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RESEARCH ARTICLE

Suppression of a Thermosensitive *zipA* Cell Division Mutant by Altering Amino Acid Metabolism

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ABSTRACT ZipA is essential for cell division in *Escherichia coli*, acting early in the process to anchor polymers of FtsZ to the cytoplasmic membrane. Along with FtsA, FtsZ and ZipA form a proto-ring at midcell that recruits additional proteins to eventually build the division septum. Cells carrying the thermosensitive *zipA1* allele divide fairly normally at 30°C in rich medium but cease dividing at temperatures above 34°C, forming long filaments. In a search for suppressors of the *zipA1* allele, we found that deletions of specific genes involved in amino acid biosynthesis could partially rescue cell growth and division at 34°C or 37°C but not at 42°C. Notably, although a diverse group of amino acid biosynthesis gene deletions could partially rescue the growth of *zipA1* cells at 34°C, only deletions of genes related to the biosynthesis of threonine, glycine, serine, and methionine could rescue growth at 37°C. Adding exogenous pyridoxal 5-phosphate (PLP), a cofactor for many of the enzymes affected by this study, partially suppressed *zipA1* mutant thermosensitivity. For many of the deletions, PLP had an additive rescuing effect on the *zipA1* mutant. Moreover, added PLP partially suppressed the thermosensitivity of *ftsQ* and *ftsK* mutants and weakly suppressed an *ftsI* mutant, but it failed to suppress *ftsA* or *ftsZ* thermosensitive mutants. Along with the ability of a deletion of *metC* to partially suppress the *ftsK* mutant, our results suggest that perturbations of amino acid metabolic pathways, particularly those that redirect the flow of carbon away from the synthesis of threonine, glycine, or methionine, are able to partially rescue some cell division defects.

IMPORTANCE Cell division of bacteria, such as *Escherichia coli*, is essential for their successful colonization. It is becoming increasingly clear that nutritional status and central metabolism can affect bacterial size and shape; for example, a metabolic enzyme (OpgH) can moonlight as a regulator of FtsZ, an essential cell division protein. Here, we demonstrate a link between amino acid metabolism and ZipA, another essential cell division protein that binds directly to FtsZ and tethers it to the cytoplasmic membrane. Our evidence suggests that altering flux through the methionine-threonine-glycine-serine pathways and supplementing with the enzyme cofactor pyridoxal-5-phosphate can partially compensate for an otherwise lethal defect in ZipA, as well as several other cell division proteins.

KEYWORDS *Escherichia coli*, FtsZ, ZipA, amino acid biosynthesis, cell division

To divide, *Escherichia coli* cells use a number of essential proteins to form a cytokinetic ring at midcell and synthesize a cell division septum (1). Three of these, FtsZ, FtsA, and ZipA, are required for assembly of the initial ring structure, called the proto-ring (2). The tubulin-like FtsZ protein forms dynamic treadmilling polymers that are attached to the inner surface of the cytoplasmic membrane by FtsA and ZipA (3). Together, these three proteins form several mobile complexes that recruit additional cell division proteins, including enzymes that synthesize septal peptidoglycan, which build the division septum (3–6).

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FtsZ can still localize to the ring structure in the absence of either FtsA or ZipA, but the rings are not able to proceed in division, indicating that FtsA and ZipA have partially overlapping functions (7–10). Notably, the essential function of ZipA can be completely bypassed in certain cases, including in mutants of FtsA or FtsL, or when FtsN is overproduced, suggesting that ZipA is not an indispensable structural component of the septal ring but perhaps part of a checkpoint mechanism (11–15). FtsA is attached to the membrane with an amphipathic helix (16), theoretically allowing it to bind reversibly, while ZipA is bound permanently to the membrane through an N-terminal transmembrane domain (17). ZipA interacts with FtsZ through a C-terminal FtsZ-binding domain (18, 19); its two functional domains are connected by an intrinsically disordered peptide linker (20). ZipA enhances the bundling of FtsZ protofilaments *in vitro* (17, 21). Consistent with this idea, a single amino acid substitution in FtsZ that increases self-bundling can also bypass the need for ZipA (22).

Studies of essential bacterial cell division genes have been facilitated by conditional mutants, particularly thermosensitive (ts) mutants. Such mutants can divide at lower temperatures, such as 30°C, but fail to divide at nonpermissive temperatures, such as 42°C, conveniently allowing the rapid inactivation of cell division by a simple temperature shift. There is only one known ts mutant of *zipA*, called *zipA1*, which consists of one missense mutation in the N-terminal transmembrane domain and three missense mutations in the FtsZ binding domain (9). Cells harboring the *zipA1* allele divide fairly normally at 30°C in rich medium, although not as well as wild-type (WT) cells, but rapidly cease division when shifted from 30°C to 42°C, forming long filaments with multiple nucleoids (9).

In this study, we isolated and characterized thermoresistant suppressors of the *zipA1* allele that are not in other cell division genes but are instead in genes involved in amino acid metabolism. In particular, we find that inactivating genes involved in methionine-threonine-glycine biosynthesis or adding pyridoxal-5-phosphate cofactor suppresses the thermosensitivity of the *zipA1* mutant and some, but not all, thermosensitive cell division mutants. Our evidence supports a novel connection between amino acid biosynthesis and cell division.

RESULTS

The original *zipA1* mutant in W3110 exhibits medium-dependent growth and division defects at the permissive temperature that are partially suppressed by added Casamino Acids, glycine, or L-threonine. Cells of the wild-type *E. coli* parent strain W3110 and its *zipA1* mutant derivative WM2991 grew and divided normally at the permissive temperature of 30°C, although some WM2991 cells were slightly longer (Fig. 1A and G), probably because of residual defects of the *zipA1* allele at this temperature. However, when we grew these two strains in minimal medium (either morpholinepropanesulfonic acid [MOPS] or M9), we were surprised to find that both grew poorly (Fig. 1M). Although W3110 cells remained short under these growth conditions (Fig. 1B and C), WM2991 cells became highly filamentous (Fig. 1H and I), indicating that any small cell division defect seen in LB was greatly exacerbated in minimal medium.

These medium-dependent defects in cell growth and division prompted us to ask whether adding nutrients back to M9 medium could suppress these defects. We found that the addition of Casamino Acids partially suppressed the filamentation of WM2991 cells (Fig. 1J). We then added back individual amino acids to WM2991 cells grown in M9 and found that only glycine (Fig. 1K) or L-threonine (Fig. 1L) could significantly suppress the cell division and growth defects (Fig. 1M). Even though WM2991 and the W3110 parent exhibited similar growth defects in M9, the fact that cell division defects in the *zipA1* mutant were medium dependent and specifically suppressed by glycine or L-threonine prompted us to consider a possible link between cell division and the glycine-threonine biosynthesis pathway.

To determine whether the observed medium-dependent effects of the *zipA1* allele on growth and division were specific to the W3110 strain background, we used a linked

