Each eluted fraction was analyzed by SDS-PAGE visualized using CBB. Peak fractions were collected and dialyzed against K100 buffer for more than 3 h. After dialysis, purified PanD protein was stored at −80°C.

**Purification of PanZ-His and PanZ(N45A)-His**

SN202 harboring pBAD24-PanZ-his or panZ(N45A)-his was cultured in L medium containing 50 μg/ml ampicillin until the OD600 reached 0.6, and then L-arabinose was added to a final concentration of 0.2%. After further incubation for 3 h, cells were harvested by centrifugation and resuspended by ultracentrifugation. Ammonium sulfate was added to the supernatant to a final 50% saturation. After the precipitate was removed by centrifugation, further ammonium sulfate was added to 80% saturation. After ultracentrifugation, the precipitate was resuspended in His-binding buffer (50 mmol/L HEPES/KOH, 100 mmol/L potassium acetate, 20 mM imidazole, pH 7.6) and loaded on HisTrap FF column (GE Healthcare) using the AKTA prime plus liquid chromatography system (GE Healthcare). Stepwise elution chromatography was carried out using Elution buffer (50 mmol/L HEPES/KOH, 100 mmol/L potassium acetate, 500 mmol/L imidazole, pH 7.6). Eluted proteins were analyzed by SDS-PAGE visualised using CBB. Peak fractions were collected and dialyzed against K100 buffer containing 10% glycerol. After dialysis, purified proteins were stored at −80°C.

**Assay of PanD-FLAG cleavage in vivo and in vitro**

To assay in vivo PanD-FLAG cleavage, exponentially growing cells in LB broth at 37°C were harvested, and washed once with 0.85% (w/v) NaCl. The cells were lysed in 1× SDS-PAGE loading buffer and subjected to SDS polyacrylamide (20%) gel electrophoresis. After blotting of the proteins to a nitrocellulose membrane (GE Healthcare), immunodetection using anti-FLAG M2 antibody (SIGMA) was carried out. To express His-PanZ protein in SN216 cells harboring pCA24N(-GFP)-his-PanZ, cells were incubated in LB broth at 37°C until the OD600 reached 0.2, then IPTG was added to a final concentration of 0, 1, 10 and 100 μM. The cells were incubated for an additional 60 min. SN216 cells harboring pCA24N(-GFP) were used as control.

For assay of in vitro PanD-FLAG cleavage, purified PanD-FLAG (2.5 μmol/L) was incubated with purified His-PanZ (0, 0.04, 0.16, 0.32, 0.63, 2.5, 10 and 40 μmol/L) in 50 μl of TBSE buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1mmol/L EDTA, pH 7.5) for 3 h. The reaction was quenched with SDS-PAGE loading buffer and the samples were boiled for 5 min and subjected to SDS polyacrylamide (20%) gel electrophoresis. Western blotting was carried out as described above except that anti-Penta-His antibody (QIAGEN) was used for detection of His-PanZ.

**Bacterial two-hybrid assay**

The bacterial two-hybrid system was applied as described by Karimova et al.(Karimova et al. 1998). All combinations of pUT18C derivatives (pUT18C, pUT18C-PanZ or pUT18C-PanD) and pKT25 derivatives (pKT25, pKT25-PanZ or pKT25-PanD) were co-transformed into the reporter strain, DHM1 on LB plates containing 50 μg/ml ampicillin and 15 μg/ml kanamycin. The positive control was the pair of pUT18C-PanD and pKT25-PanD vectors and the negative control was the empty vectors, pUT18C and pKT25. The transformed cells were spread on M9 glucose plates containing 50 μg/ml ampicillin and 15 μg/ml kanamycin and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The plates were incubated at 30°C for 2 days.

**Phylogenetic tree of panD**

A phylogenetic tree of PanD was generated using ClustalX (Thompson 1997) and drawn using TreeView X software (Page 1996).

**Gel filtration assay**

Purified proteins were subjected to gel filtration column using the AKTA prime plus liquid chromatography system (GE Healthcare). A Superose 6 10/300 GL gel filtration column (GE Healthcare) was used at a flow rate of 0.2 ml/
min of GF buffer (50 mmol/L Hepes/KOH, 150 mmol/L KCl, pH 7.6) and each 0.3 ml of eluate was fractionated from the void volume. Elution profiles of the column were calibrated using a Gel Filtration Calibration Kits (GE Healthcare) containing aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), ovalbumin (44 kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and Blue Dextran 2000 (2000 kDa). For in vitro binding assay of PanD and PanZ-His (or PanZ(N45A)-His), 60 µmol/L of PanD and 15 µmol/L PanZ-His (or PanZ(N45A)-His) were mixed and incubated for 60 min at 4°C, and then 100 µl of sample was subjected to gel filtration chromatography.

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Conflict of Interest

None declared.

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Supporting Information

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

Table S1. Oligonucleotide primers used for PCR.

Figure S1. Sequence alignment of panD from representative bacterial species. panZ-conserved species are indicated in a gray box. The amino acid residues are color coded as follows: Blue = Ala, Val, Leu, Ile, Met, Trp, Phe and Cys, Green = Ser, Thr, Asn and Gln, Magenta = Asp and Glu, Red = Lys and Arg, Cyan = His and Tyr, Orange = Gly, Yellow = Pro.

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