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The *dgt* gene of *E. coli* facilitates thymine utilization in thymine-requiring strains

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Summary

The *Escherichia coli* dGTP triphosphohydrolase (dGTPase) encoded by the *dgt* gene catalyzes the hydrolysis of dGTP to deoxyguanosine and triphosphate. The recent discovery of a mutator effect associated with deletion of *dgt* indicated participation of the triphosphohydrolase in preventing mutagenesis. Here, we have investigated the possible involvement of *dgt* in facilitating thymine utilization through its ability to provide intracellular deoxyguanosine, which is readily converted by the DeoD phosphorylase, to deoxyribose-1-phosphate, the critical intermediate that enables uptake and utilization of thymine. Indeed, we observed that the minimal amount of thymine required for growth of thymine-requiring (*thyA*) strains decreased with increased expression level of the *dgt* gene. As expected, this *dgt*-mediated effect was dependent on the DeoD purine nucleoside phosphorylase. We also observed that *thyA* strains experience growth difficulties upon nutritional shift-up and that the *dgt* gene facilitates adaptation to the new growth conditions. Blockage of the alternative *yjjG* (dUMP phosphatase) pathway for deoxyribose-1-phosphate generation greatly exacerbated the severity of thymine starvation in enriched media, and under these conditions the *dgt* pathway becomes crucial in protecting the cells against thymineless death. Overall, our results suggest that the *dgt*-dependent pathway for deoxyribose-1-phosphate generation may operate under various cell conditions to provide deoxyribosyl donors.

Keywords

dGTPase; *E. coli dgt*; *thyA* strains; thymine starvation; nutritional shift; salvage pathways

Introduction

The pathways for biosynthesis of the deoxyribose-5'-triphosphates (dNTPs), the precursors for DNA synthesis, are important for both basic and applied sciences, as the quality and quantities in which these compounds are available for the replication fork directly impact the maintenance of genetic stability and cell viability.

The concentrations of the four canonical dNTPs are carefully regulated, as disturbances of the dNTP pools are mutagenic due to increased replication errors (Kunz *et al*., 1994). A well-studied case where an extreme imbalance in DNA precursors severely impacts cell physiology is thymineless death, named for the rapid loss of cell viability of thymine-requiring (*thyA*) strains upon thymine deprivation (Cohen and Barner, 1954; Ahmad *et al*., 1998). A key factor in the proper balance of dNTPs is the enzyme ribonucleotide reductase (RNR), which reduces ribonucleotides (most often NDPs) to the corresponding deoxynucleotides (Eriksson and Sjöberg, 1989), the so-called *de novo* pathway for

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deoxynucleotide synthesis. While dATP, dGTP, and dCTP are generated from the corresponding NDPs by RNR and nucleoside diphosphate kinase, dTTP is synthesized from the non-canonical dNTP, dUTP (Fig. 1). The latter, potentially toxic compound is rapidly converted to dUMP by dUTPase (Kouzminova and Kuzminov, 2004), preventing its incorporation into DNA. dUMP is then converted to dTMP by the enzyme thymidylate synthetase (encoded by *thyA*), followed by the two-step conversion to dTDP and dTTP.

Alternatively, deoxynucleotides can be generated via salvage pathways (Fig.1). Such pathways may involve phosphorylation of deoxynucleosides by deoxynucleoside kinases yielding dNMPs. The number of deoxynucleoside kinases varies widely throughout nature. Human cells have a variety of deoxynucleoside kinases with different specificities (Arner and Eriksson, 1995), while insects have only one with broad specificity (Knecht *et al*., 2000). Much less is known about the diversity of deoxynucleoside kinases in prokaryotic cells (Ives and Ikeda, 1998; Andersen and Neuhard, 2001). *Escherichia coli* possesses only one type of deoxynucleoside kinase - thymidine kinase. Thus, among the four conventional deoxynucleosides only thymidine can serve as a specific DNA precursor in this organism (Neuhard and Kelln, 1996).

In addition to the activities described above ensuring adequate amounts of DNA precursors, a second issue important for genetic stability is the purity of the dNTP pools. For example, cells possess several dNTP-hydrolyzing enzymes that are essential for sanitizing the dNTP pool from damaged or modified derivatives (Galperin *et al*., 2006). Incorporation of such alternative dNTPs into the DNA can cause increases in mutation rate due to their ambiguous base-pairing properties (Kamiya, 2003).

One unusual class of dNTP-hydrolyzing enzymes is the dNTP triphosphohydrolases, which hydrolyze dNTPs to the corresponding deoxynucleoside and triphosphate (PPP_i). Four members of this group have been investigated in some detail: the *E. coli dgt* gene product, also referred to as dGTPase (Beauchamp and Richardson 1988; Seto *et al*., 1988), the *Thermus thermophilus* TT1383 protein (Kondo *et al*., 2004), and two triphosphohydrolases from *Pseudomonas aeruginosa* (*P. aeruginosa*) PA1124 and PA3043 (Mega *et al*., 2009).

E. coli dGTPase and *P. aeruginosa* PA1124 have a preference for hydrolyzing dGTP. The physiological importance of these enzymes is still unclear. However, it has been shown that loss of *E. coli dgt* leads to a 2-fold increase in the cellular dGTP level (Quirk *et al*., 1990), while its overexpression (in *optA1* strains, which carry a (up) mutation in the *dgt* promoter) leads to a 30- to 50-fold increased amount of dGTPase (Quirk *et al*., 1990; Wurgler and Richardson, *et al*., 1990) and a 5-fold decrease in the dGTP level (Meyers *et al*., 1987). Recently, our laboratory showed that deletion of the *dgt* gene results in a mutator phenotype leading, in particular, to an enhancement of G·C→C·G and A·T→G·C base-pair substitutions on an F' episome (Gawel *et al*., 2008). This observation suggested the likely involvement of the *dgt* gene in DNA replication fidelity, presumably through its effect on the cellular dNTP pools (Gawel *et al*., 2008).

In the present study, we have investigated another possibility for the cellular function of the *dgt* gene, namely, involvement of the dGTPase reaction in thymine salvage. Thymine salvage is the critical pathway for dTTP production in *thyA* strains, which lack the enzyme thymidylate synthetase of the *de novo* synthesis pathway. The utilization of external thymine involves its condensation with the internal precursor deoxyribose-1-phosphate by thymidine phosphorylase (*deoA* gene product) yielding thymidine (see Fig. 1). A major source of the intracellular deoxyribose-1-phosphate in *thyA* strains has been reported to be the dUMP phosphatase pathway encoded by the *yjjG* gene (Weiss, 2007a). YjjG hydrolyzes dUMP to deoxyuridine (U-dRib), which is subsequently converted to deoxyribose-1-phosphate by

DeoA or uridine phosphorylase (Udp) (Fig. 1). The importance of this pathway in *thyA* strains reflects, in part, the accumulation of dUMP (Kwon *et al*. 2010) in these strains resulting from the block of the *thyA* thymidylate synthetase pathway (Fig. 1).