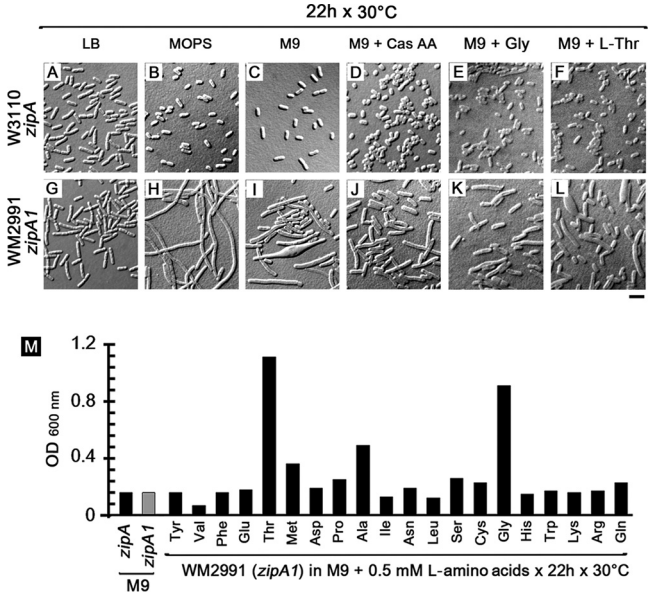

FIG 1 The *zipA1* mutant in W3110 displays a medium-dependent cell division defect at the permissive temperature. Wild-type *E. coli* W3110 exhibited normal cell morphology when grown at 30°C in LB (A), MOPS (B), M9 (C), M9 plus 1% Casamino Acids (Cas AA) (D), M9 plus 0.5 mM glycine (E), or M9 plus 0.5 mM L-threonine (F). The thermosensitive *zipA1* mutant (WM2991) was slightly elongated in LB (G), filamented in MOPS (H), and filamented with some misshapen cells in M9 (I), and these defects were partially suppressed when M9 was supplemented with 1% Casamino Acids (J), glycine (K), or L-threonine (L). (M) The bar graph represents the growth of W3110 (wild-type *zipA*) and WM2991 (*zipA1* allele) in M9 and the effects of different L-amino acids on the growth of WM2991 after 22 h at 30°C. Scale bar, 4 μm.

nupC::Tn*10* marker to transduce the *zipA1* allele by P1 transduction into MG1655, creating WM5337. Cells of WM5337 were moderately filamentous at 30°C in both M9 (see Fig. S1D in the supplemental material) and in LB at 30°C (Fig. S1E), presumably because of residual thermosensitivity of the *zipA1* allele. As expected, WM5337 cells were severely filamentous in LB at the nonpermissive temperature of 42°C (Fig. S1F), and they grew well in a spot assay in LB only at 30°C (Fig. S1G) but not at 42°C (Fig. S1H). A plasmid expressing wild-type *zipA* was able to complement WM5337 for growth at 42°C (Fig. S1H), while the same plasmid expressing the *zipA1* gene failed to complement. Conversely, when we converted WM2991 (W3110 *zipA1*) to *zipA*$^+$ by transduction creating WM5322, the filamentation phenotype either in M9 (Fig. S1A) or LB (Fig. S1B and C) was abolished. We conclude that *zipA1* confers a thermosensitive cell division phenotype on MG1655 as well as W3110.

However, we also found that when the *zipA1* allele was introduced into the MG1655 background (WM5337), the strain grew more robustly in M9 compared to the equivalent W3110 derivative, WM2991. This, along with the lack of a strong medium-dependent cell division phenotype in WM5337, led us to consider that W3110 itself is auxotrophic under nutrient deprivation conditions. To test this more definitively, we measured the cell densities of W3110, WM2991, MG1655, and WM5337 grown in parallel at 30°C for 24 h in LB, MOPS, and M9. Even in rich LB medium, MG1655 and its *zipA1* mutant derivative WM5337 grew slightly better than W3110 and its *zipA1* mutant derivative WM2991 (Fig. S2A). However, these strain-background growth differences were much more evident in MOPS (Fig. S2B) and, at least for W3110, in M9 (Fig. S2C). The growth defects of W3110 and WM2991 were greatly suppressed when glycine or L-threonine was added to MOPS (Fig. S2D and E) or M9 (Fig. S2G and H) but not when L-methionine was added (Fig. S2F and I), indicating that W3110 itself is starved for glycine or L-threonine. Although the reasons for this are not known, we chose to use the MG1655 background for all further studies of the *zipA1* allele to eliminate confounding effects on cell division and growth caused by the W3110 background.

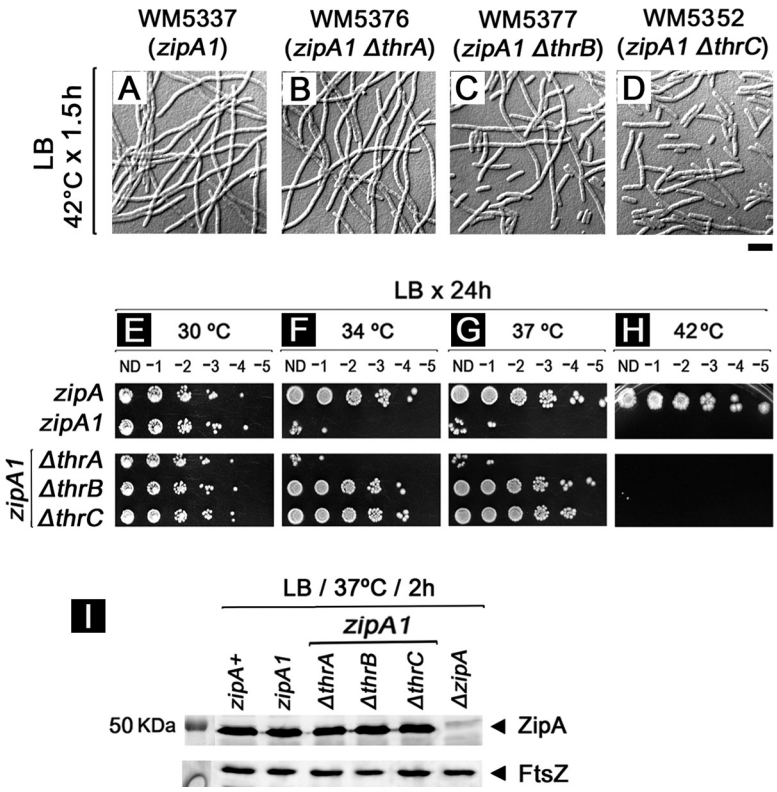

FIG 2 Defects in L-threonine biosynthesis partially suppress *zipA1* mutant thermosensitivity. (A to D) WM5337 and mutants lacking *thrA*, *thrB*, or *thrC* were grown for 1.5 h at 42°C in LB. (E to H) Viability was measured by spotting assay on LB at 30°C (E), 34°C (F), 37°C (G), or 42°C (H) for 24 h. ND, no dilution. (I) The levels of ZipA and FtsZ protein in indicated cells grown at 37°C for 2 h were determined by Western blotting using antibody against ZipA or FtsZ.

Blocking L-threonine biosynthesis partially suppresses *zipA1* mutant growth and division defects. Prior to our realization that the *zipA1* mutant strain WM2991 (and its parent W3110) seemed to be auxotrophic for threonine and glycine and thus differed in important ways from MG1655 derivatives, we asked whether L-threonine had any effect on the *zipA1* allele in the MG1655 strain background by deleting genes required for threonine biosynthesis (*thrA*, *thrB*, and *thrC*) (Fig. S3). Although we could not test effects in M9 medium because cells cannot grow without added L-threonine, we decided to explore whether there were any effects of these deletions on the *zipA1* mutant in rich medium. In LB broth at the permissive temperature (30°C), we found that the *zipA1* strain and all three *thr* mutant derivatives of the *zipA1* strain displayed normal growth and morphology (data not shown). In LB at 42°C, cells of the *zipA1* parent (Fig. 2A) and the *zipA1* Δ*thrA* double mutant (Fig. 2B) grew to high density but were very filamentous, as expected. However, we noticed many more short cells in the *zipA1* Δ*thrB* (Fig. 2C) and *zipA1* Δ*thrC* (Fig. 2D) double mutants than in the *zipA1* and *zipA1* Δ*thrA* mutants, which were mostly filaments (Fig. 2A and B). Despite this hint of suppression in broth at 42°C, none of the double mutants formed colonies on LB plates in spot assays at 42°C (Fig. 2H), indicating that the Δ*thrB* and Δ*thrC* mutants could not completely bypass ZipA at the most nonpermissive temperature.

