This supplemental note is an extended consideration of the data presented in Supplemental Fig 1. The same data is summarized in Table 1 in the main paper.

Cell division, DNA replication, and DNA repair

Given the morphological anomalies associated with the lack of ppGpp, it is perhaps unsurprising that 15 cell division genes showed differential expression in the $ppGpp^0$ strain (Fig 7A). Of these, 9 were expressed >2-fold higher in the $ppGpp^0$ strain, while 6 were expressed >2-fold lower. Three genes involved in cell division were induced >2-fold in the WT in response to isoleucine starvation. These genes, *fic, dacC,* and *bolA*, are members of the RpoS-dependent general stress response (Utsumi *et al.*, 1993, Santos *et al.*, 2002, Lange & Hengge-Aronis, 1991). Among them, the *bolA* and *dacC* gene products are known to play a role in determining the coccoid morphology associated with stationary phase cells (Lange & Hengge-Aronis, 1991, Santos et al., 2002). None of these genes were induced in the $ppGpp^0$ strain, likely because of failed induction/accumulation of RpoS and/or impacted ability of σ^S to compete for core RNAP (Gentry et al., 1993, Kvint *et al.*, 2000).

Most of the remaining cell division genes showed no change or modest down regulation in response to isoleucine starvation in the WT. Two genes were down regulated >2-fold in the WT (*yibP* and *tig*) in response to isoleucine starvation, but both of these genes were expressed >2-fold higher in the $ppGpp^0$ strain. Also notable among the cell division genes expressed higher in the $ppGpp^0$ strain, is *cgtA* (12.4-fold higher), which encodes an essential GTPase known to interact with SpoT in *Vibrio cholerae* (Raskin et al., 2007). In that system, the activity of CgtA apparently keeps the hydrolytic activity of SpoT high in order to maintain low amounts of ppGpp in nutrient rich environments. Similar results for *E. coli* have been recently reported (Jiang *et al.*, 2007). Furthermore, depletion of CgtA in *E. coli* was shown to result in defective chromosome partitioning and filamentous morphology (Foti *et al.*, 2007). Though the implications of

heightened expression of *cgtA* in the ppGpp^0 strain are not entirely clear, we note that since the lack of ppGpp results in filamentation even in the presence of presumably high levels of CgtA, it is possible that CgtA and ppGpp function in the same regulatory cascade to modulate normal cell division during times of nutrient limitation.

Sixteen genes involved in DNA replication showed a different pattern of expression in the ppGpp^0 strain, by comparison to the WT, with all but one gene (*cspD*) expressed more highly in the mutant (Fig. 7B). Many of these genes encode products directly involved in DNA replication including helicases (*recG, hrpB*), primase (*dnaG*, *priB*, *priA*), topoisomerases (*topB*, *parC*, *topA*, and *parE*), and DNA polymerase III, the primary replicative DNA polymerase (*dnaN*, *holD*, *holB*). *dnaA*, whose product controls replication initiation, was also expressed >2-fold higher in the ppGpp^0 strain. Taken together, the expression levels of these genes show a downward trend in the WT, consistent with decreased chromosome replication. No such trend is apparent in the ppGpp^0 strain with most of these genes being modestly or significantly induced. Recently, ppGpp has been shown to regulate DNA elongation through direct interaction with DNA primase in *B. subtilis* (Wang *et al.*, 2007). The higher levels of expression observed in our experiments for genes involved in DNA replication, combined with possible direct effects of ppGpp on DNA primase, are consistent with continued DNA replication in the ppGpp^0 strain despite the growth limitations imposed by amino acid starvation.

The WT strain did not exhibit increased expression of genes associated with the SOS response to DNA damage, however, this trend was quite apparent in the ppGpp^0 strain (Fig. 7C). Twenty-three DNA repair genes showed altered expression in the ppGpp^0 strain. The ppGpp^0 strain induced 16 DNA repair genes >2-fold in response to isoleucine starvation, including 13 members of the LexA regulon (Fernandez De Henestrosa *et al.*, 2000), i.e., *recN, uvrD, dinD*, *dinF, recA, ruvA, lexA*, *dinG, ydjQ, umuD*, *umuC, yebG,* and *sbmC*. Only 2

genes were more highly expressed in the WT, including *sbmC*, which encodes a DNA gyrase inhibitor known to protect cells against DNA damage (Baquero *et al.*, 1995). Interestingly, stationary phase induction of *sbmC* has been shown to be RpoS-dependent (Oh *et al.*, 2001). Whereas the WT likely terminates DNA replication via a combination of DNA gyrase inhibition and lowered transcription of DNA replication genes, it appears that chromosome replication continues abnormally in the ppGpp^0 strain, ultimately resulting in DNA damage.