Previous studies in *thyA* mutants supplemented with thymine have indicated that it is the size of the deoxyribose-1-phosphate pool that ultimately limits the rate of conversion of thymine to thymidine and, hence, the rate of incorporation into DNA (Kammen, 1967; Beacham *et al*., 1971; Jensen *et al*. 1973). Thus, the intracellular deoxyribose-1-phosphate pool determines the external thymine concentration minimally required needed to support growth of *thyA* mutants. It has also been shown that thymine incorporation can be stimulated by exogenous deoxynucleosides like G-dRib or deoxyadenosine (Kammen, 1967; Jensen *et al*. 1973), an effect presumably related to the ability of these agents to elevate the deoxyribose-1-phosphate pool (via the DeoD purine nucleoside phosphorylase reaction) (see Fig. 1). Within the context of this model, one may hypothesize that the internal deoxyribose-1-phosphate pool may also be determined, at least in part, by the production of G-dRib through the *dgt* pathway, and that the minimal external thymine concentration required for *thyA* mutants would be dependent on the Dgt activity. To test this hypothesis, we generated Δ*thyA* mutants in strains with different *dgt* expression levels and investigated their thymine requirements. Our results show that the ability of cells to take up and metabolize extracellular thymine correlates with *dgt* expression level, most logically due to its ability to generate intracellular deoxyribosyl donors.

Results

Thymine requirements of thy*A* strains with different *dgt* expression levels

To test the hypothesis that the *dgt* gene provides an intracellular G-dRib source that can be used to generate deoxyribose-1-phosphate for thymine incorporation (Fig. 1), we investigated several *dgt thyA* strains, with different *dgt* expression levels, for the minimum thymine concentration required for their growth. Specifically, we compared the dgt^+ wild-type strain to a *dgt* null mutant (*dgt*::mini-Tn10*kan*) or the *dgt* overexpressor specified by the *optA1* allele. The latter carries a single base change in the *dgt* promoter creating an enhanced expression allele (Beauchamp, *et al*., 1988; Quirk *et al*., 1990). We also tested the interaction between the *dgt* alleles and the *deoD* gene encoding the purine nucleoside phosphorylase responsible for the downstream conversion of G-dRib to deoxyribose-1-phosphate and guanine (see Fig. 1).

A continuous range of thymine concentrations was tested using thymine gradient plates. As shown in Fig. 2A, these plates clearly showed a positive correlation of growth at lower thymine concentrations with increased *dgt* expression: the least growth was seen in the *dgt* strain, while the most growth occurred for the overproducing *optA1* strain. In contrast, no such correlation was seen in the corresponding *deoD* mutants, where the streak lengths do not vary significantly with the *dgt* level and coincide approximately with that of *dgt* $deoD^+$ strain. The data are presented quantitatively in the histogram of Fig. 2C. In contrast, no growth differences were observed on plates containing a gradient of thymidine instead of thymine (Fig. 2B).

The same trend was seen in a series of experiments using minimal-medium liquid batch cultures supplied with increasing thymine concentrations (Fig. 3). In the $deoD^+$ series, the *optA1* strain attained the largest final yield after 19 hours, followed by the dgt^+ strain, and the *dgt* strain showing the lowest yield. In the Δ*deoD* series, the *optA1* and dgt^+ derivatives reached a similar low level, while the *dgt* derivative attained the lowest yield, which coincides with that of the *dgt* $deoD^+$ strain.

These combined data clearly suggest that the dGTP triphosphohydrolase encoded by the *dgt* gene (along with the DeoD purine nucleoside phosphorylase) is an important factor in facilitating thymine incorporation through the production of deoxyribose-1-phosphate from dGTP.

Thymine requirements in the ΔyjjG background

The thymine requirements of *thyA* strains were also tested under potentially more stringent conditions. For that purpose, we additionally introduced a *yjjG* deletion into the strains. The *yjjG* gene encodes a (d)NMP phosphatase (nucleotidase), which has been shown to be responsible, at least in part, for the hydrolysis of the dUMP that accumulates in *thyA* mutants, yielding deoxyuridine (U-dRib) (Weiss 2007a). The U-dRib is subsequently used to generate deoxyribose-1-phosphate by DeoA (see Fig. 1). In our experiments, we observed little or no effect of the *yjjG* deletion under the standard conditions of minimal medium (in fact, a slight enhancement of growth may be seen) (Fig. 4A left panel), suggesting that under these conditions the *yjjG* pathway may, in fact, be minor relative to the *dgt* pathway. However, when the medium was enriched with casamino acids (CAA) to speed up bacterial growth, the thymine requirements of all *yjjG* strains were clearly increased, consistent with the previous report (Weiss 2007a) and indicating the importance of this pathway. Importantly, also in this Δ*yjjG* background, the *optA1* derivative shows the most growth, followed by dgt^+, with the least growth observed for the *dgt* strain (Fig. 4A right panel). Thus, also under conditions of rapid growth the *dgt* pathway makes an important contribution to the intracellular generation of deoxyribose-1-P.

Viability of thymineless *dgt* strains upon nutritional shift-up

The experiments described above reveal the extra demands that casamino acid-enrichment of the medium places on the thymine requirements of *thyA* strains. To further investigate this phenomenon, we applied a nutritional shift-up to a series of minimal medium batch cultures growing at steady state (Fishov *et al*., 1995). A thymine concentration of 20 μg/ml was chosen for this experiment. We followed both the total biomass (OD_{630}) and the total viable cell count. Each of the strains responded to the addition of casamino acids by an immediate 1.5- to 1.9-fold increase in rate of biomass growth (OD_{630}) (Fig. 5 circles). For example, the wild-type (dgt^+) strain increased its biomass growth rate from 0.86 doublings/hour to 1.28 doublings/hour upon addition of the casamino acids. However, only the *optA1* mutant (panel C) succeeded ultimately to increase its rate of cell division (increase in viable count, closed squares) commensurate with the new nutritional conditions. This acceleration of division rate was observed after a 4-hr period of maintaining the original division rate (rate maintenance, see Kjelgaard *et al*., 1958, and Discussion). In contrast, no increase in the rate of cell division is seen in the dgt^+ and *dgt* strains (panels A and B). In fact, in the *dgt* strain cell divisions are completely halted after two hours (panel A), while in the dgt^+ strain they slow down and manage to regain the pre-shift rate only after several hours (panel B). Addition of Δ*yjjG* to the above mutants greatly exacerbates the deleterious consequences of the medium enrichment. The *dgt* Δ*yjjG* strain likely undergoes lysis, reflected by the decrease in optical density of the culture approximately two hours after shift-up and a corresponding drop in cell viability (panel D). As thymine starvation is known to lead to SOS induction and prophage induction (Korn and Weissbach, 1962), the strains were checked for the presence of phage under various conditions, including after UV irradiation. No phage was found. Cell divisions of the dgt^+ Δ*yjjG* strain (panel E) stop two hours after the shift-up, and the viable count shows a very slight decrease after four hours, switching to a slow rise after 8 hours possibly due to amplification of preexisting mutants or suppressors. Only the *optA1* Δ*yjjG* strain keeps dividing (panel F), although with the pre-shift rate, and it fails to accelerate the division rate to the level seen in the $yjjG^+$ counterpart. Thus, health and viability of the strains under these conditions is clearly affected by the status of *dgt*.