Because they showed some suppression of filamentation at 42°C, we then tested whether the Δ*thrB* or Δ*thrC* mutation might suppress the *zipA1* mutant at potentially less restrictive temperatures (34 and 37°C). Although both temperatures were still lethal for the *zipA1* parent strain, they allowed partial suppression of *zipA1* mutant thermosensitivity in the Δ*thrB* and Δ*thrC* derivatives (Fig. 2F and G). The rates of exponential growth of the wild-type parent and the partially suppressed *zipA1* Δ*thrB* and *zipA1* Δ*thrC* derivatives at 37°C were similar to those of nonsuppressed *zipA1* and *zipA1* Δ*thrA*

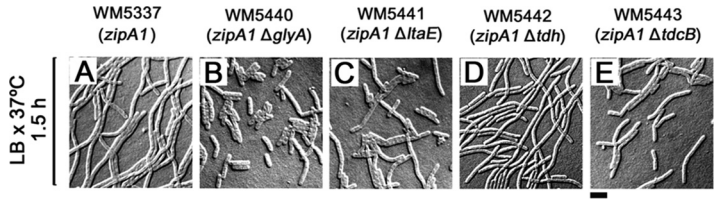

FIG 3 Deletion of *glyA*, *ltaE*, and *tdcB* but not *tdh* partially suppresses *zipA1* mutant thermosensitivity. The effects of suppressing *zipA1* mutant thermosensitivity were determined by growing WM5337 (A) and *zipA1* mutants lacking *glyA* (B), *ltaE* (C), *tdh* (D), or *tdcB* (E) in LB broth for 1.5 h at 37°C. Scale bar, 4 μm.

mutant strains, although the *zipA1* and *zipA1* Δ*thrA* mutant strains reached lower cell densities, as expected (Fig. S4). At the permissive temperature of 30°C, no differences in viability (Fig. 2E) or cellular ZipA protein levels (Fig. 2I) were detected among the *zipA*$^+$, *zipA1*, or the Δ*thrA*, Δ*thrB*, or Δ*thrC* derivatives of *zipA1*. Together, these results suggest that deletion mutations in *thrB* and *thrC*, the last two steps of L-threonine biosynthesis from aspartate after the synthesis of L-homoserine, can partially suppress the thermosensitivity of the *zipA1* allele.

Deletions of other genes directly or indirectly involved in L-threonine or glycine metabolism also partially suppress cell *zipA1* mutant thermosensitivity. Based on the unexpected effects on suppressing the thermosensitivity of the *zipA1* strain caused by deleting genes involved in the L-threonine metabolism, we asked whether other genes encoding enzymes in related amino acid biosynthesis pathways (Fig. S3) had similar effects. First, using the *zipA*$^+$ or *zipA1* MG1655 derivative, we deleted *glyA*, encoding serine hydroxymethyltransferase (SHMT), which catalyzes the interconversion of serine and glycine (23, 24); *ltaE*, encoding low-specificity L-threonine aldolase, which converts L-threonine or L-allo-threonine to glycine (23–25); *tdcB*, encoding a catabolic threonine dehydratase, the first step in L-threonine and L-serine degradation (23, 26); or *tdh*, encoding threonine dehydrogenase, which catalyzes the conversion of L-threonine to L-2-amino-3-oxobutanoate, yielding either aminoacetone or conversion to glycine (27). In the *zipA*$^+$ (MG1655) background, none of the single deletions affected cell division significantly (data not shown), but in a *zipA1* (MG1655) background, mutants lacking *glyA*, *ltaE*, or *tdcB*, but not those lacking *tdh*, partially suppressed cell filamentation at 37°C (Fig. 3B, C, and E; compare with Fig. 3A and D), similar to the effects of *thrB* and *thrC* deletions.

Next, based on the observation that except for *thrB*, all the deleted genes that partially suppressed *zipA1* mutant thermosensitivity required pyridoxal-5-phosphate (PLP) as a cofactor (Fig. S3), we asked whether deleting other PLP-dependent genes near or far from the glycine-serine-threonine pathway could suppress the thermosensitivity of the *zipA1* allele. Using the *zipA1* MG1655 derivative (WM5337) as a host, we generated 19 new single-deletion mutants in amino acid biosynthesis genes. Including our original mutants, we could now test a total of 15 PLP-dependent and 11 non-PLP dependent genes (Table 1).

To determine the effects of single deletions on suppression of the thermosensitivity of the *zipA1* allele, spot assays were carried out in LB at 30, 34, 37, and 42°C by using all the mutants generated in a *zipA1* background. All 26 single-deletion mutants grew well at 30°C (Table 1, +, and Fig. 4P), with spots out to the 10^{-4} dilution, although the Δ*glyA* and Δ*ltaE* mutant spots were weaker. At 34°C, essentially no viability was observed for the parental *zipA1* strain and mutants lacking *thrA*, *trpA*, *trpB*, *metA*, *metE*, *metL*, *lysA*, and *malY* (Table 1, −, and Fig. 4Q), partial viability was observed for mutants lacking *metH*, *mmuM*, *argD*, *aspC*, *hisC*, *hisD*, and *asnB* (Table 1, +/−, and Fig. 4Q), and growth equivalent to that at 30°C was observed in mutants lacking *thrB*, *thrC*, *glyA*, *tdcB*, *tdh*, *ilvA*, *kbl*, *ltaE*, *metB*, *metC*, and *lysC* (Table 1, +, and Fig. 4Q).

The less permissive temperature of 37°C caused a general reduction in viability compared with 34°C for most of the mutants. Mutants that previously grew well at 34°C showed partial viability at 37°C, and those with partial viability at 34°C were generally

TABLE 1 Capacity of single-deletion mutants to partially suppress thermosensitivity of the *zipA1* allele

Strain	Relevant genotype	PLP dependence	Suppression of thermosensitivity by temp[a]			
			30°C	34°C	37°C	42°C
WM5163	MG1655		+	+	+	+
WM5337	MG1655 *zipA1* Δ*nupC*::*tet*		+	−	−	−
WM5376	WM5337 Δ*thrA*::*kan*	No	+	−	−	−
WM5377	WM5337 Δ*thrB*::*kan*	No	+	+	+	−
WM5352	WM5337 Δ*thrC*::*kan*	Yes	+	+	+	−
WM5354	WM5337 Δ*glyA*::*kan*	Yes	+	+	+/−	−
WM5378	WM5337 Δ*tdh*::*kan*	No	+	+	−	−
WM5379	WM5337 Δ*tdcB*::*kan*	Yes	+	+	+/−	−
WM5473	WM5337 Δ*ilvA*::*kan*	Yes	+	+	+/−	−
WM5698	WM5337 Δ*kbl*::*kan*	Yes	+	+	+/−	−
WM5433	WM5337 Δ*ltaE*::*kan*	Yes	+	+	+/−	−
WM5695	WM5337 Δ*trpA*::*kan*	Yes	+	−	−	−
WM5696	WM5337 Δ*trpB*::*kan*	Yes	+	−	−	−
WM5388	WM5337 Δ*metA*::*kan*	No	+	−	−	−
WM5389	WM5337 Δ*metB*::*kan*	Yes	+	+	+	−
WM5390	WM5337 Δ*metC*::*kan*	Yes	+	+	+	−
WM5430	WM5337 Δ*metE*::*kan*	No	+	−	−	−
WM5431	WM5337 Δ*metH*::*kan*	No	+	+/−	−	−
WM5432	WM5337 Δ*mmuM*::*kan*	No	+	+/−	−	−
WM5475	WM5337 Δ*metL*::*kan*	No	+	−	−	−
WM5477	WM5337 Δ*lysC*::*kan*	No	+	+	+	−
WM5693	WM5337 Δ*argD*::*kan*	Yes	+	+/−	−	−
WM5694	WM5337 Δ*lysA*::*kan*	Yes	+	−	−	−
WM5697	WM5337 Δ*malY*::*kan*	Yes	+	−	−	−
WM5705	WM5337 Δ*aspC*::*kan*	Yes	+	+/−	−	−
WM5706	WM5337 Δ*hisC*::*kan*	Yes	+	+/−	−	−
WM5707	WM5337 Δ*hisD*::*kan*	No	+	+/−	−	−
WM5708	WM5337 Δ*asnB*::*kan*	No	+	+/−	−	−

[a]+, full viability; +/−, partial viability, unable to form normal spots at all dilutions; −, weak viability, unable to grow outside the "no dilution" spot (see also Fig. 4P to S).

unable to survive at 37°C (Table 1). Only mutants lacking *thrB*, *thrC*, *metB*, *metC*, and *lysC* were fully viable either at 34 or 37°C (Table 1 and Fig. 4R). However, despite the suppressing effects on thermosensitivity observed on plates at 37°C, cells of most of the mutants were filamentous when grown in LB broth at the same temperature (Fig. 4C to O), suggesting that the filaments could divide enough to form colonies under these conditions. As expected, no growth was observed on spot plates at 42°C for any of the double mutants (Table 1 and Fig. 4S). These data support the idea that inactivation of some genes involved in amino acid biosynthesis can partially suppress the thermosensitivity of the *zipA1* allele.