Nucleotide biosynthesis and degradation

Down regulation of nucleotide biosynthesis has been associated with the stringent response (Chang *et al.*, 2002, Cashel et al., 1996, Turnbough, 1983), and as expected, we observed this trend in isoleucine-starved WT cultures: 21 genes down regulated >2-fold. Nucleotide biosynthetic genes showed some of the greatest levels of down regulation in the entire data set, including *pyrI, pyrB,* and *carA* (down regulated 79-, 93-, and 78-fold, respectively). Twenty-seven nucleotide biosynthetic genes showed abnormal expression in the strain lacking ppGpp (Fig. 7D); only 3 genes (*nrdA*, *nrdB*, and *thyA*) were expressed at a lower level, while the remaining 24 were expressed at levels >2-fold higher in the ppGpp^0 strain compared to the WT. While 9 of these genes were down regulated at least 2-fold in the ppGpp^0 strain, the greatest level of down regulation was only 23-fold (observed for *pyrI*) compared to 79-fold in the WT. Overall, the ppGpp^0 strain exhibited a greatly diminished ability to stringently down regulate genes involved in nucleotide biosynthesis. The strong down regulation of nucleotide biosynthetic genes in the WT reflects the greatly decreased need for nucleotides as cellular processes that consume nucleotides are curtailed (namely ribosome and chromosome synthesis). Given the continued production of rRNA and DNA in the ppGpp^0 strain, it might be expected that nucleotide pools would be diminished. In keeping with this notion, expression of *purR*, whose product autorepresses its own transcription (Rolfes & Zalkin, 1990), as well as a large number of genes involved in purine biosynthesis (Meng *et al.*, 1990), was

induced 4.5-fold, compared to the modest repression observed in the WT. The derepression of *purR* suggests that the hypoxanthine levels are low in the ppGpp^0 strain, implying that the supply of nucleotides had dwindled. Moreover, decreased nucleotide availability in the ppGpp^0 strain might stall DNA replication, thus prompting the DNA damage response noted above.

The WT showed both induction and down regulation of genes involved in nucleotide salvage/degradation (Fig. 7E), including 12 that were abnormally expressed in the ppGpp^0 strain. Several of these genes were induced in the WT in response to isoleucine starvation, including *amn*, *udp*, *deoA*, and *add*. These four genes, and two more, *dgt* and *deoC*, were expressed >2-fold higher in the WT strain. All six of these genes are involved in degradation, i.e., catabolism of nucleotides to intermediates of central metabolism. Induction of these nucleotide degradation genes, down regulation of nucleotide biosynthesis genes (discussed above), and the observation that nucleotide utilization was enhanced as a result of isoleucine starvation in our Biolog assays (Fig. 5), lead us to conclude that the WT shifted from funneling precursors into nucleotide production during rapid growth, to catabolism of excess nucleotides upon growth arrest. Furthermore, the induction of the non-oxidative branch of the pentose phosphate pathway observed in the WT might further enhance the flux of nucleotide degradation products into central metabolism. These observations are consistent with stationary phase-induced nucleotide degradation and byproduct excretion described elsewhere (Kim *et al.*, 2006, Rinas *et al.*, 1995). The genes *codA*, *upp*, *cmk*, *gpt*, *apt*, and *gsk* were expressed >2-fold higher in the ppGpp^0 strain. Interestingly, all 6 of these genes are involved in the salvage of endogenous nucleotide precursors for the production of new nucleotides, as opposed to catabolism of nucleotides via the non-oxidative branch of the pentose phosphate pathway. This trend suggests that the ppGpp^0 strain actively attempted to salvage nucleotides in keeping with continued synthesis of nucleic acid. Thus, the expression data, combined with the loss of ability to utilize nucleotides as C-sources in the Biolog assays, suggest that the switch from nucleotide production

for nucleic acid synthesis to degradation of nucleotides is a ppGpp-dependent process.

Fatty acid biosynthesis, β-oxidation, cell wall/LPS biosynthesis, and glycogen synthesis

Phospholipids and their constituent fatty acids (FA) form a significant portion of the dry weight of *E. coli* (~9%) (Neidhardt *et al.*, 1990), and accordingly, cells invest a corresponding amount of carbon and energy in phospholipid biosynthesis (see (DiRusso & Nystrom, 1998) for a review). Seventeen genes involved in phospholipid biosynthesis showed abnormal expression in the ppGpp^0 strain (Fig. 7F). Considered in context, the *accC* and *accD* genes are involved in the initial production of malonyl-CoA from acetyl-CoA and CO_2, while *fabD*, *fabH*, *fabF*, and *acpT* gene products are involved in the cyclical elongation of FA. The product of the *plsC* gene catalyzes the second step in phosphatidic acid biosynthesis from glycerol-3-P. Phosphatidic acid is then activated to CDP-diacylglycerol by the *cdsA* gene product. The *plsX* gene product is also thought to play a role in phosphatidic acid synthesis. Of the 17 phospholipid metabolism genes under consideration here, 12 showed either modest down regulation or no change in the WT (*accC*, *mdoB*, *cdh*, *acpT*, *fabH*, *plsX*, *fabF*, *fabA*, *accD*, *fabD*, *plsC*, and *cdsA*). In the ppGpp^0 strain, all 12 of these genes were expressed at levels >2-fold higher than in the WT, including 5 (*plsX*, *fabF*, *fabA*, *plsC*, and *cdsA*) that were induced >2-fold in response to isoleucine starvation. Of the remaining 5 genes, 4 were significantly induced in the WT in response to isoleucine starvation (*pgsA, ybhO*, *uspA* and *cfa*), but were expressed at lower levels in the ppGpp^0 strain. Induction of the *cfa* and *ybhO* genes in the WT reflects the conversion of unsaturated FAs in membrane lipids into cyclopropane derivatives and the accumulation of cardiolipin, both of which are associated with entry into stationary phase (Taguchi *et al.*, 1980a, Wang & Cronan, 1994, Hiraoka *et al.*, 1993).