Discussion

In this study we describe a second *in vivo* function of the *dgt*-encoded dGTPase, in addition to its previously reported role in reducing the frequency of spontaneous mutations (Gawel *et al*., 2008). This second role is its key participation in a newly described pathway for maintaining the intracellular pool of deoxyribose-1-phosphate (dRib-1-P), the availability of which is the critical parameter for thymine uptake and utilization in thymine-requiring cells.

There is no active transport system for thymine in *E. coli* (Crawford, 1958; Jensen *et al*. 1973), and its uptake by diffusion and its utilization is directly coupled to its condensation with intracellular dRib-1-P yielding thymidine. Wild-type cells have a very small pool of dRib-1-P and are, therefore, not able to utilize exogenously provided thymine effectively. In *thyA* strains, the pool of dRib-1-P is increased due to the accumulation and breakdown of deoxyuridine-5'-phosphate (dUMP) (Fig. 1) (Jensen *et al.* 1973; Kwon *et al.* 2010). The minimal thymine concentration required for growth of this kind of strain is about 20 μg/ml. Blockage of the catabolism of dRib-1-P by defects in the *deoB* or *deoC* genes (*thyA deoB* or *thyA deoC* strains) results in further enlargement of the dRib-1-P pool size (Jensen *et al*. 1973) and a correspondingly lower minimally required concentration of thymine (as low as 2 μg/ml) (Neuhard and Kelln, 1996). (These strains are called thymine low-requirers) (Ahmad and Pritchard, 1971; Neuhard and Kelln, 1996). The thymine requirement may be further lowered by increasing the expression level of the *deo* operon (*deoCABD*). This operon is under double negative control by the DeoR and CytR repressor proteins (Valentin-Hansen *et al*., 1978). Inactivating these repressors, such as in a triple *thyA deoB deoR* or *thyA deoB cytR* mutant, lowers the thymine requirement to only 0.2 μg/ml (Ahmad and Pritchard, 1971; Neuhard and Kelln, 1996) (thymine super-low requirers), as these mutants have both an increased dRib-1-P pool and elevated level of thymidine phosphorylase (DeoA).

The present study demonstrates that the thymine requirement of *thyA* mutants is controlled additionally by two genes, *dgt* and *deoD*, that participate in the two sequential steps converting dGTP into dRib-1-P (Fig. 1). Successive increases in the *dgt* expression level in the order *dgt*, *dgt*$^{+}$, *optA1* lead to a corresponding decline in thymine requirements of a *thyA* strain, consistent with a progressively increased dRib-1-P pool mediated by the intracellular production of deoxyguanosine (G-dRib). Furthermore, blockage of the downstream conversion of G-dRib into guanine and dRib-1-P in the *deoD* mutant negates the effects of the *dgt* expression and returns the thymine requirement to approximately the same, high level (Fig. 2). The slightly higher cell yield obtained in *dgt*$^{+}$Δ*deoD* and *optA1*Δ*deoD* strains compared to the *dgt* Δ*deoD* strain in the liquid growth experiments (Fig. 3) may be reasonably explained by the activity of some non-specific phosphorylase capable of cleaving accumulated G-dRib, thus contributing to the improvement in bacterial growth on low thymine.

The importance of the dGTP pathway is also readily visible in the nutritional shift-up experiments. When a culture growing in steady state (Fishov *et al*., 1995) is suddenly enriched by the addition of extra nutrients, the culture undergoes an orderly and highly reproducible series of changes (Neidhardt *et al*.,1990). The growth rate for total biomass increases immediately to a higher value that is consistent with the new nutritional value of the medium. However, the rate of cell division does not change for a period of $C + D$ min, where C (chromosome time) is the time needed to complete a full round of chromosome replication and D (division time) is the time interval between chromosome termination and cell division. This phenomenon is called “rate maintenance” (Kjeldgaard *et al*., 1958). It is due to the fact that, while nutritional shift-ups accelerate total DNA synthesis by increasing the frequency of initiations at the chromosomal origin, the corresponding cell divisions are

not triggered until these new rounds of replication have completed (C + D) (Helmstetter *et al*., 1968). In our case of the *thyA* mutants, only the *optA1* derivative demonstrates the expected phenomenon: the pre-shift division rate is maintained for a period of 4–5 hours, after which it accelerates (Fig. 5A). [We also note that this period of rate maintenance is rather long, compared to wild-type strains (Helmstetter *et al*., 1968; Helmstetter, 1996). We may assume this is due to the very long *C* time period (slow intrinsic replication rate) that is characteristic of thymineless mutants under conditions of limiting thymine (Pritchard and Zaritsky, 1970)]. In contrast, the *dgt* and dgt^+ derivatives of the *thyA* strain do not show rate maintenance and actually reduce their division rate 90–120 min after shift-up (Fig. 5A and B, filled squares), with some slow recovery in the dgt^+ strain (Fig. 5B) and a complete arrest in the *dgt* derivative (Fig. 5A). These observations are indicative of the difficulties faced by these strains in maintaining their dTTP pools and DNA synthesis rate when additional replication forks are initiated by the shift up.

A large portion of the pool of dRib-1-P in *thyA* strains has been reported to be derived from dUMP mediated by the dUMP phosphatase encoded by the *yjjG* gene (Weiss, 2007a) (see also Fig. 1). Indeed, both our enriched medium experiments (Fig. 4) and nutritional shift-up (Fig. 5) experiments clearly show the negative consequences of loss of the *yjjG* pathway upon thymine limitation. The progressive loss of viable count that we observe for the *dgt* Δ*yjjG* double mutant likely represents a case of thymineless death (Cohen and Barner, 1954; Ahmad *et al*., 1998). In this case, the extreme lack of thymine leads to persistent damage to the chromosomal origins, along with irreversible SOS-dependent inhibition of cell division (Fonville *et al*., 2010; Kuong and Kuzminov, 2010; Martin and Guzman, 2010; Sangurdekar *et al*., 2010). Fewer cells succumb to this mechanism in the dgt^+ Δ*yjjG* derivative, while killing is essentially prevented in the *optA1* Δ*yjjG* derivative, presumably due to the much increased dRib-1-P pool.