We then asked whether combinations of some of these deletions had an additive effect on suppression. Deletions of *metB*, *glyA*, *ltaE*, *tdcB*, or *tdh* were individually introduced by P1 transduction into a *zipA1* background lacking *metC*, which exhibits partial suppression of *zipA1* mutant thermosensitivity (Fig. 4). No differences in the viability of the parental strain and the double-deletion mutants were detected in a comparison of spot assays on LB at 30, 34, 37, or 42°C (data not shown). Therefore, at least in the case of the *metC* deletion, combining it with deletions that individually can partially suppress *zipA1* thermosensitivity suggests that the effects are not additive. The possibility remains that combining more than two deletions, or deletions in other pathways, is additive.

Effects on other cell division genes. We next asked whether these gene deletions had a general stimulatory effect on cell division versus a more specific effect on *zipA1*. To test this, we chose deletions of *thrC* or *metC*, because both could suppress *zipA1* strongly at 37°C and were not located upstream of other amino acid genes where they could potentially have indirect effects. The Δ*thrC*::*kan* and Δ*metC*::*kan* alleles were introduced by P1 transduction into *ftsA12*, *ftsZ84*, *ftsK44*, *ftsQ1*, and *ftsI23* thermosen-

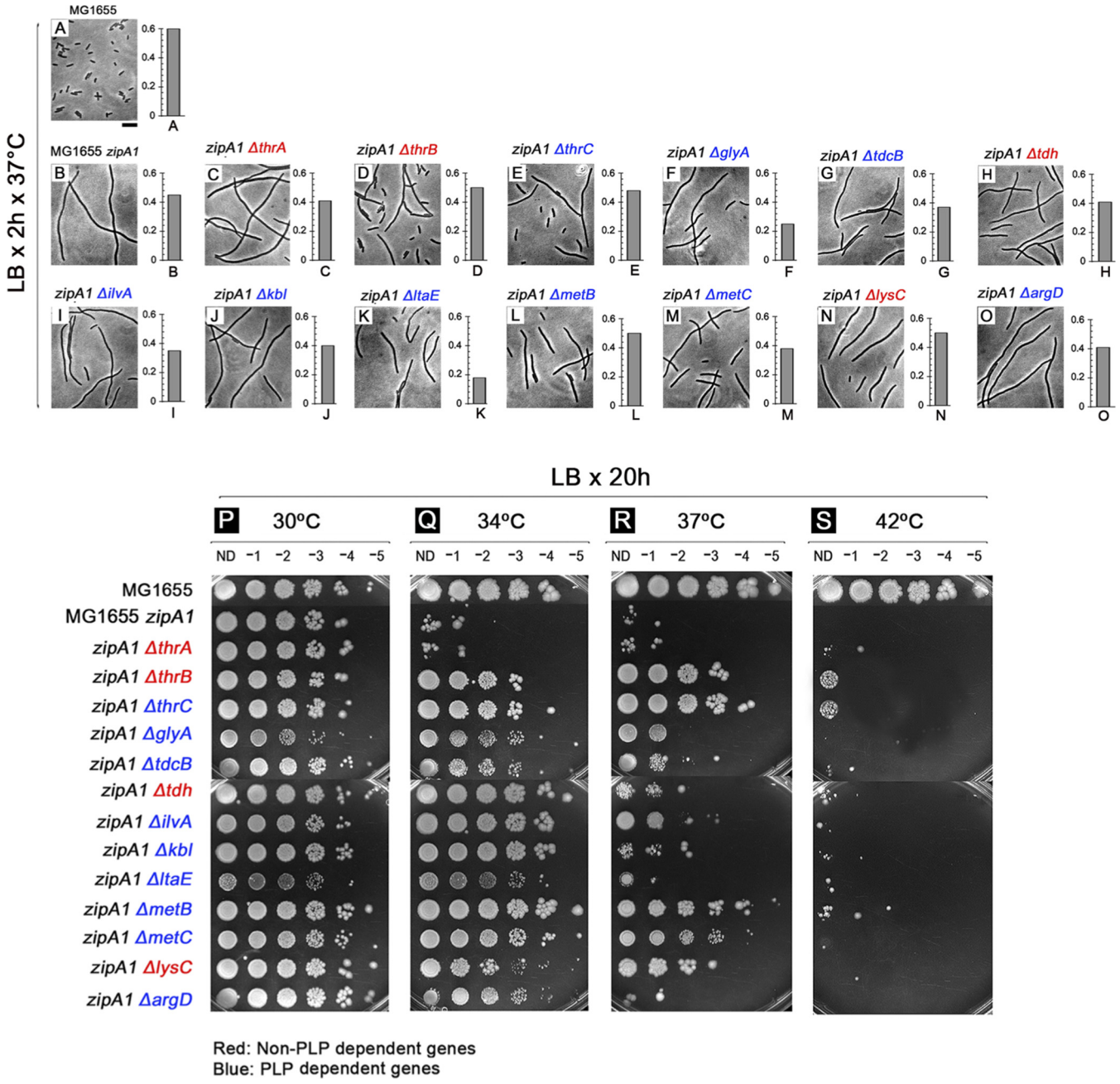

FIG 4 Deletion of many genes involved in amino acid biosynthesis partially suppresses *zipA1* mutant thermosensitivity. To determine the effects of suppressing *zipA1* mutant thermosensitivity, MG1655 (A), WM5337 (MG1655 *zipA1*) (B), and *zipA1* mutants lacking *thrA* (C), *thrB* (D), *thrC* (E), *glyA* (F), *tdcB* (G), *tdh* (H), *ilvA* (I), *kbl* (J), *ltaE* (K), *metB* (L), *metC* (M), *lysC* (N), or *argD* (O) were grown in LB broth for 2 h at 37°C, at which time cell density was measured by optical density and cell morphology examined by phase-contrast microscopy. Scale bar, 4 μm. Spot assays were used to measure cell viabilities in LB at 30°C (P), 34°C (Q), 37°C (R), and 42°C (S). ND, no dilution.

sitive cell division mutants, either in an MG1655 background (the first three) or in an MC4100 background (the last two). The double mutants were grown in LB at 37°C for 2 h and checked for cell filamentation. None of the combined mutant cells divided significantly better than the single mutants based on a qualitative examination of multiple micrographs (Fig. 5A to O).