Six genes involved in β-oxidation of FA were aberrantly expressed in the ppGpp^0 strain (Fig. 7G), 3 of which (*yfcX*, *aidB*, and *fadE*) were induced in the WT, but not in the ppGpp^0 strain. The only gene expressed higher in the ppGpp^0 strain than in the WT was *fadD*, which encodes acyl-CoA synthetase. These data fit the known shift from large scale phospholipid synthesis to FA breakdown (DiRusso & Nystrom, 1998), concomitant with the assumption of smaller cell sizes during transition to stationary phase. Gene expression in the ppGpp^0 strain points in the opposite direction, with possibly enhanced synthesis of FA (described above) and lower-than-normal levels of FA degradation.

Though stringent control of peptidoglycan synthesis at the level of enzyme inhibition has been observed (Ishiguro & Ramey, 1976), the genes involved in synthesis of peptidoglycan were not comprehensively down regulated in the WT. However, 13 genes involved in peptidoglycan biosynthesis were abnormally regulated in the ppGpp^0 strain, with 9 of them expressed >2-fold higher than the WT (Fig. 7H). These included two genes for penicillin binding proteins (*mrdA* and *dacD*), and two N-acetylmuramoyl-L-alanine amidases (*amiC* and *amiA*) involved in post-division cell separation. *mltA* and *mpl,* whose products are involved with murein turnover, were also expressed higher in the ppGpp^0 strain. *mreC* which encodes a subunit of the MreBCD transmembrane complex was down regulated in the WT, but not in the ppGpp^0 strain. Genes expressed lower in the ppGpp^0 strain include *murE* and *murF*, which catalyze the final cytoplasmic steps in the formation of peptidoglycan precursors. Taken together, these results suggest the ppGpp^0 strain may have an abnormally high rate of peptidoglycan turnover. We hypothesize that this response of the ppGpp^0 culture may be linked to the exhaustion of glutamate, which serves as a precursor for peptidoglycan synthesis.

Twenty-four genes involved in the synthesis of lipopolysaccharide and other components of the outer membrane (OM) were expressed abnormally in the ppGpp^0 strain and all but one of these (*gutQ*) were expressed >2-fold higher in

the mutant when compared to the WT. Most of these genes are involved in O-antigen synthesis, lipid A synthesis, or synthesis of other LPS precursors or transporters. Moreover, 8 of these genes (*rfaS*, *lpxC*, *rfaH*, *htrB*, *rfaK*, *ddg*, *lpxK*, and *msbB*) were induced >2-fold in the ppGpp^0 strain in response to isoleucine starvation. Fourteen surface antigen and OM protein encoding genes were down regulated >2-fold in the WT in response to isoleucine starvation, while only one of these (*ugd*) was significantly down regulated in the ppGpp^0 strain. These results indicate that the down regulation of genes involved in LPS synthesis and other OM structures in response to isoleucine starvation is directly or indirectly dependent on ppGpp. When considered with the expression patterns observed for FA metabolism genes, the down regulation of OM component genes, including those for LPS synthesis, suggests that inner and outer membrane assembly is largely halted in the WT in response to isoleucine starvation. Moreover, these processes likely continue unabated in the ppGpp^0 strain, adding to the abnormally high biomass produced by the strain lacking ppGpp.

If excess carbon is available during the transition to stationary phase, glycogen can be accumulated (as is the case under isoleucine starvation). Glycogen can account for up to 3% of *E. coli* dry cell weight, and serves as a carbon source during periods of carbon starvation. Accumulation of glycogen is known to be a ppGpp-dependent process (Taguchi *et al.*, 1980b, Ishiguro & Ramey, 1978), and this trend is clearly observable in the array data, with seven genes involved in glycogen metabolism being expressed >2-fold lower in the ppGpp^0 strain (Fig. 7J). Moreover, *glgS*, known to be a member of the RpoS-dependent general stress response (Hengge-Aronis & Fischer, 1992), was not induced in the ppGpp^0 strain as it was in the WT. Taken together, these results suggest that glycogen probably does not contribute to the higher biomass produced by the ppGpp^0 strain.

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