The demonstration by Weiss (2007a) of the importance of the *yjjG* pathway for thymine salvage was based on experiments conducted in casamino acid-enriched medium. Our experiments in such media (Figs. 4 and 5) fully support this role. On the other hand, our experiments in non-enriched medium indicate that the *yjjG* gene does not play a major role in permitting growth under these conditions. This contrasts to the clear role played by *dgt* under the same conditions. Possibly, the *yjjG* expression or activity levels are modulated by the growth conditions compared to other possible dUMP hydrolyzing enzymes, such as YfbR and SurE (Proudfoot *et al*. 2004, Weiss 2007b). Indeed, it was noted (Weiss, 2007a) that a *yjjG* mutant still possessed some 50% of its dUMP phosphatase activity. It is also intriguing that the absence of *yjjG* under minimal conditions slightly improves thymine-limited growth (Fig. 4A, left), regardless of *dgt* status, suggesting that YjjG can also have a deleterious effect. The specificity of the enzyme is rather broad (Proudfoot *et al*., 2004; Titz *et al*., 2007) and includes its ability to hydrolyze dTMP, which might be deleterious under some conditions.

The observed enhancement of thymine starvation in *thyA* strains by increased protein synthesis (i.e., in rich media) has parallels in other situations. For example, an enhancement of thymine starvation in enriched medium was observed with *dcd* mutants defective in dCTP deaminase (Weiss and Wang, 1994). As this enzyme supplies most of the dUTP used for thymidylate synthesis (Neuhard and Thomassen, 1971) (see also Fig. 1), *dcd* mutants are thymidine deficient and, like the *dgt* Δ*thyA* mutants, display a greater growth defect when the medium is supplemented with casamino acids (Weiss and Wang, 1994).

The present study clearly demonstrates a role of the *dgt*-mediated reaction in potentiating thymine incorporation in thymine-requiring strains. A remaining question is the role of *dgt* in the salvage pathway of wild-type (thy^+) strains. Dgt is expected to be equally active in

wild-type cells. This is evidenced by the *dgt* mutator effect caused by its absence (Gawel *et al*., 2008), as well as a demonstration of *dgt*-dependent excretion of deoxyguanosine into the medium under conditions when the DeoD purine phosphorylase pathway is blocked (results to be described elsewhere). Thus, also in wild-type cells, Dgt will contribute to the production and maintenance of a dRib-1-P pool. While this pool is reportedly small (Jensen *et al*., 1973; Munch-Petersen, 1970), it should still be available for salvage, which could provide metabolic flexibility under conditions of change. Although wild-type cells generally use exogenous thymine poorly (Crawford, 1958; Reinhart and Copeland, 1973), which is due to a number of factors (lack of active transport, low dRib-1-P pool, and a limited level of DeoA thymine phosphorylase), nevertheless, detectable thymine usage has been observed upon addition of exogenous deoxynucleosides (Jensen *et al*., 1973), confirming that indeed the dRib-1-P pool is available for thymine salvage. Maintenance of a modest dRib-1-P pool might also be advantageous for controlling expression of the *deoCABD* operon, which is under control of the DeoR repressor (Valentin-Hansen *et al*., 1978). The inducer for the operon is dRib-5-P, which is the next catabolic product down from dRib-1-P (see Fig. 1). Thus, one of the functions of *dgt*-mediated hydrolysis of dGTP may be aimed at ensuring a sufficient basal expression level of the *deo* operon, providing metabolic flexibility and adaptability. To our knowledge, thymine utilization has not been investigated under various conditions of stress, including DNA stress, where salvage reactions may be beneficial.

Experimental procedures

Bacterial strains

The *Escherichia coli* strains used are derivatives of *E. coli* K-12 (λ^-) and are listed in Table 1. All strains used in the experiments testing thymine requirements are derivatives of strain NR16947 [*ara*, *thi*, *trpE9777*, Δ*prolac*, F'*prolac* (F'CC106)] (Gawel *et al*., 2008) and also contain the *zad-220*::Tn*10 panD tonA* markers, as described in Table 1. Deletions of the chromosomal *thyA*, *deoD*, and *yjjG* genes were created by the method of Datsenko and Wanner using strain BW25113 and the kanamycin resistance cassette specified by plasmid pKD13 or the chloramphenicol resistance specified by plasmid pKD3 (Datsenko and Wanner, 2000). Primers used for generating the required PCR product are described in Table 2. The kanamycin resistance markers of the Δ*thyA*::*kan* and Δ*deoD*::*kan* alleles were eliminated using the temperature-sensitive replicon pCP20 (Datsenko and Wanner, 2000). The deletion/insertion constructs were confirmed by PCR reactions. Transfer of genetic markers was accomplished by P1 transduction using P1*virA.* Due to the lack of a direct selection for *optA1* (*dgt* overexpressor), this allele was co-transduced with the *zad-220*::*Tn10* transposon insertion (Singer *et al*., 1989) to which it is genetically linked (25–30 %). To check for co-transfer of *optA1*, tetracycline-resistant transductants were tested for their ability to support growth of the bacteriophage T4 *tsL141* mutant (Muzyczka *et al*., 1972). Clones that restrict growth of this phage at 30°C are *optA1*, while permissive clones are dgt^+ (Gauss *et al*., 1983). Wild-type phage T4D was used as a positive control. The T4 phages were obtained from Dr. J. W. Drake, NIEHS.

Media

Strains were selected and maintained on LB medium supplemented with thymidine (200 μg/ml), and also containing kanamycin (50 μg/ml) for strains with a *kan* marker, chloramphenicol (25 μg/ml) for strains with a *cam* marker, or tetracycline (15 μg/ml) for strains with a *Tn10* marker. Minimal Medium (Min) contained Vogel-Bonner (VB) salts (Vogel and Bonner, 1956), glucose (0.4%), proline (50 μg/ml), tryptophan (50 μg/ml), thiamine (5 μg/ml), D-pantothenic acid (5 μM), various concentrations of thymine (5–20 μg/ml), and, where indicated, 1% casamino acids (CAA).

Thymine requirements

Thymine requirements on solid media were determined by measuring the growth of bacterial streaks on thymine gradient plates. The gradient plates were generated as follows: 31 ml of minimal liquid agar containing 20 or 40 μg/ml of thymine (or 16 μg/ml of thymidine) was poured into square Petri dishes, which were immediately tilted on the Petri dish lid. After agar solidification, the Petri dishes were returned to the horizontal position, and 36 ml of MM liquid agar without thymine was poured on top of the skewed surface. Streaks were generated by spreading 10 μl overnight cultures - 10-fold diluted in VB buffer - along the length of the gradient using an inoculating loop (Fisherbrand). The plates were incubated at 37°C for 24hr (minimal medium) or 16hr (minimal medium with CAA) until clearly visible zones of growth were apparent and demarcation lines could be placed. No manipulation of the images in Photoshop or other image-processing program was performed.