To obtain a more complete picture of how well the Δ*metC* or Δ*thrC* mutant might suppress cell division defects, we tested their effects on the viability of *fts* mutants in spot assays on LB plates at several temperatures. To ensure that any effects on viability were not a result of differences between the MG1655 and MC4100 backgrounds, the

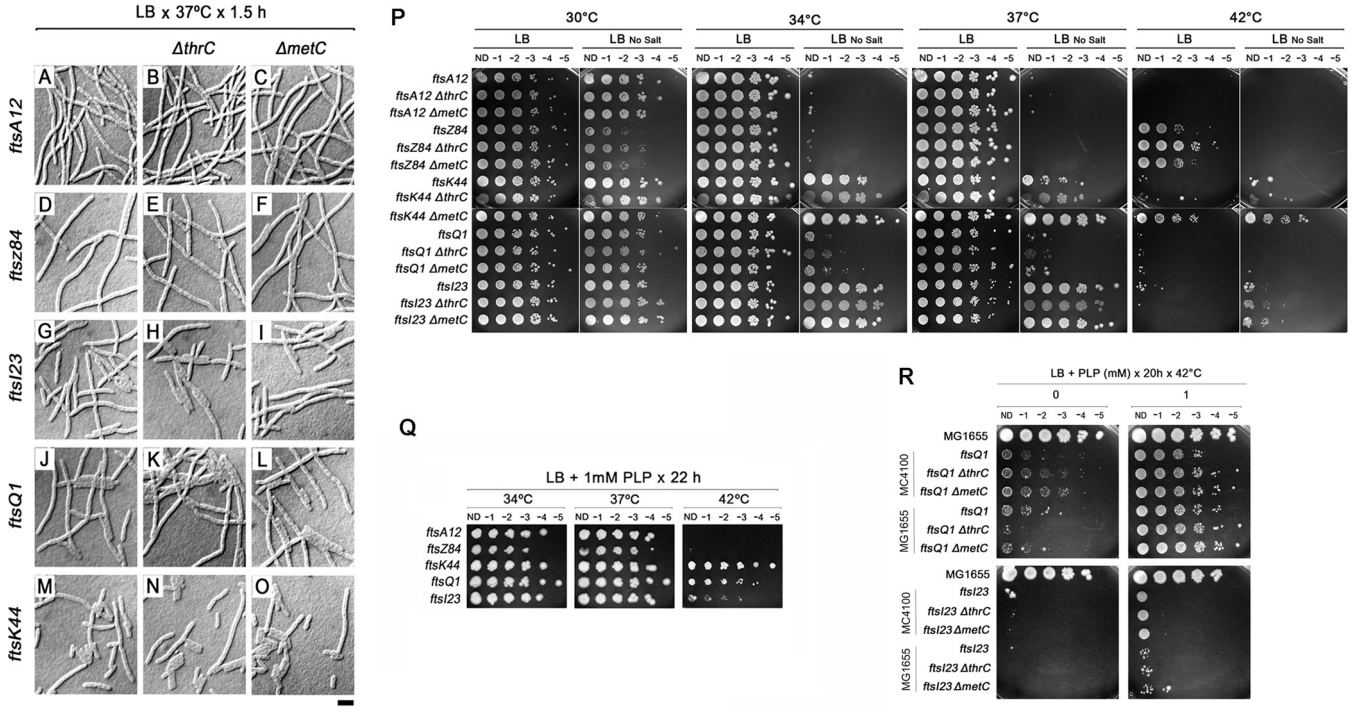

FIG 5 Effects of pyridoxal-5-phosphate and deletions of *thrC* or *metC* in suppressing thermosensitivity of *fts* cell division mutants. Thermosensitive mutants *ftsA12*, *ftsZ84*, *fts123*, *ftsQ1*, and *ftsK44* (A, D, G, J, and M, respectively) and single deletions of *thrC* (B, E, H, K, and N) or *metC* (C, F, I, L, and O) generated in each *fts* strain background were grown for 1.5 h in LB at the semipermissive temperature of 37°C and examined by microscopy (A to O). After transferring the *ftsQ1* and *ftsI23* alleles to the MG1655 strain background, *fts* mutant cells with or without Δ*metC* or Δ*thrC* were spotted on LB or LB with no added NaCl at four different temperatures (P). Five *fts* mutants were tested for the ability of 1 mM PLP to suppress their thermosensitivity after 22 h of incubation at the temperatures indicated; the *ftsQ1* and *ftsI23* derivatives shown here are in MC4100, while the others are in MG1655 (Q). To rule out effects due to strain background, derivatives of *ftsQ1* or *ftsI23* mutants with or without Δ*metC* or Δ*thrC* in either the MC4100 strain background or the MG1655 background were compared for the ability of 1 mM PLP to rescue viability after 20 h of incubation at 42°C (R). ND, no dilution. Scale bar, 4 μm.

ftsQ1 and *ftsI23* mutants were introduced into the MG1655 background harboring the other *fts* mutants. We tested all of the MG1655-derived *fts* mutants at 30°C, 34°C, 37°C, or 42°C on LB or LB with no added NaCl, which is a more nonpermissive condition for many *fts* mutants that might allow the detection of more subtle suppression effects. As expected, the *ftsZ84*, *ftsA12*, and *ftsQ1* mutants were thermosensitive at intermediate temperatures on LB with no salt, making it possible to detect small suppression effects by the Δ*metC* or Δ*thrC* mutation. Notably, the Δ*metC* mutation suppressed the *ftsK44* mutant quite strongly (Fig. 5P). The Δ*thrC* mutation also suppressed the *ftsZ84* mutant, but very weakly. The levels of FtsZ in the *zipA1* strain or in the *zipA1* strain with a Δ*thrA*, Δ*thrB*, or Δ*thrC* mutation were unchanged (Fig. 2I), ruling out the possibility that the suppression of *zipA1* was due to enhanced expression of the *ftsZ* or *zipA1* mutant gene, at least in those deletion strains. The ability of the *ftsK44* mutant to be suppressed by the Δ*metC* mutation suggests that components of the cell division machinery other than ZipA can be affected by amino acid metabolism.

Exogenous PLP partially suppresses *zipA1* mutant thermosensitivity. Although we observed partial suppression of *zipA1* mutant thermosensitivity by deleting either PLP or non-PLP dependent genes (Table 1 and Fig. 4), we nevertheless were interested to know whether PLP plays a common role in that thermosensitivity. PLP serves as a cofactor for a large number of essential enzymes which catalyze more than 140 distinct reactions, including important steps in amino acid metabolism (28, 29).

We determined the effect of PLP on *zipA1* mutant viability in spot assays on LB plates by supplementing with 0.25 or 1 mM PLP and growth at 30, 34, 37, and 42°C for 20 h. We added 1 mM thiamine hydrochloride (vitamin B_1) to LB under the same conditions as a negative control. At 30°C, all cells grew well in the presence of either

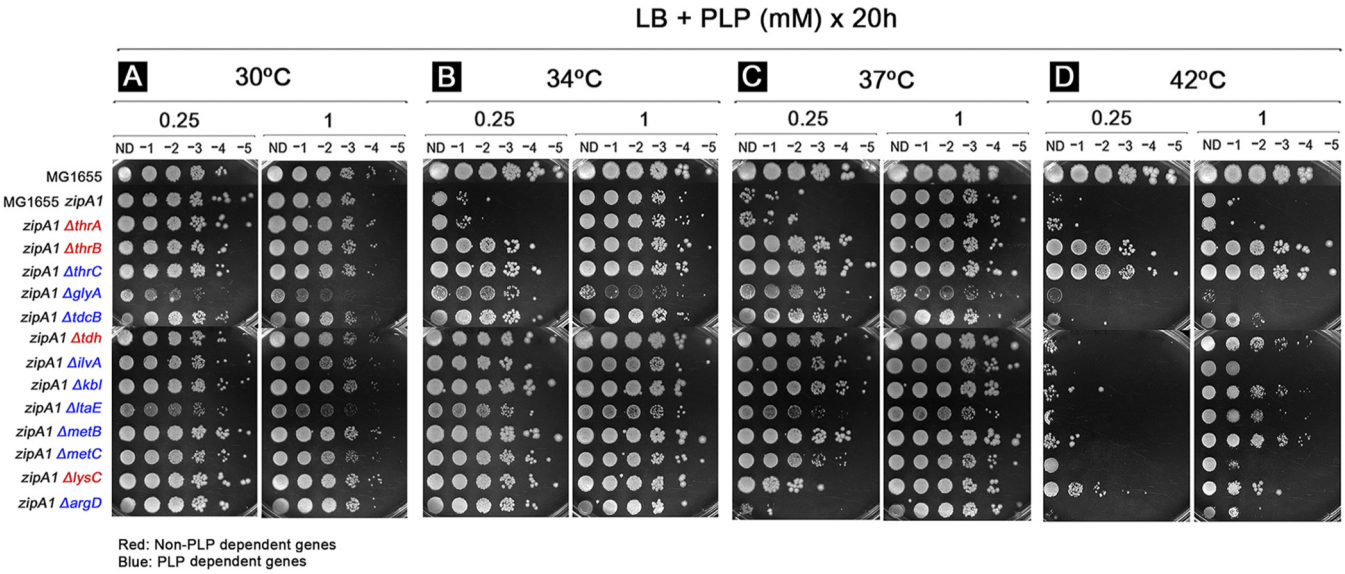

FIG 6 Exogenous pyridoxal-5-phosphate partially suppresses *zipA1* mutant thermosensitivity and has an additive effect with many of the gene deletions. To determine the effect of pyridoxal-5-phosphate (PLP) on suppressing *zipA1* mutant thermosensitivity, cells used for the spot assay in Fig. 3 were also spotted on LB plates supplemented with 0.25 mM or 1 mM PLP and incubated for 20 h at 30°C (A), 34°C (B), 37°C (C), and 42°C (D). ND, no dilution.