Because of the possibility of prophage induction during thymine starvation, the culture supernatants were checked carefully for the presence of phage. None were found. We also checked our strains for phage induction following UV light exposure. Again, none were found.

Thymine requirements in liquid batch culture were tested by measuring biomass growth (optical density at 630 nm) with a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc.).

Viable cell counts

Cell viability was determined by colony-forming ability on LB plates (with 200 μg/ml of thymidine) following appropriate dilutions. The number of colonies was counted after 24 h incubation at 37°C.

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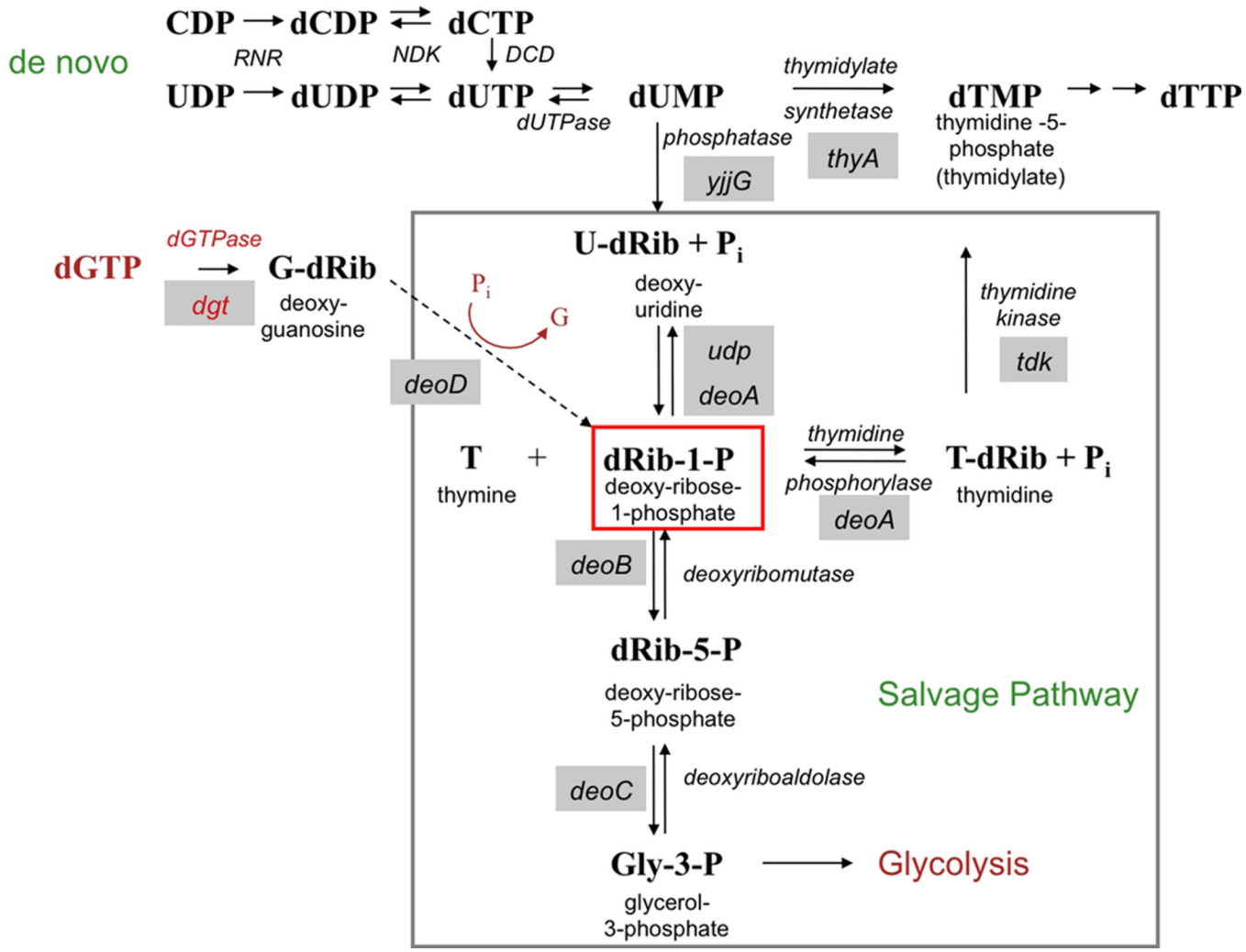

Fig. 1.

de novo and salvage pathways for thymidylate synthesis (adapted from Zaritsky *et al*., 2006) as present in *E. coli*. *thyA* mutants cannot produce thymidylate by the *de novo* route [mediated by ribonucleotide reductase (RNR), nucleotide diphosphate kinase (NDK) and dCTP deaminase (DCD)] but, in contrast to wild-type cells, they have increased potential for using intra- and extracellular thymine for DNA incorporation. The mechanism involves accumulation of deoxyribose-1-phosphate (dRib-1-P) (central red square) because of increased dUMP hydrolysis due to the *thyA* block. dUMP phosphatase (encoded by *yjjG*) converts dUMP to deoxyuridine (U-dRib), which undergoes phosphorolysis to U and dRib-1-P by uridine or thymidine phosphorylase (*udp* or *deoA* gene, respectively). dRib-1-P can condense with thymine to generate thymidine in a reaction catalyzed by thymidine phosphorylase (encoded by *deoA*). Deoxyguanosine (the direct product of the reaction catalyzed by dGTPase (produced by the *dgt* gene) can further enlarge the dRib-1-P pool through purine nucleoside phosphorylase activity encoded by *deoD*, thus enhancing thymine incorporation.

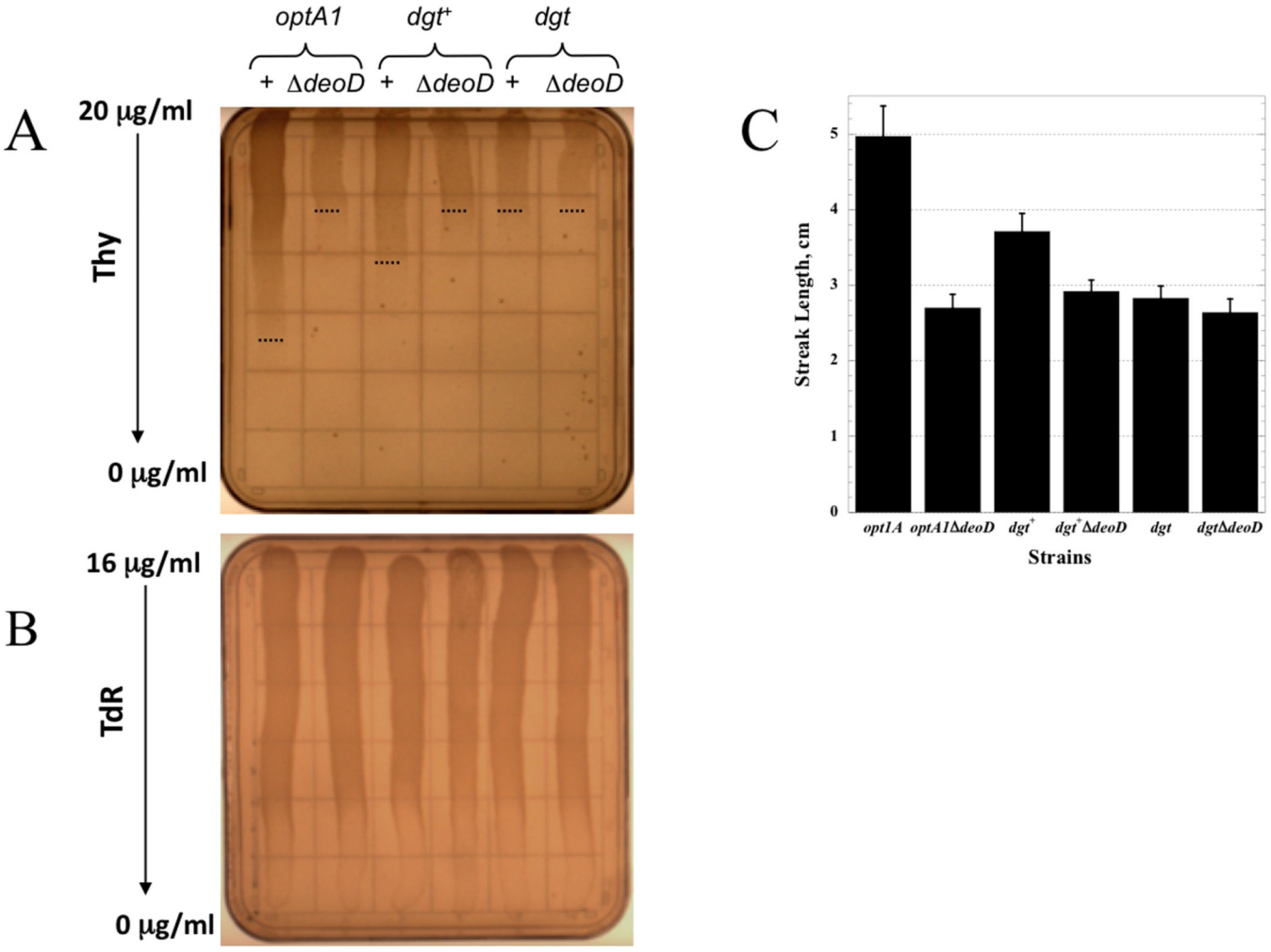

Fig. 2.

Thymine requirements of *thyA* and *thyA deoD* strains with different *dgt* expression levels as determined on thymine gradient plates. Suspensions of the indicated cultures were streaked on minimal-medium plates containing a gradient from top to bottom of decreasing thymine or thymidine concentrations. A. Thymine gradient from 20 to 0 μg/ml. B. Thymidine gradient from 16 to 0 mg/ml. The thymidine gradient plate also contained CAA. C. Histogram of observed growth (streak lengths) of the corresponding strains on thymine gradient plates. A total of ten plates was used for standard error calculation.

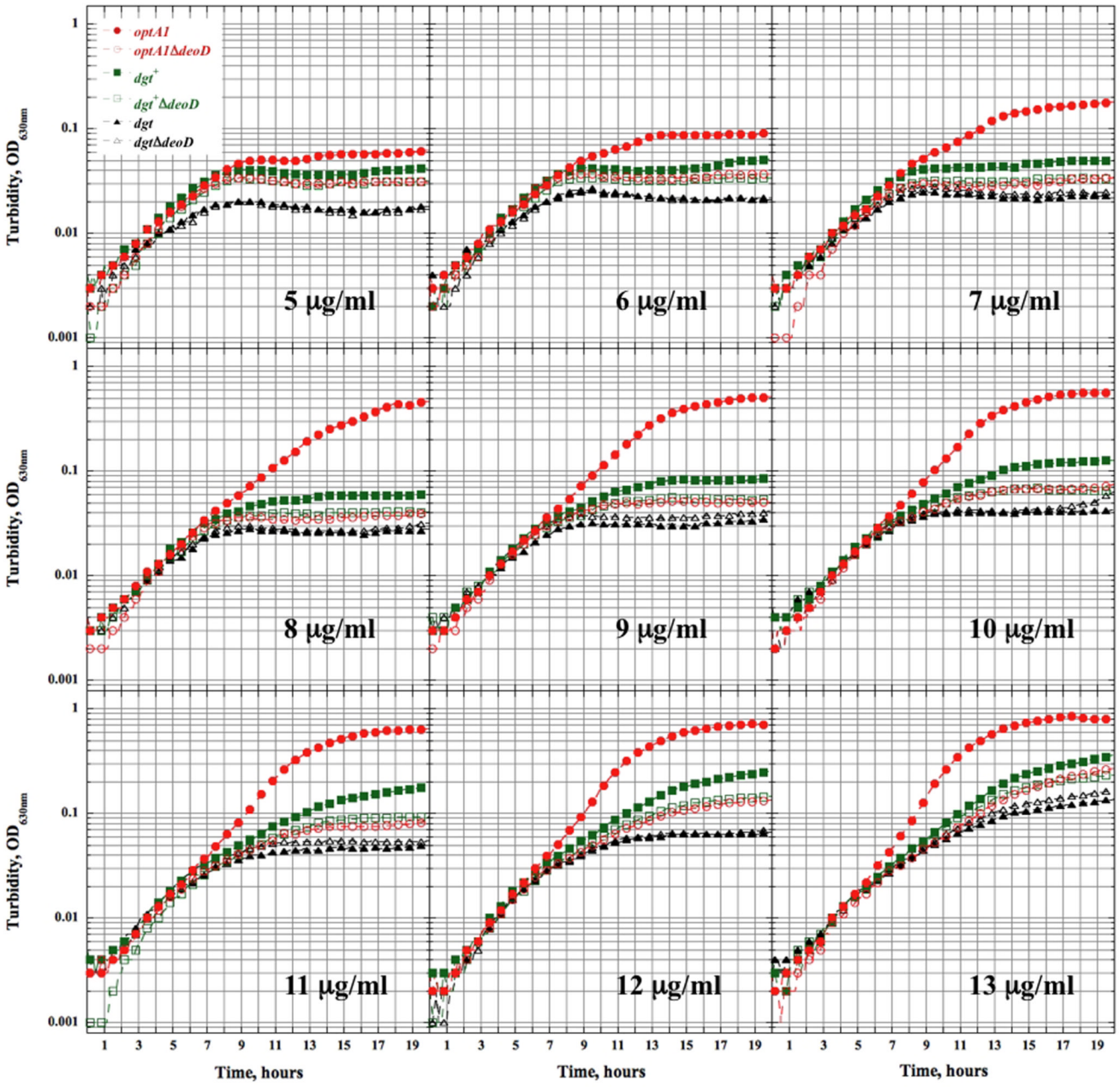

Fig. 3.