0.25 or 1 mM PLP (Fig. 6A), although as before, Δ*glyA* and Δ*ltaE* cell spots were weaker. Strikingly, adding 1 mM PLP greatly suppressed the thermosensitivity of the parental *zipA1* strain at 34°C (Fig. 6B) and 37°C (Fig. 6C) and even had a weak suppressing effect at 42°C. Moreover, 1 mM PLP improved the growth of several double mutants at 34°C and 37°C, and with the exception of the *glyA zipA1* mutant, permitted partial to full growth rescue even at 42°C (Fig. 6D). The lower (0.25 mM) concentration of PLP was unable to support viability at 42°C except for the Δ*thrB zipA1* and Δ*thrC zipA1* mutants, which were viable 42°C with either 0.25 or 1 mM PLP.

Added PLP can partially suppress other cell division mutants. Based on these unexpected results, we wanted to know if this ability of PLP to rescue the *zipA1* mutant would translate to the suppression of other thermosensitive cell division mutants. Strikingly, the *ftsK44* and *ftsQ1* mutants were able to grow at 42°C when LB was supplemented with 1 mM PLP (Fig. 5Q), with the *ftsK44* mutant being more strongly suppressed. The *ftsI23* mutant was very weakly suppressed at 42°C. In contrast, the growth of the *ftsA12* and *ftsZ84* mutants at 42°C was not rescued (Fig. 5Q), suggesting that the inactivation of the *ftsA12* and *ftsZ84* mutants at 42°C may be more complete than that of the other *fts* mutants, or perhaps that PLP affects a later stage of cell division than the proto-ring. We confirmed that the MC4100 strain background of the *ftsQ1* and *ftsI23* mutants shown in Fig. 5Q was not a significant factor in suppression by PLP, as MG1655 derivatives of these two mutants showed similar PLP suppression profiles (Fig. 5R). Moreover, the presence or absence of a Δ*metC* or Δ*thrC* mutation in the *ftsQ1* or *ftsI23* mutant strain did not appreciably affect the ability of PLP to suppress thermosensitivity.

Overall, these data support a role of PLP in partially suppressing the thermosensitivity of the parental *zipA1* mutant. Such suppressing effects were more evident when *zipA1* was combined with single deletions of genes involved in amino acid biosynthesis. The ability of PLP to partially suppress the thermosensitivity of the *ftsK44* and *ftsQ1* mutants indicates that this effect is not exclusive to the *zipA1* allele.

DISCUSSION

In this study, we initially found that a thermosensitive *zipA* (*zipA1*) strain in W3110 (WM2991) exhibited a growth and division defect in minimal medium at the permissive temperature. This was not surprising, as *zipA1* cells were reported to have a mild cell

division defect at 30°C when grown in LB (9). However, it was somewhat surprising that the *zipA1* mutant defect persisted under slow-growth conditions in minimal medium, because some cell division defects are suppressed at lower growth rates. For example, Δ*ftsN* mutants that are partially suppressed by mutations in *ftsB* or *ftsL* divide better in minimal medium than in rich medium (12). We were further surprised to find that the parent W3110 strain had a similar growth defect in minimal medium, although the cells did not form filaments like the *zipA1* mutant derivatives. Further examination of this phenomenon indicated that both W3110 and its *zipA1* derivative WM2991 behaved as if they were starved for L-threonine or glycine, as the addition of either of these two amino acids could rescue the growth defect. Although we do not understand the reason for this unexpected auxotrophic phenotype, we wanted to work in a strain background that did not have these defects, so we moved the *zipA1* allele into MG1655 (WM5337). This strain grew and divided fairly normally in M9 and LB at 30°C and formed the expected filaments at 42°C. We also found that WM5337 formed filaments and was not viable at temperatures as low as 34°C, which allowed us to look for suppressors of the *zipA1* allele that could survive at this and other temperatures lower than 42°C.

As our initial studies with WM2991 were focused on glycine and L-threonine, we made deletions in genes involved in glycine and threonine biosynthesis to see if limiting the cells for these amino acids might exacerbate thermosensitivity of the *zipA1* mutant. The fact that we found the opposite effect was clearly serendipitous. We then deleted other genes in the methionine-threonine-glycine-serine pathways and found that most, but not all, also partially suppressed *zipA1* mutant thermosensitivity at 34° and 37°C but not at the most nonpermissive temperature of 42°C. This partial suppression was not a general effect on all stages of cell division, as two deletions that rescued the *zipA1* mutant (Δ*thrC* and Δ*metC*) failed to significantly rescue cell division in *ftsZ*, *ftsA*, *ftsQ*, and *ftsI* mutants at the nonpermissive temperature. However, we were surprised to find that Δ*metC* could suppress the *ftsK44* mutants quite efficiently. Little is known about the defect in the *ftsK44* mutant, but this ability of the Δ*metC* (but not Δ*thrC*) mutation to suppress its thermosensitivity suggests that other aspects of cell division may be sensitive to perturbations in amino acid metabolism.

One curious aspect of the suppression was the ability of the Δ*thrB* and Δ*thrC* mutations but not the Δ*thrA* mutation, to suppress *zipA1* thermosensitivity. One mechanism for this specificity might involve an increase in L-homoserine levels. L-Aspartate is converted into L-aspartate-4-semialdehyde (ASA) by the three aspartate kinase (AK) genes *thrA* (AK-I), *metL* (AK-II), and *lysC* (AK-III) and the aspartate semialdehyde dehydrogenase gene (*asd*). ASA is subsequently converted into L-homoserine by homoserine dehydrogenases encoded by *thrA* or *metL*. Interestingly, deletion of either *thrA* or *metL* failed to suppress *zipA1* thermosensitivity. In the absence of *thrA*, less L-homoserine should be synthesized, whereas in the absence of either *thrB* or *thrC*, which encode the enzymes needed to convert L-homoserine to L-threonine, cellular L-homoserine levels should increase.

Our work also revealed a role for PLP in partial rescue of the *zipA1* allele's phenotypic defects. Of the 17 genes we deleted that were in pathways within or related to threonine metabolism, eight of these were capable of partially suppressing *zipA1* thermosensitivity in LB (Table 1). Of these eight, seven (*thrC*, *glyA*, *ltaE*, *tdcB*, *metB*, *metC*, and *ilvA*) are known to require PLP as a cofactor; only *thrB* is PLP independent. We therefore initially hypothesized that the *zipA1* allele might perturb PLP homeostasis, which was supported by the ability of extra PLP to rescue the growth and division of the *zipA1* mutant at 37°C in LB. To test this hypothesis, we deleted a number of other amino acid biosynthesis genes, both PLP dependent and PLP independent. In addition to the Δ*thrB* mutation, we found that deletions of other PLP-independent amino acid biosynthesis genes in related pathways, including *tdh*, *metH*, *mmuM*, and *lysC*, could partially suppress thermosensitivity of the *zipA1* allele to various degrees. Any one of these deletions might indirectly perturb PLP homeostasis because they affect the threonine-glycine-serine and methionine pathways and thus could affect PLP-dependent enzymes indirectly. Moreover, deletions of amino acid biosynthesis genes,

such as Δ*hisD* and Δ*asnB*, in seemingly unrelated pathways were able to weakly suppress the *zipA1* allele at 34°C but are not PLP dependent. The reason why the PLP-independent Δ*thrB* mutation can suppress *zipA1* thermosensitivity is unclear, but perhaps the accumulation of L-homoserine as described above might alter flux through various pathways (see below), indirectly causing increased demand for PLP. ThrB can also synthesize L-4-phosphohydroxythreonine, a precursor of PLP, via an alternative pathway that has the potential to compete with serine biosynthesis (30), so potential diversion of metabolites into the serine biosynthesis pathway because of the absence of *thrB* might play a role in the effects.