Growth of *thyA* and *thyA deoD* strains with different *dgt* expression levels in liquid minimal medium containing indicated concentrations of thymine.

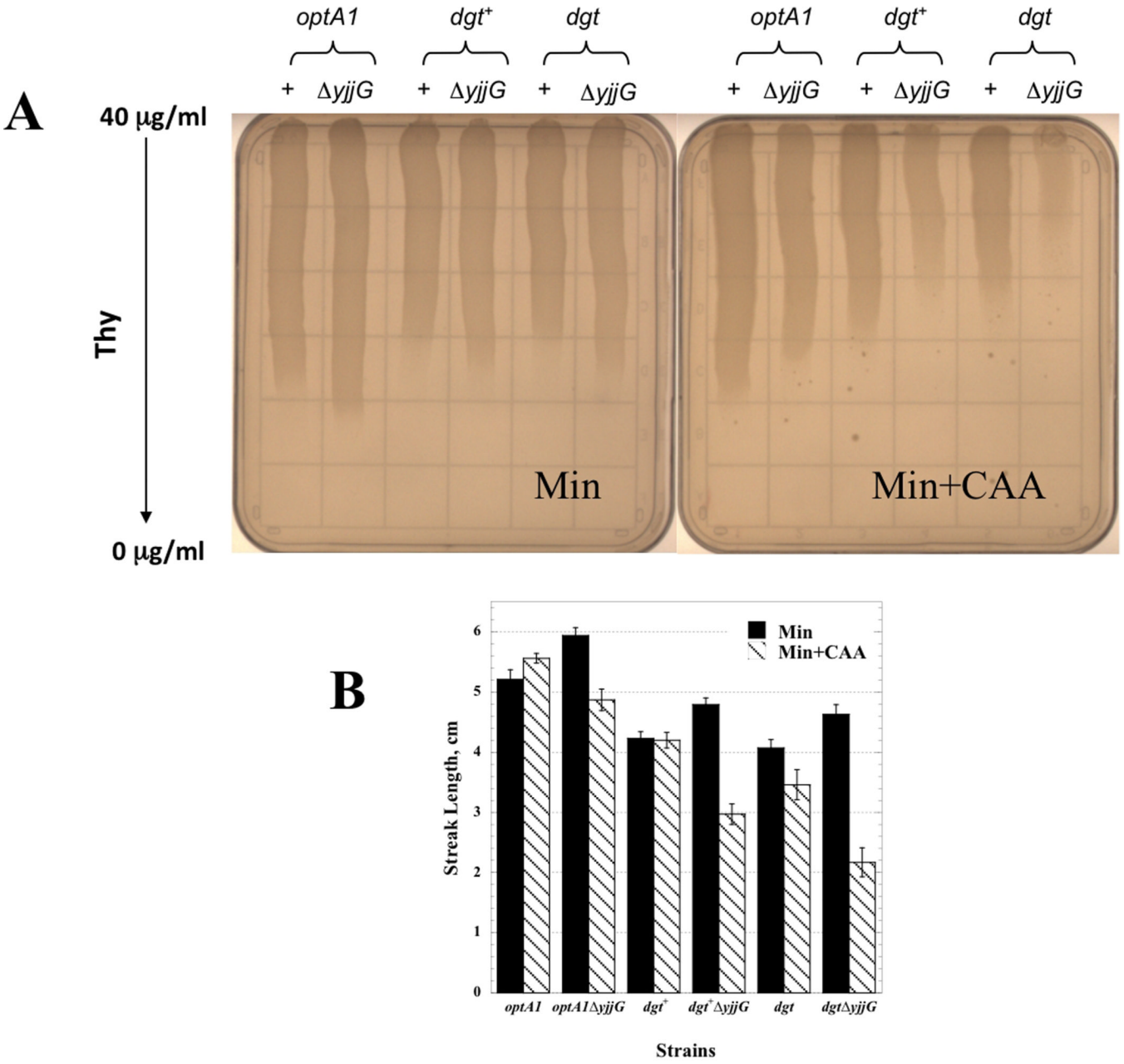

Fig. 4.

Thymine requirements of *thyA* and *thyA yjjG* strains with different *dgt* expression levels determined on thymine gradient plates. A. Suspensions of the indicated cultures were streaked on minimal-medium plates (Min) or minimal-medium plates supplemented with 0.5% casamino acids (Min + CAA). The plates contained a gradient from top to bottom of decreasing thymine concentration from 40 to 0 μg/ml. B. Histogram of streak lengths (extent of growth) for each of the strains: black bars, (Min); dashed bars, (Min+CAA). A total of ten plates was used for standard error calculation. The modest difference between the *dgt* and *dgt*$^+$ strains on the Min plate (top left) (0–40 μg/ml gradient) contrasts with the very obvious difference observed between the two strains on the (0–20 μg/ml) gradient plate (Fig. 2A). This phenomenon, observed repeatedly, is likely related to the difference in steepness of the

gradients, where a steeper gradient is predicted to diminish the apparent difference in growth (i.e., streak length).

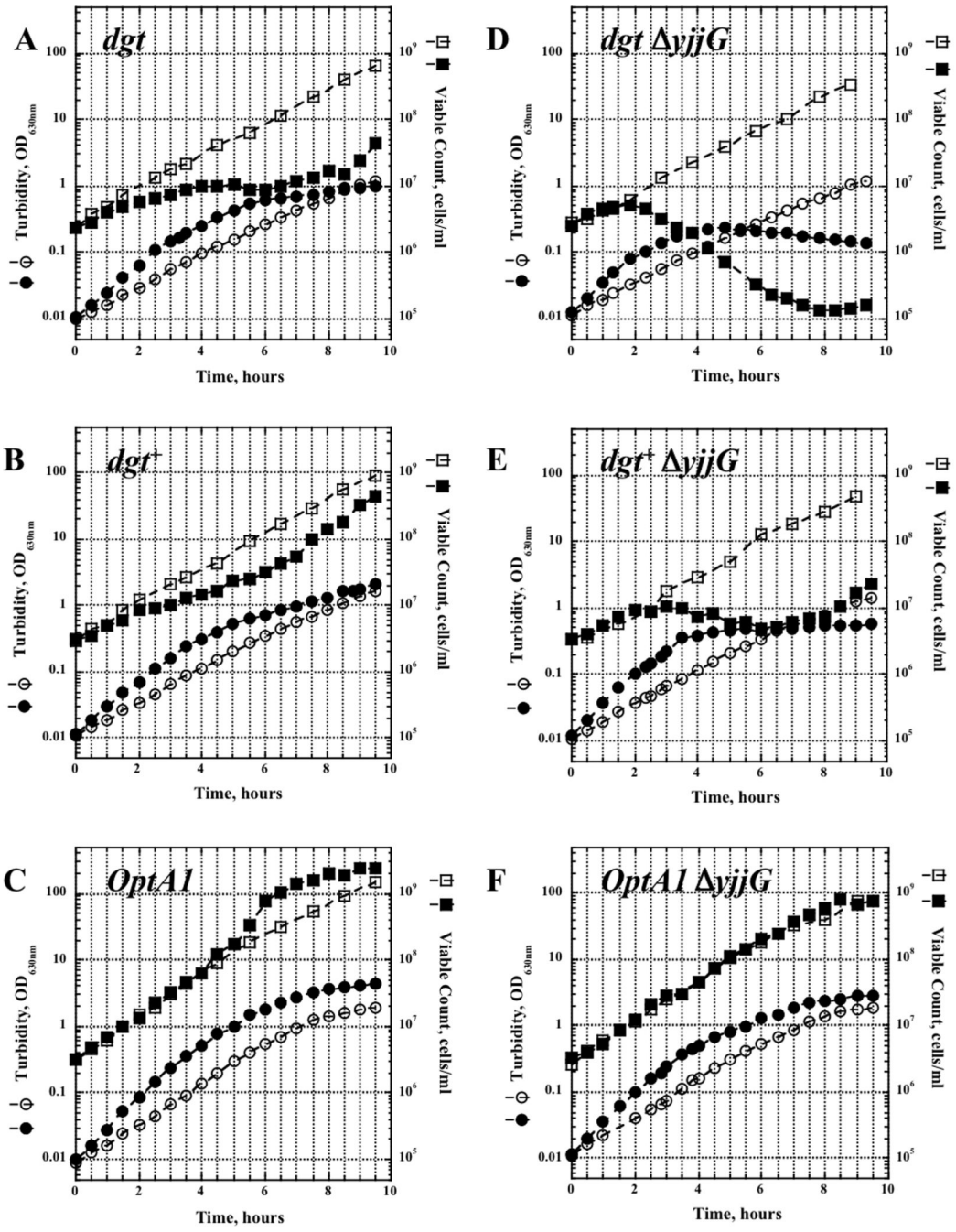

Fig. 5.

Biomass growth (OD_{630}) (circles) and viable count (squares) of various indicated *thyA* strains upon a nutritional shift-up from minimal medium (open) to minimal medium supplemented with 1% casamino acids (closed) at time 0. The thymine concentration was 20 μg/ml in all cases.

Table 1

E. coli strains used in this study[a]

Strain	Relevant genotype	Reference or source
BW25113	*lacI*^{q} *rrnB* $_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD* $_{AH33}$ Δ*rhaBAD* $_{LD78}$	Datsenko and Wanner, 2000
CAG12025	*zad-220::*Tn*10 panD*	Singer *et al*., 1989
JD20450[b]	*dgt::*mini-Tn*10kan*	Nat. Inst. Genet., Japan[c]
NR16947	*ara*, *thi*, Δ*prolac*, *trpE9777*, F'CC106	Gawel *et al*., 2008
NR17063 (HR44 but *optA1*)	*thi-1 argH1 his-1 leu-6 lys-25 thr-1 ara-13 gal-6 lacY1 malA1 mtl xylA1 strR tonA optA1*	J. W. Drake
NR17624	*optA1 zad-220*::Tn*10 tonA panD*	NR17063 × P1/CAG12025
NR17642[d]	BW25113, but Δ*thyA*::*kan*	This work
NR17647	*optA1 zad-220*::Tn*10 panD tonA*	NR16947 × P1/NR17624
NR17648	*zad-220*::Tn*10 panD tonA*	NR16947 × P1/NR17624
NR17649	*optA1 zad-220*::Tn*10 panD tonA* Δ*thyA*::*kan*	NR17647 × P1/NR17642
NR17650	*zad-220*::Tn*10 panD tonA* Δ*thyA*::*kan*	NR17648 × P1/NR17642
NR17673	NR17649, but kan^{s}	NR17649 × pCP20
NR17674	NR17650, but kan^{s}	NR17650 × pCP20
NR17685[e]	BW25113, but Δ*deoD*::*kan*	This work
NR17688	*optA1 zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*deoD*::*kan*	NR17673 × P1/NR17685
NR17689	*zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*deoD*::*kan*	NR17674 × P1/ NR17685
NR17698	NR17688, but kan^{s}	NR17688 × pCP20
NR17699	NR17689, but kan^{s}	NR17689 × pCP20
NR17726	*dgt*::mini-Tn*10kan zad-220*::Tn*10 panD tonA* Δ*thyA*	NR17674 × P1/JD20450
NR17738	*dgt*::mini-Tn*10kan zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*deoD*	NR17699 × P1/JD20450
NR17755[f]	BW25113, but Δ*yjjG*::*cam*	This work
NR17756	*optA1 zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*yjjG*::*cam*	NR17673 × P1/NR17755
NR17757	*zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*yjjG*::*cam*	NR17674 × P1/NR17755
NR17758	*dgt*::mini-Tn*10kan zad-220*::Tn*10 panD tonA* Δ*thyA* Δ*yjjG*::*cam*	NR17726 × P1/NR17755

[a] All strains are *E. coli* K-12 (λ^{-})

[b] mini-Tn*10kan* insertion located at 228 nucleotides from start of *dgt* gene (insertion site 179,465)

[c] National BioResource Project (NIG, Japan) (http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp)

[d] The mutant lacks 87% of *thyA* gene; *kan* cassette replaces nucleotides 2,962,433–2,963,127.

[e] The mutant lacks 86% of *deoD*; *kan* cassette replaces nucleotides 4,618,906–4,619,625.

[f] The mutant lacks 92% of *yjjG*; *cam* cassette replaces nucleotides 4,606,629–4,607,284.

Table 2

Primers used for chromosomal gene deletions.

Target gene	Pairs of primers (5'-3')	Template[b]
thyA	ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCTCGACGAAGGCACACAGTGTAGGCTGGAGCTGCTTC TTAGATAGCCACCGGCGCTTTAATGCCCGGATGCGGATCGTAGCCTTCAAATTCCGGGGATCCGTCGACC	pKD13
deoD	ATGGCTACCCCACACATTAATGCAGAAATGGGCGATTTCGCTGACGTAGTGTGTAGGCTGGAGCTGCTTC TTACTCTTTATCGCCCAGCAGAACGGATTCCAGTGCGATTTTGATCATGTATTCCGGGGATCCGTCGACC	pKD13
yjjG[a]	TCATGGCGTTGCCAATCAGTATGTAATACAAGGTGGAATAGTGTAGGCTGGAGCTGCTTC CCAGTTCGTGCAACGAAGAAACGGTCCAGGTGGGCGCGATCATATGAATATCCTCCTTAG	pKD3

[a] Primers were designed according to Weiss, 2007a.

[b] Datsenko and Wanner, 2000.