The relationship between central metabolism and cell shape/division in bacteria has emerged in recent years, with several notable examples of metabolic enzymes moonlighting as cell division regulators (31), including direct interaction between glucolipid enzymes or NAD(H) oxidoreductases and FtsZ (32–35). Amino acid biosynthesis enzymes have not yet been implicated in directly regulating cell division proteins, although a deficiency of shikimate kinase 1 (encoded by *aroK*), part of the aromatic amino acid pathway, can partially suppress the thermosensitivity of the *ftsZ84* mutant by an unknown mechanism (36). In addition, a deficiency of *S*-adenosylmethionine (SAM) has been shown to inhibit cell division in *E. coli* (37). However, the present study does not support the idea of SAM involvement in the suppression of the *zipA1* phenotype, particularly as blocking several steps in the synthesis of L-methionine helps to rescue division in *zipA1* cells (and in the case of Δ*metC*, even *ftsK44* cells). Another potential mechanism of suppression might be slower growth, which could compensate for a deficiency in cell division. Such a mechanism may be why Δ*min* Δ*slmA* mutants can divide in M9 medium but not LB (38), or, as mentioned earlier, why growth in minimal medium can enhance the cell division of *ftsN* mutants in certain cases (12). However, we think this possibility is unlikely, because the suppressed strains all had colony sizes similar to the nonsuppressed strains and grew as fast as or faster than the nonsuppressed strains in LB broth when switched from 30°C to 37°C.

By analogy to the role of pyruvate in regulating cell division in *Bacillus subtilis* (39), it is possible that the *zipA1* allele can perturb PLP homeostasis if PLP itself is a regulator of ZipA. For example, ZipA may require PLP for its proper function in cell division, and the ZipA1 mutant protein may be defective in utilizing PLP. This defect would be relieved, and ZipA function partially restored, either by directly providing extra PLP to the growth medium, or by shunting amino acid biosynthesis pathways away from PLP-intensive enzymes, such as those in the threonine-methionine-glycine pathways. Such shunting could be accomplished by rerouting biosynthesis by blocking specific PLP-intensive steps. Consistent with this idea, a modest deficiency of pyridoxine 5′ oxidase (PdxH), a key enzyme that converts pyridoxine 5′-phosphate to PLP, was reported to inhibit *E. coli* cell division (40). However, because deletions of genes encoding PLP-independent enzymes suppress the *zipA1* mutant, and added PLP can partially rescue later cell division defects of *ftsK44* and *ftsQ1* mutants, any model in which PLP regulates ZipA is likely too simplistic. Although it is known that ZipA is required for recruiting later cell division proteins (10), it is also not clear which step(s) of cell division is being rescued by the suppressing factors. Future dissection of these pathways and their relationships with cell division proteins should provide more mechanistic insights into the relationship between PLP, amino acid biosynthesis, and cell division.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and chemicals. The *Escherichia coli* strains used are listed in Table 2. Plasmid pDSW210 (a pBR322 derivative with a weakened *trp-lac* promoter and a *gfp* gene) was used to clone the *zipA* or *zipA1* mutant genes by EcoRI/SalI digestion. Cells were grown at 30, 34, 37, and 42°C in either Luria-Bertani (LB) broth with 0.5% NaCl, LB with no NaCl added, MOPS-glucose, or M9-glucose (41). For experiments in minimal medium, cells were first inoculated in LB and incubated for 16 h at 30°C, after which cells were pelleted, washed twice, resuspended in minimal medium, and diluted to 1:100 or 1:1,000 in M9 or MOPS. For all growth assays in liquid medium, cells were incubated with shaking in a BioTek Synergy MX microplate reader for the times indicated in Fig. 1M, 4A to O, and S2 and S4 in the supplemental material prior to measuring the cell optical density at 600 nm (OD_{600}). For

TABLE 2 *E. coli* strains used in this study[a]

Strain	Description	Source
W3110	*rpoS*$_{am}$ *rph-1* INV *(rrnD-rrnE)*	Lab collection
WM1074	Wild-type MG1655 F$^-$ λ$^-$ *ilvG rfb50 rph-1 lacU169*	Lab collection
MC4100	F$^-$ [*araD139*]$_{B/r}$ Δ(*argF-lac*)169 λ$^-$ e14$^-$ *flhD5301* Δ(*fruK-yeiR*)725 (*fruA25*) *relA1 rpsL150*(Str^r) *rbsR22* Δ(*fimB-fimE*)*632*(::IS*1*) *deoC1*	Lab collection
WM2991	W3110 *zipA1* (M44V, T211L, Y229C, and N281S)	
WM5322	WM2991 *zipA*$^+$ Δ*nupC*::Tn*10*	*zipA*$^+$, *nupC*::Tn*10* × WM2991
WM5337	MG1655 *zipA1* Δ*nupC*::Tn*10*	*zipA1*, *nupC*::Tn*10* × MG1655
WM5339	MG1655 *nupC*::Tn*10*	*zipA*$^+$, *nupC*::Tn*10* × MG1655
WM5372	WM5339 Δ*thrA*::*kan*	Δ*thrA*, JW0001-1[a] × WM5339
WM5376	WM5337 Δ*thrA*::*kan*	Δ*thrA*, JW0001-1 × WM5337
WM5373	WM5339 Δ*thrB*::*kan*	Δ*thrB*, JW0002-3 × WM5339
WM5377	WM5337 Δ*thrB*::*kan*	Δ*thrB*, JW0002-3 × WM5337
WM5351	WM5339 Δ*thrC*::*kan*	Δ*thrC*, JW0003-2 × WM5339
WM5352	WM5337 Δ*thrC*::*kan*	Δ*thrC*, JW0003-2 × WM5337
WM5369	WM5339 Δ*glyA*::*kan*	Δ*glyA*, JW2535-1 × WM5339
WM5354	WM5337 Δ*glyA*::*kan*	Δ*glyA*, JW2535-1 × WM5337
WM5429	WM5339 Δ*ltaE*::*kan*	Δ*ltaE*, JW0854-1 × WM5339
WM5433	WM5337 Δ*ltaE*::*kan*	Δ*ltaE*, JW0854-1 × WM5337
WM5374	WM5339 Δ*tdh*::*kan*	Δ*tdh*, JW3591-4 × WM5339
WM5378	WM5337 Δ*tdh*::*kan*	Δ*tdh*, JW3591-4 × WM5337
WM5375	WM5339 Δ*tdcB*::*kan*	Δ*tdcB*, JW3088-2 × WM5339
WM5379	WM5337 Δ*tdcB*::*kan*	Δ*tdcB*, JW3088-2 × WM5337
WM5385	WM5339 Δ*metA*::*kan*	Δ*metA*, JW3973-1 × WM5339
WM5388	WM5337 Δ*metA*::*kan*	Δ*metA*, JW3973-1 × WM5337
WM5386	WM5339 Δ*metB*::*kan*	Δ*metB*, JW3910-1 × WM5339
WM5389	WM5337 Δ*metB*::*kan*	Δ*metB*, JW3910-1 × WM5337
WM5387	WM5339 Δ*metC*::*kan*	Δ*metC*, JW2975-1 × WM5339
WM5390	WM5337 Δ*metC*::*kan*	Δ*metC*, JW2975-1 × WM5337
WM5426	WM5339 Δ*metE*::*kan*	Δ*metE*, JW3805-1 × WM5339
WM5430	WM5337 Δ*metE*::*kan*	Δ*metE*, JW3805-1 × WM5337
WM5474	WM5339 Δ*metL*::*kan*	Δ*metL*, JW3911-1 × WM5339
WM5475	WM5337 Δ*metL*::*kan*	Δ*metL*, JW3911-1 × WM5337
WM5427	WM5339 Δ*metH*::*kan*	Δ*metH*, JW3979-1 × WM5339
WM5431	WM5337 Δ*metH*::*kan*	Δ*metH*, JW3979-1 × WM5337
WM5428	WM5339 Δ*mmuM*::*kan*	Δ*mmuM*, JW0253-1 × WM5339
WM5432	WM5337 Δ*mmuM*::*kan*	Δ*mmuM*, JW0253-1 × WM5337
WM5472	WM5339 Δ*ilvA*::*kan*	Δ*ilvA*, JW3745-2 × WM5339
WM5473	WM5337 Δ*ilvA*::*kan*	Δ*ilvA*, JW3745-2 × WM5337
WM5476	WM5339 Δ*lysC*::*kan*	Δ*lysC*, JW3984-1 × WM5339
WM5477	WM5337 Δ*lysC*::*kan*	Δ*lysC*, JW3984-1 × WM5337
WM5693	WM5337 Δ*argD*::*kan*	Δ*argD*, JW3322-1 × WM5337
WM5694	WM5337 Δ*lysA*::*kan*	Δ*lysA*, JW2806-1 × WM5337
WM5695	WM5337 Δ*trpA*::*kan*	Δ*trpA*, JW1252-1 × WM5337
WM5696	WM5337 Δ*trpB*::*kan*	Δ*trpB*, JW1253-1 × WM5337
WM5697	WM5337 Δ*malY*::*kan*	Δ*malY*, JW1614-1 × WM5337
WM5698	WM5337 Δ*kbl*::*kan*	Δ*kbl*, JW3592-2 × WM5337
WM5705	WM5337 Δ*aspC*::*kan*	Δ*aspC*, JW0911-1 × WM5337
WM5706	WM5337 Δ*hisC*::*kan*	Δ*hisC*, JW2003-1 × WM5337
WM5707	WM5337 Δ*hisD*::*kan*	Δ*hisD*, JW2002-1 × WM5337
WM5708	WM5337 Δ*asnB*::*kan*	Δ*asnB*, JW0660-2 × WM5337
WM5440	WM5337 Δ*glyA*::*frt*	WM5354 cured by pCP20
WM5441	WM5337 Δ*ltaE*::*frt*	WM5433 cured by pCP20
WM5442	WM5337 Δ*tdh*::*frt*	WM5378 cured by pCP20
WM5443	WM5337 Δ*tdcB*::*frt*	WM5379 cured by pCP20
WM5444	WM5337 Δ*metC*::*frt*	WM5390 cured by pCP20
WM5462	WM5337 Δ*ltaE*::*frt* Δ*tdcB*::*kan*	Δ*tdcB*, JW3088-2 × WM5441
WM5463	WM5337 Δ*ltaE*::*frt* Δ*metC*::*kan*	Δ*tdcB*, JW3088-2 × WM5444
WM5464	WM5337 Δ*glyA*::*frt* Δ*ltaE*::*kan*	Δ*ltaE*, JW0854-1 × WM5440
WM5485	WM1125 (*ftsZ84*) Δ*thrC*::*kan* in WM1074	Δ*thrC*, JW0003-2 × WM1125
WM5486	WM1125 (*ftsZ84*) Δ*metC*::*kan* in WM1074	Δ*metC*, JW2975-1 × WM1125
WM5487	WM3993 (*ftsI23*) Δ*thrC*::*kan* in MC4100	Δ*thrC*, JW0003-2 × WM3993
WM5488	WM3993 (*ftsI23*) Δ*metC*::*kan* in MC4100	Δ*metC*, JW2975-1 × WM3993
WM5489	WM3994 (*ftsQ1*) Δ*thrC*::*kan* in MC4100	Δ*thrC*, JW0003-2 × WM3994
WM5490	WM3994 (*ftsQ1*) Δ*metC*::*kan* in MC4100	Δ*metC*, JW2975-1 × WM3994
WM5491	WM2101 (*ftsK44*) Δ*thrC*::*kan* in WM1074	Δ*thrC*, JW0003-2 × WM2101
WM5492	WM2101 (*ftsK44*) Δ*metC*::*kan* in WM1074	Δ*metC*, JW2975-1 × WM2101

(Continued on next page)

TABLE 2 (Continued)

Strain	Description	Source
WM4649	WM3993 (*ftsI23*) in WM1074	*ftsI23 leuO*::Tn*10* × WM1074
WM4661	WM3994 (*ftsQ1*) in WM1074	*ftsQ1 leuO*::Tn*10* × WM1074
WM5817	WM4649 (*ftsI23*) Δ*thrC*::*kan* in WM1074	Δ*thrC*, JW0003-2 × WM4649
WM5818	WM4649 (*ftsI23*) Δ*metC*::*kan* in WM1074	Δ*metC*, JW2975-1 × WM4649
WM5819	WM4661 (*ftsQ1*) Δ*thrC*::*kan* in WM1074	Δ*thrC*, JW0003-2 × WM4661
WM5820	WM4661 (*ftsQ1*) Δ*metC*::*kan* in WM1074	Δ*metC*, JW2975-1 × WM4661

[a]Phage P1 lysates were made from the indicated JW strains in the KEIO collection and used as donors in transductions.

microscopic analysis, 10 μl of culture was spread onto an agarose-covered glass microscope slide and topped with a glass coverslip.

Where noted, M9 or MOPS medium was supplemented with one of the following compounds (Sigma-Aldrich): Casamino Acids, L-tyrosine, L-valine, L-phenylalanine, L-glutamic acid, L-threonine, L-methionine, L-aspartic acid, L-proline, L-alanine, L-isoleucine, L-asparagine, L-leucine, L-serine, L-cysteine, glycine, L-histidine, L-tryptophan, L-lysine, L-arginine, L-glutamic acid, or pyridoxal 5-phosphate. Kanamycin (50 $\mu g \cdot ml^{-1}$), ampicillin (50 $\mu g \cdot ml^{-1}$), chloramphenicol (20 $\mu g \cdot ml^{-1}$), or tetracycline (10 $\mu g \cdot ml^{-1}$) was added to the plates or broth as needed. For spotting assays, an overnight culture was diluted 250-fold, followed by growth at 30°C for 2 h. Five microliters of 10-fold serial dilutions was spotted onto prewarmed plates and incubated at selected temperatures.

Strain construction. Single-deletion mutant strains listed in Table 1 were generated by growing P1 phage on strains from the KEIO collection (42) and then transducing the kanamycin resistance (Kan^r) markers into the appropriate recipient. For the construction of double mutants, the Kan^r cassette was removed by using plasmid pCP20 (43); the normal plasmid curing protocol was modified by using 30°C instead of higher temperatures to avoid thermoinactivating the *zipA1* allele. The presence of each mutation was confirmed by diagnostic PCR. The *ftsQ1* and *ftsI23* mutants were transferred from the MC4100 background to the MG1655 background (WM1074) by P1 transduction using the linked *leuO*::Tn*10* marker present in the MC4100 strains, followed by a screen for thermosensitivity. The Δ*metC*::*kan* and Δ*thrC*::*kan* alleles were introduced into these strains by transduction and confirmed by PCR, as described above.

Immunoblotting. Equivalent OD_{600} units of cell lysate were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with standard procedures using a 1:10,000 dilution of polyclonal ZipA or FtsZ primary antibody (14). Secondary horseradish peroxidase-conjugated goat anti-rabbit and chemiluminescent substrate were applied prior to developing and imaging.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00535-17.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

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